To the Chairman of the Scientific Jury,
determined by Order No. 276 of 06.10.2025
of the Director of the NCIPD

REVIEW

OF THE ASSOC. PROF. **IVAYLO ALEKSIEV IVANOV**, PhD,

DISSERTATION THESIS

on the topic **«ANTIRETROVIRAL RESISTANCE AND MOLECULAR EPIDEMIOLOGY OF HIV-1 IN BULGARIA: AN INTEGRATED ANALYSIS OF GENETIC DIVERSITY, PHYLODYNAMICS, AND DEMOGRAPHIC CORRELATIONS»**

for awarding the scientific degree "DOCTOR OF SCIENCES" in the
Specialty: Virology, Field of higher education: 4. Natural sciences, mathematics and informatics;
Professional direction: 4.3. Biological sciences

Reviewer: Prof. NELI KORSUN, MD, DSc

Biography

Assoc. Prof. Ivaylo Aleksiev Ivanov is a graduate of the Faculty of Biology at Sofia University "St. Kliment Ohridski," where he obtained his master's degree in 1994. Since 2003, he has been working at the National HIV Reference Laboratory of the National Center of Infectious and Parasitic Diseases (NCIPD). He acquired a specialization in *Clinical Virology* in 2008. From June 2008 to September 2011, he was a PhD student at NCIPD, where, in 2011, he defended his dissertation titled *"Molecular and Virological Characterization of the HIV-1 Epidemic in Bulgaria through Sequencing and Phylogenetic Analysis"* and received the educational and scientific degree *Doctor of Virology*. In 2014, he was elected *Associate Professor*. Since 2016, he has served as Head of the National HIV Reference Laboratory, and since 2024, he has been Head of the Department of Virology at NCIPD. He has completed six international specializations in molecular biological methods and bioinformatics at leading scientific centers in Athens, Greece (2006), Cairo, Egypt (2009), CDC Atlanta, USA (2013), Rome, Italy (2014), Luxembourg (2016), and Gainesville, USA (2016). The specialization at the University of Florida in Gainesville lasted five months and was supported by a Fulbright fellowship for teaching and research.

Relevance of the Dissertation Topic

The HIV/AIDS pandemic remains one of the most devastating global public health crises in recent decades. According to UNAIDS data, since the beginning of the epidemic in 1981, more than 91.4 million people have been infected with HIV, and approximately 44.1 million have died from AIDS-related diseases. In the absence of an effective preventive vaccine, the only available means to inhibit disease progression and reduce viral transmission is combination antiretroviral therapy

(ART). Owing to its widespread use, both morbidity and mortality related to AIDS have markedly decreased, and the life expectancy of infected individuals has substantially increased. Timely administration of antiretroviral agents suppressing HIV replication, together with prophylaxis and treatment of opportunistic infections, allows for immune system restoration, halts disease progression, maintains working capacity, and improves the quality of life of people living with HIV.

The vast scale of the HIV/AIDS pandemic, the remarkable genetic diversity of circulating viral forms worldwide, and the high mutational capacity of HIV—leading to rapid emergence of drug resistance—necessitate comprehensive research on antiretroviral resistance in Bulgaria. This includes the characterization of genetic and epidemiological mechanisms that influence resistance formation at both the individual and population levels. The need for a better understanding of the state of antiretroviral resistance and the molecular epidemiology of HIV infection in Bulgaria determines the indisputable relevance and scientific importance of the dissertation topic.

General Characteristics of the Dissertation

The dissertation is structured according to the requirements of the *Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB)*. It comprises 310 pages and includes: title page; table of contents (2 pages); list of abbreviations (1 page); list of tables (1 page); list of figures (1 page); introduction (3 pages); literature review (87 pages); aim and objectives (2 pages); materials and methods (63 pages); results (92 pages); discussion (28 pages); conclusions (4 pages); contributions (3 pages); bibliography (15 pages, including 332 literature sources and 10 online resources and databases); and appendices (5 pages), including a list of publications related to the dissertation, specializations and training abroad, international collaborations, awards, memberships in scientific organizations, and acknowledgments. The balance among the individual sections meets accepted academic standards. The dissertation is illustrated with 43 tables and 58 figures.

Review and Evaluation of the Dissertation Thesis

In the introduction to his dissertation, Assoc. Prof. Aleksiev outlines the history of HIV discovery, the scale of the global HIV/AIDS pandemic, the classification of HIV-1 into groups, subtypes, circulating and unique recombinant forms, and their global distribution. He identifies the risk of drug resistance development and substantiates the need for integrated analysis of resistance mutations, subtype diversity, phylogenetic relationships, and demographic correlations to optimize therapy and guide national strategies for antiretroviral resistance prevention.

The literature review is comprehensive, well-structured, clearly written, richly illustrated, and engaging to read. It examines the history of human retrovirus discovery, their biology, and detailed classification. The structure of HIV-1, its genome, viral proteins (structural, regulatory, and accessory), and stages of the viral replication cycle are described in depth. An important part of the review is dedicated to antiretroviral drugs—their discovery, mechanisms of action, and the current state of ART. The dissertation focuses on three main classes of antiretroviral agents: NRTIs, NNRTIs, and PIs. The central section of the review deals with antiretroviral resistance—patterns of resistance (transmitted, acquired, and primary), mechanisms of resistance formation, and methods for resistance testing. Common resistance mutations for each of the three drug classes are described in detail, including their mechanisms and impact on drug susceptibility. The influence of HIV-1 subtypes on resistance development is thoroughly discussed.

The section on HIV-1 genetic diversity includes several detailed subsections: the origin and nomenclature of HIV; the classification and nomenclature of HIV-1; the global and regional distribution of genetic variants; their clinical and epidemiological significance; challenges in vaccine development; and evolutionary dynamics and future perspectives. The review concludes with a detailed discussion of subtyping and phylogenetic analysis methods. Four web-based subtyping tools are described (COMET, REGA, jpHMM, and RIP), along with the principles and stages of manual phylogenetic and cluster analysis. This section demonstrates the author's excellent command of modern molecular biology and bioinformatics methods applied in his research.

The review is competently written and demonstrates the author's thorough knowledge of the literature, as well as his ability to systematize, summarize, and interpret existing— including the most recent—scientific information on the topic.

The author's own research begins with Chapter 2, ***Aims and Objectives***. The dissertation aims to conduct a comprehensive analysis of the molecular epidemiology and antiretroviral resistance of HIV-1 in Bulgaria through an integrated approach that combines genotyping, phylogenetic analysis, characterization of resistance mutations, examination of transmission networks, and phylodynamic modeling, to optimize therapeutic strategies and improve public health policies.

Twenty-one specific research tasks are clearly and precisely formulated, grouped into five thematic areas that encompass the main directions of the research process.

The materials and methods used to achieve the study's aim and objectives are described in exceptional detail and clarity. The section begins with a general characterization of the cohort of individuals infected with HIV-1 in Bulgaria between 1986 and the end of 2023, categorized by sex and mode of transmission. The study is based on plasma samples from 1,654 HIV-1–infected patients, in which a 1.2 bp fragment of the *pol* gene was sequenced. Of the analyzed sequences, 970 have been deposited in GenBank, attesting to their high quality.

Two sequencing platforms were employed: the *TRUGENE HIV-1 Genotyping Kit* (Siemens) and the *ViroSeq™ HIV-1 Genotyping System v2.0* (Abbott), with each methodological step described in sequence. Resistance mutations were identified by comparing the obtained sequences with the reference HXB2 sequence of HIV-1. The *Stanford HIVDB* program was used for phenotypic resistance analysis, in which each resistance mutation is assigned penalty scores for individual antiretroviral drugs based on experimental data and clinical studies. The cumulative score for all mutations provides an overall assessment of resistance to a given drug. Within this program, five levels of drug resistance are defined: potential low, low, intermediate, and high.

The dissertation presents a classification of resistance mutations divided into polymorphic, nonpolymorphic (resistance-associated) mutations, and mutations related to phenotypic characteristics, as well as the grouping of mutations by drug class.

Another major group of applied methods includes HIV-1 subtyping techniques. Several web-based tools are described in detail—COMET, REGA, HMM-based jpHMM, RIP, and BLAST Search—along with the manual approaches used for phylogenetic analysis. A significant methodological achievement is the application of Bayesian phylogenetics and molecular clock calibration to date evolutionary events.

The use of the *BEAST*, *ClusterPicker*, and *MicrobeTrace* programs for identifying and visualizing complex networks of connections among different demographic and risk groups represents another modern methodological advancement. Together, these three approaches constitute an integrated framework for studying the molecular epidemiological characteristics of HIV-1 infection in Bulgaria.

A powerful impression in this section is the application of cutting-edge molecular biological methods and bioinformatics tools, which are presented with precision, competence, and methodological excellence.

At the beginning of the ***Results*** section, Assoc. Prof. Aleksiev presents a detailed and in-depth analysis of the studied cohort of patients, categorized by sex, age, country of origin and country of infection, region of residence, and transmission group.

The first major focus of the conducted research is the analysis of resistant mutations. The author presents a summarized overview of the number of samples with proven resistant mutations among various categories of patients, grouped by sex, transmission group, country of origin, probable country of infection, region of residence, pregnancy status, presence of AIDS, engagement in sex work, and incarceration. Statistically significant differences were found in the number of samples with resistant mutations among certain patient categories. A higher prevalence was observed in the groups of men who have sex with men (MSM) and people who inject drugs (PWID). The author concludes that these populations should be prioritized in efforts to prevent and control the spread of resistant HIV strains.

All identified resistance mutations to each of the three classes of antiretroviral agents are presented in detail, including their number (as a percentage) and participation in different mutation combinations. The author analyzes the levels of resistance—potential low, low, intermediate, and high—to individual drugs, assessing the phenotypic impact of the mutations using a scoring system. The frequency of resistant mutations to the various drug classes (major and accessory PIs, NRTIs, and NNRTIs) is presented across different patient categories within the studied population, and all comparisons are supported by statistical analysis.

The second major research direction involves subtyping and phylogenetic analysis of HIV-1 strains identified in 1,654 infected individuals. A high degree of genetic diversity was established, with 26 distinct subtypes, CRFs, and URFs detected, among which subtype B predominates, followed by CRF01_AE and CRF02_AG. The author analyzes the number and proportion of resistant mutations across the individual subtypes, CRFs, and URFs. The distribution of all identified subtypes, CRFs, and URFs is presented by patient categories according to sex, transmission group, place of infection, and region within the country.

Another major focus of the research is the analysis of transmission clusters. Phylogenetic analysis of subtype B identified 127 transmission clusters containing 298 sequences. The author provides a detailed characterization of resistance mutations to each of the three classes of antiretroviral drugs (major and accessory PIs, NRTIs, and NNRTIs) identified within the individual clusters. Similar analyses were carried out for other subtypes (F1, A1, A6, and C) and recombinant forms (CRF01_AE, CRF02_AG, and URF).

A separate section presents an analysis of antiretroviral resistance, subtype diversity, and geographic distribution of HIV-1 among injection drug users (IDUs). To clarify the origin and evolutionary dynamics of the main HIV-1 subtypes among IDUs in Bulgaria, Assoc. Prof. Aleksiev performs Bayesian molecular clock analysis. The constructed phylogenetic trees depict the most recent common ancestor and the geographical spread of CRF01_AE and CRF02_AG. A similar analysis is conducted for subtype B among heterosexual patients.

A detailed analysis of resistance mutations within the subtype B subepidemic is presented. The author first outlines the general characteristics and origin of subtype B infections in Bulgaria, followed by a detailed examination of the spread of 70 identified resistance mutations. Comparable analyses are performed for the CRF01_AE subepidemic and subtype C. The phylogenetic trees illustrate the position of Bulgarian sequences in the global context. Of particular interest are the figures showing the trends in the spread of subtype C and the conclusion that the most recent common ancestor of this subtype dates back to 1954.

At the end of the *Results* section, the author analyzes transmitted drug resistance (TDR) in Bulgaria during the period 2012–2020, based on 1,053 clinical samples from treatment-naïve individuals with HIV-1 infection. Mutations associated with transmitted drug resistance were found in 60 (5.7%) of the infected individuals. The frequencies of all identified resistant mutations are presented, as well as the levels of resistance (potentially low, low, intermediate, and high) for each specific drug. Using the MicrobeTrace program, the author characterizes transmission clusters within the individual subtypes and recombinant forms, as well as the resistance mutations identified in each cluster.

The *Results* section encompasses numerous studies and analyses that together create a multifaceted picture of the HIV-1 epidemic in Bulgaria, focusing on antiretroviral resistance, viral genetic diversity, phylodynamics, and transmission clusters.

The results of the conducted studies are summarized and analyzed in detail in the ***Discussion*** section. Here, the author provides a comprehensive overview of the demographic and epidemiological characteristics of the studied population, summarizing the data with respect to age structure and sex distribution, regional differences within the country, transmission categories and risk behaviors, international connections and migration factors, special populations and vulnerable groups, as well as social determinants and inequalities.

The obtained data on antiretroviral resistance are summarized: general resistance trends, transmitted drug resistance, mutation profiles and clinical significance, subtype-specific resistance, resistance within transmission clusters, and the impact of resistance on therapy. The author interprets the observed levels of resistance and proposes specific diagnostic and therapeutic approaches for the different drug classes.

The exceptionally high genetic diversity of HIV-1 in Bulgaria is analyzed, significantly exceeding that observed in most European countries. The dynamics of the spread of individual subtypes (B, C, F1, A1, and A6) and recombinant forms (CRF01_AE, CRF02_AG, and URF) in Bulgaria are presented in the context of their introduction into the country, epidemiological connections with other regions, affected transmission groups, and identified resistance mutations.

Based on phylodynamic analysis, the author outlines the overall course of the HIV-1 epidemic in Bulgaria: an exponential increase before 1990, a plateau between 1990 and 1996, a brief renewed exponential growth around 2000, followed by stabilization. The analysis of transmission networks and clusters reveals that, in addition to large clusters, numerous smaller ones have been identified, each containing only two to three sequences. The geographic analysis demonstrates an uneven distribution of HIV-1 subtypes across the country, which has important implications for the planning of preventive programs and the allocation of public health resources.

The author formulates valuable recommendations for clinicians, emphasizing the need for baseline resistance testing prior to the initiation of antiretroviral therapy, periodic monitoring of viral load, and timely transition to alternative treatment regimens in cases of therapeutic failure. He highlights the necessity of a personalized therapeutic approach based on viral subtype, knowledge of the transmission group, and treatment history.

He also discusses the significance of genotyping and antiretroviral resistance data for the surveillance system and public health from the perspective of monitoring trends, timely identification of emerging resistant clusters, and planning population-level interventions.

At the end of the *Discussion* section, the author comments on the limitations of his study on the one hand, and on the other, its importance for public health. This approach demonstrates his capacity for critical evaluation, which would enable the overcoming of the identified limitations and the enhancement of societal benefits in future research.

In conclusion, Assoc. Prof. Aleksiev presents 21 **conclusions** grouped into five thematic directions, which are excellently formulated and correspond precisely to the objectives of the study. The author not only derives conclusions from the obtained results but also indicates their significance and offers recommendations for future actions.

I accept the 11 **contributions** of scientific originality and the 8 of applied scientific value presented by Assoc. Prof. Aleksiev. Among the numerous achievements of the author, I would particularly emphasize the introduction of methods for phylodynamic analysis and dating of evolutionary events using a molecular clock - performed for the first time in Bulgaria.

The bibliography contains 342 references, of which 336 are in English and six in Bulgarian. The reference list includes four books and monographs in English and ten online resources and databases.

Evaluation of the abstract

The author's abstract consists of 125 pages and systematically presents the key results of the dissertation.

Publication Activity

In connection with the dissertation topic, Assoc. Prof. Aleksiev has published eight scientific papers—seven in Q1 and one in Q2 journals (total of 195 points under indicator G7)—as well as one monograph (30 points under indicator G5), not used as the main habilitation work. The total impact factor of these publications is 29.971. In six (75%) of them, he is the first author, attesting to his leading role in the research. The eight publications have been cited 54 times in international databases (Web of Science and Scopus), corresponding to 108 points under indicator D11. The citations reflect the significance of his research and the international recognition it has received. His h-index (excluding self-citations) is 12.

According to the submitted documentation, Assoc. Prof. Aleksiev meets and exceeds the minimum national requirements for the acquisition of the scientific degree *Doctor of Sciences* as defined by the *Regulations for the Implementation of the LDASRB* (amended and supplemented, State Gazette No. 15, February 19, 2019): Group A (dissertation – 50 points), Group B (dissertation – 100 points), Group G (225 points, required minimum 100), and Group D (108 points, required minimum 100). His total score is 483 points, well above the required 350.

Compliance with the Minimum National Requirements

The presented documentation and calculated indicators confirm full compliance with the *Regulation for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria* – Section III: Conditions and Procedures for the Acquisition of the Scientific Degree "Doctor of Sciences" (amended and supplemented, State Gazette No. 15 of 19.02.2019), within Area 4 "Natural Sciences, Mathematics and Informatics," Professional Field 4.3 "Biological Sciences," as well as with the internal regulations of the National Center of Infectious and Parasitic Diseases (NCIPD).

Table 1. Number of points by indicators

A group of metrics	**Indicators**	**Number of points according** to the Regulations for the implementation of the LDASRB	**Number of points based on the evidence presented**
A	1. Dissertation work for awarding the educational and scientific degree "Doctor"	50	**50**
Б	2. Dissertation work for awarding the scientific degree "Doctor of Science"	100	**100**

Г	5. Published monograph that is not presented as the main habilitation thesis	30	**30**
	7. Scientific publication in journals that are refereed and indexed in world-renowned databases of scientific information (Web of Science and Scopus), outside of a habilitation thesis	25 for publ. in Q1 20 for publ. in Q2	**175** (7 x 25) **20** (1 x 20)
Д	11. Citations in scientific publications, monographs, collective volumes, and patents, referenced and indexed in world-renowned databases of scientific information (Web of Science and Scopus)	2	**108** (54 x 2)
	Total points		**483**

Table 2. Minimum required points by groups of indicators for awarding the scientific degree "Doctor of Science" and the number of points based on the presented evidence

A group of metrics	**Indicators**	**Required minimum number of points according to the Regulations for the Implementation of the LDASRB**	**Number of points for Assoc. Prof. Aleksiev, based on the presented evidence**
А	Indicator 1	50	50
Б	Indicator 2	100	100
В	Sum of points in indicators 3 and 4	-	-
Г	Sum of points in indicators 5 to 10	100	225
Д	Sum of points in indicator 11	100	108
	Total points	350	**483**

Conclusion

The dissertation of Assoc. Prof. Ivaylo Aleksiev is highly relevant, excellently executed, and methodologically rigorous, presenting findings of significant importance for both individual and public health in relation to HIV infection. In his work, the author provides an extensive and multifaceted analysis of the HIV-1 epidemic in Bulgaria over 35 years, integrating data on antiretroviral resistance and the molecular-genetic characteristics of identified viral strains with the demographic and epidemiological correlations within the studied population. The obtained results represent a substantial and original contribution to the research on HIV infection and its treatment.

The dissertation fully meets the mandatory and specific requirements, as well as the scientometric criteria outlined in the *Law on the Development of the Academic Staff in the Republic of Bulgaria* (amended and supplemented, State Gazette No. 15 of February 19, 2019), its *Regulation for*