

**To the members of the scientific jury**  
**Defined by Order No. P109-388/28.08.2023**  
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## **R E V I E W**

**Of Prof. Dr. Iva Stefanova Christova, MD, Ph.D., D.Sc.**  
**National Center of Infectious and Parasitic Diseases**

Concerning Dissertation thesis for the award of the scientific degree "DOCTOR OF SCIENCES" in the scientific specialty "Microbiology"

**Topic of the thesis:**

ANTIBIOTIC RESISTANCE OF THE MOST COMMON ETIOLOGICAL AGENTS  
OF BACTEREMIA AND LETHALITY ASSOCIATED WITH THEM

**Author of the thesis:** Prof. Temenuga Zhekova Stoeva, MD, PhD

The dissertation work of Prof. Temenuga Stoeva is impressive with its thoroughness and precision. It is dedicated to the WHO's most relevant topic in the field of microbiology, namely the growing global antibacterial resistance, categorized by the WHO among the ten biggest threats to human health. The overuse and misuse of antimicrobials is a major cause of the emergence of resistant microbial pathogens.

The work covers studies on the ESKAPEEc pathogens: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp., *E. coli*, as etiological agents of bacteremia in hospitalized patients over a 10-year period. These are pathogens of increasing importance, causing severe nosocomial infections. There is a global trend towards a rapid increase in the frequency of infections caused by ESKAPEEc pathogens, as well as in the development of multiple resistance in them. That is why, Prof. Stoeva's in-depth studies on the etiological structure, risk factors and lethality of

infections caused by these pathogens are of utmost importance for surveillance and antibiotic policy.

My general impression of the dissertation is that it is a large-scale, well-conceived, precisely executed at a high methodical level and excellently designed work, very intelligently written, with comprehensive information in all its sections, with many merits and contributions. It contains a wealth of evidentiary and well-illustrated material from its own research.

The dissertation work is voluminous, it is written on 295 pages together with the literature used and the appendices. The research was conducted at the Department of Microbiology and Virology at the University of Varna.

The literature review is one of the main merits of the dissertation work. It is written on 73 pages based on a huge number of literary sources, 77% of which are from the last 10 years. The literature review is excellently written, thorough and comprehensive. Modern information is presented, described in detail and at the same time concisely presented.

The overview gives an idea of the excellent theoretical preparation of Prof. Stoeva. It begins by defining the concept of blood infections, their variants and criteria for differentiation, and presents their classification. Peculiarities in the epidemiology of blood infections - factors affecting their frequency, sources of infection, risk factors and frequency of adverse outcome depending on the specific microbial pathogen - are reviewed. A main place is logically devoted to the etiology of bacterial infections of the blood - the most common bacterial etiological agents, most of which refer to the ESKAPEE<sub>c</sub> group, are presented thoroughly and with knowledge of many years of international population and hospital studies. Presentation of etiological spectrum of blood infections according to the affected patient population is particularly valuable - patients over 65 years old, patients with liver cirrhosis, patients with HIV, patients with solid tumors, patients after transplantation, patients in intensive care units. Blood infections in patients with oncohematological diseases are presented in detail - their frequency, etiological spectrum, lethality depending on the causative pathogen. Modern microbiological diagnostics of blood infections, sensitivity testing methods with an emphasis on molecular genetic methods - PNA FISH (Peptide Nucleic Acid Fluorescence In Situ Hybridization), PCR, multiplex PCR, DNA sequencing, as well as genetic

mechanisms of resistance to antimicrobial agents among the leading causes of bacterial blood infections.

The literature review very aptly concludes with a summary of the importance of blood infections and the need to conduct hospital epidemiological and microbiological studies on the etiological spectrum of blood infections, antibiotic resistance of the most common agents, risk factors and mortality over a long period of time, which logically leads to the goal of the dissertation work. 5 large-scale tasks are formulated, which outline the main areas of research.

The study is retrospective, covering all microbiologically proven and clinically established episodes of bacteremia in "St. Marina" UMBAL Varna for a 10-year period (2011-2020) and, respectively, an 11-year period (2010-2020) in patients with oncohematological diseases. Additionally, in 798 patients with bacteremia for the period 2016-2020, information on 27 variables (demographic, clinical, microbiological) was analysed. The rich set of methods used in the dissertation is presented comprehensively, clearly, concisely. It includes microbiological methods for proving pathogens in blood, species identification, antimicrobial susceptibility testing, and statistical methods. An impressive array of genetic methods has been used for epidemiologic typing (ERIC-PCR, RAPD-PCR, REP-PCR, MLST) and to demonstrate genes encoding ESBLs (TEM, SHV, CTX-M, KPC, VIM, IMP, NDM, OXA-23/24/51/58/143/235). The rich set of modern methods and the manner of their presentation are proof of the high methodical level at which the dissertation work was completed.

The results of her own research are presented and discussed in 100 pages. The dissertation is richly illustrated with 36 tables and 27 figures. The description of the results is accurate, clear and informative. The obtained huge volume of multifaceted results together with their discussion is divided into 5 sections, following the logical sequence of the tasks.

The first section of the dissertation is dedicated to the results and analysis of the etiological spectrum of bacterial blood infections in hospitalized patients for the period 2011-2020. An increasing share of Gram-positive bacteria was found (from 33.3% in 2011 to 45.7% in 2020) and a decrease in the share of Gram-negatives (from 57.4% in 2011 to 50.7% in 2020). The proportion of ESKAPEE<sub>c</sub> isolates in this study was 66.8% of the total number of invasive isolates over the 10-year period. The results achieved are

analysed in light of published data from the two largest international antimicrobial resistance surveillance networks – EARS Net for Europe and the global SENTRY Antimicrobial Surveillance Program.

The second section of the dissertation is the most extensive. Here Prof. Stoeva analyses the antibiotic resistance of the most common etiological agents of bacteremia, comparing the obtained results with European and world data.

With regard to *S. aureus* isolates, a share of MRSA close to the European average was found in line with the permanent tendency to decrease their relative share in the country. Of all 462 *S. aureus* isolates tested, glycopeptide antibiotics were active in 99.8% of them. However, increasing resistance to gentamicin, macrolides and clindamycin is found in the study.

In *E. faecium*, a statistically significant trend for increasing resistance to the glycopeptide group of antibiotics was established, with all vancomycin-resistant strains also being teicoplanin-resistant, suggesting that the resistance is encoded by the *vanA* gene. In such strains, multiple resistance to all groups of antibiotics tested, except linezolid, make it the last choice for therapy. A significantly lower resistance to glycopeptide antibiotics was found in *E. faecalis* compared to *E. faecium*, in line with the data from Southern and Eastern Europe and at the same time it proved very high levels of quinolone and HLAR (high level aminoglycoside resistance).

In *E. coli*, she expectedly found a very high level of resistance to 3rd generation cephalosporins, significantly higher than the European average. According to ECDC data, Bulgaria has the highest level of this resistance, while in the years of the COVID-pandemic in other European countries, this level decreased. Prof. Stoeva found a low level of carbapenem-resistant *E. coli*, but a high proportion of aminopenicillin-resistant *E. coli*, which is in line with the national data, as well as higher than European resistance to fluoroquinolones, although with a decreasing trend in the last 5 years of the survey.

In studying the resistance to 3rd generation cephalosporins and carbapenems of *K. pneumoniae* strains, genetic techniques have been skilfully applied. By PCR and subsequent sequencing to demonstrate the most common genes encoding ESBLs, a high frequency of the *bla<sub>CTX-M-15</sub>* and *bla<sub>KPC-2</sub>* genes was found. Epidemiological typing of these strains by ERIC PCR and MLST and establishment of the main branches is also of

great importance. The study demonstrated ST15 as the dominant type associated with carbapenem-resistant *K. pneumoniae* and KC-2 carbapenemase as the most common carbapenemase mediating carbapenem resistance.

In *Enterobacter* spp. the study found a high level of resistance to 3rd generation cephalosporins, gentamicin and fluoroquinolones. Genetic techniques identified one *E. cloacae* isolate producing NDM-1 metallo-carbapenemase, CTX-M-3 ESBL and ACT-68 AmpC assigned to MLST type ST23.

Among *P. aeruginosa* isolates, the proportion of carbapenem-resistant ones varied greatly over the years of the study, while for the country, their proportion rose continuously, significantly exceeding the European average. Over 25% of the tested strains were resistant to anti-pseudomonas agents, except for amikacin.

In *Acinetobacter baumannii* – *calcoaceticus* complex, the mechanisms of resistance to carbapenems have been studied using genetic techniques. Multiplex PCR was used to search for the genes for the most common OXA-carbapenemases. Epidemiological typing by *gyrB* PCR was done and 4 clusters were identified.

In a separate section, the studies on the etiology of bacteremia in patients with oncohematological diseases for an 11-year period are distinguished. These are patients with significant neutropenia and an increased risk of developing severe infections. A dominance of Gram-negative bacteria was found - most often *E. coli*, followed by *Enterobacter* spp., *Klebsiella* spp., *P. aeruginosa* and *A. baumannii*. Interesting results were obtained, showing a decrease in the proportion of *S. aureus* and an increase in the proportion of CoNS and *Enterococcus* spp. during the second five-year study period.

Logically, the next section is dedicated to the antibiotic resistance of the most common etiological agents of bacteremia in patients with oncohematological diseases. Here, Prof. Stoeva points out a number of dependencies. She establishes a trend for a stable increase in the share of infections caused by MDR bacteria, which follows global trends. The results obtained in the study show a high relative share of enterobacteria resistant to third generation cephalosporins, with the highest share of *K. pneumoniae*, followed by *E. cloacae* and *E. coli*. When followed over time, the proportion of *E. cloacae* resistant to 3-generation cephalosporins increased, while that of *E. coli* decreased. Prof. Stoeva also proved high levels of resistance of enterobacteria to fluoroquinolones and found a permanent tendency to increase this resistance in *E.*

*cloaceae* complex isolates, most likely due to the wide use of these antibiotics for prophylactic purposes. And to another key group of antibiotics - the carbapenems - Prof. Stoeva found a high and growing resistance in *A. baumannii* - *calcoaceticus* complex isolates. She concludes that these isolates are highly resistant to almost all antibacterial preparations. She also alarms for high multiple resistance in *E. faecium* isolates.

The last, fifth section is devoted to the assessment of 30-day mortality in hospitalized patients with bacteremia caused by the ESKAPEEc group of bacteria. The study demonstrated a high 30-day mortality rate (26%). When evaluating the 30-day mortality in relation to the microorganism/antibiotic combination, Prof. Stoeva found that the highest proportion in the group of deceased were those with blood infections caused by aminoglycoside, quinolone and meropenem-resistant *A. baumannii* - *calcoaceticus* complex, followed by ampicillin-resistant *E. coli* and *K. pneumoniae* resistant to 3rd generation cephalosporins. Regression analysis was performed to establish the relationship between the probability of survival to 30 days and 7 risk factors, and a model was constructed including specific leading risk factors for infection with a particular pathogen, which is an interesting, innovative approach and represents an undoubted contribution.

Summarizing everything written so far, I can confidently state that a large array of valuable data has been obtained and analysed. Significant contributions have been made. Contributions of an original nature are of essential importance, among which are clarification and analysis of the etiological spectrum of bacteremia in a non-selected group of patients and in patients with oncohematological diseases; in-depth studies on the antimicrobial resistance of the most common etiological agents of bacteremia in both groups of patients; regression analysis and modelling of risk factors – studies that I value as significant contributions to public health. I also highly appreciate contributions of a confirmatory and scientific-applied nature.

A list of 18 publications related to the dissertation, 12 of which are in reputable international publications with an impact factor or impact rank is attached.

In conclusion, the dissertation work of Prof. Temenuga Stoeva impresses with its thoroughness, precise implementation and analysis, with the application of modern molecular genetic methods for epidemiological typing and detection of resistance genes.

Very important results were obtained, brilliant analyses were made for a very important and understudied problem as the blood infections.

I believe that the peer-reviewed dissertation fully meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for its Application. Giving my positive assessment, I confidently suggest to the members of the scientific jury to award Prof. Temenuga Zhekova Stoeva the scientific degree "Doctor of Sciences".

Reviewer:



Prof. Iva Christova, MD, Ph.D., DSc.