ATTENTION:

MEMBERS OF THE SCIENTIFIC JURY /SJ/,

APPOINTED BY ORDER No. 276/06.10.2025,

ISSUED BY THE DIRECTOR OF THE NATIONAL

CENTER OF INFECTIOUS AND PARASITIC DISEASES,

SOFIA

R E V I E W

On the dissertation work of Assoc. Prof. Ivaylo Aleksiev Ivanov, Head of the HIV Confirmation and Research Laboratory and Head of the Virology Department at the National Center for Infectious and Parasitic Diseases on the topic: "Antiretroviral resistance and molecular epidemiology of HIV-1 in Bulgaria: an integrated analysis of genetic diversity, phylodynamics and demographic correlations" for the acquisition of the scientific degree "Doctor of Sciences" in the scientific specialty "Virology" in the field of higher education: 4. "Natural Sciences, Mathematics and Informatics", direction 4.3. "Biological Sciences", for the needs of the Department "Virology" of the National Center of Infectious and Parasitic Diseases

Written by by Prof. Radka Mladenova Argirova, MD, PhD, virologist, Clinical Laboratory, Tokuda University Hospital, Sofia

Respected Members of the Scientific Jury,

Assoc. Prof. Ivaylo Aleksiev graduated from the Faculty of Biology of Sofia University "St. Kliment Ohridski" in 1994. He joined the National Center for HIV Research in 2003 and in 2008 he acquired the specialty "Clinical Virology". During the period 2008-2011 he was a Ph.D. student at the National Center for the Study of Virology, in 2011 he acquired PhD after successfully defending his dissertation. In 2014 he became an Assoc. Prof., since 2016 is head of the HIV Confirmation and Research Laboratory at the National Center for Infectious and Parasitic Diseases. Sofia. Since 2024 he has been a Head of the Department of Virology at the National Center for Infectious and Parasitic Diseases.

From 2006 to 2018, the author participated in 6 specialization courses mainly in the field of sequencing, bioinformatics and similar aspects of HIV molecular biology in a number of global and European centers, among them, the Fulbright Scholarship successfully implemented as a visiting researcher at the University of Florida, USA (5 months), the training in phylogenetics and NGS, CDC, Atlanta, USA (2 months) in 2013, The 2-week training in sequencing and

analysis of resistance HIV mutations at the Luxembourg Institute of Health and 4 short-term trainings in Italy, Greece and Egypt.

Relevance of the dissertation work

The basis of this work lies in the long-term scientific research and diagnostic activity of Ivaylo Aleksiev on the confirmatory diagnosis of HIV infection with proficiency using all modern molecular biological methods, as well as bioinformatics on HIV model, including sequencing and analysis of resistance HIV mutations, emerging during antiretroviral therapy (ART). Moreover. the WHO has not yet canceled the HIV/AIDS pandemic, and even in this sense alone, the dissertation is relevant. By the very nature, in this work, the conclusions from the applied research are important and necessary both at the individual and population level, as they directly show the dynamics of the HIV-1 epidemic in Bulgaria - the spread of the virus - geographically and among individual communities, target groups, and any interactions between them. The data obtained are the basis and oblige for specifically targeted interventions by governmental and non-governmental organizations responsible for public health. On the other hand, molecular virological analyses enrich our knowledge about the evolution of HIV-1 at the local and global levels. With these words, I express my conviction in the relevance of this work.

The dissertation is written on 310 pages, has the generally accepted structure for such work, contains a literature review - 87 pages, results - 92 pages, the bibliography covers 15 pages with 342 literary sources, official documents and databases, 43 tables and 58 figures.

The literature review includes historical and contemporary data on the Retroviridae family and HIV, a detailed history and description of modern ART, also used in our country, data on transmission and acquired drug resistance, genetic diversity of HIV-1, subtyping and phylogenetic analysis with a detailed description of the tools used. The existence of multiple HIV-1 subtypes and the continuous emergence of new viral variants is striking. This diversity, combined with regional differences in the prevalence of specific genetic forms, creates significant challenges for the diagnosis, treatment, and prevention of HIV infection. Of particular importance is the influence of subtype links on the development of drug resistance, which varies significantly between different geographical regions and population groups. The review is very rich, citing the most recent studies and drawing out perspectives for therapy and epidemiology. At the end of the literature review, the author pays special attention to gaps in our knowledge, the lack of data and a comprehensive analysis of the genetic diversity of circulating HIV-1 variants, detailed studies of drug resistance, as well as in-depth phylogenetic studies of the evolutionary dynamics and international connections of the local epidemic. According to the author, "understanding transmission networks and clusters is particularly important, which can outline priorities in preventive interventions and provide guidance for targeted approaches to specific risk groups." The purpose and objectives of the dissertation work logically follow from the literature review.

The goal is clearly formulated, and in its definition, one can see not only the goal of this work, but also the nationally responsible research aimed at optimizing therapeutic strategies and improving policies in the field of public health prevention. **The tasks** are described in detail in 5 sections, reflecting the historical continuity and development in the work of the laboratory. The exceptional complexity of the tasks goes far beyond pure virology, as they affect epidemiology, behavioral sciences, primary prevention, the relationship "viral variants - drug resistance - epidemic spread", and here we can already foresee the contribution of this work.

Materials and methods. The detailed description of the methods used with their advantages, disadvantages and prospects inspire complete confidence in the conclusions and recommendations of the dissertation work. In the work, fragments of the HIV-1 pol gene isolated from blood samples from 1654 individuals (38.9%) out of the total number of 4277 individuals with HIV-1 infection registered between January 1986 and December 2023 were successfully sequenced and subjected to detailed analysis. The inclusion criterion for molecular genetic analysis was the presence of a viral load above 1000 copies/ml. The group of individuals whose samples were sequenced consisted of 1396 (84.4%) men and 258 (15.6%) women. Among them, 762 men (46.1%) were MSM, 670 (40.5%) were cases with heterosexually transmitted infections, those using IDUs were 199 cases (12.0%) and 23 cases were from the mixed category MSM+IDUs with 23 cases (1.4%). A detailed comparative analysis of the two main genotyping systems used - Applied Biosystems ViroSeq™ HIV-1 genotyping system v2.0 and Siemens Visible Genetics TRUGENE™ HIV-1 - reveals the specific characteristics and capabilities of each platform. Comparison between the two systems shows that ViroSeq provides broader coverage of the pol gene, including complete protease sequence and a longer fragment of the reverse transcriptase. The analysis of resistance mutations includes the HIVDB Resistance Assessment System, which takes into account five different levels of drug resistance, described and used by the author. A classification of resistance mutations is shown, as well as their relationship to individual ART classes. Various subtyping techniques, including web-based tools and manual methods for phylogenetic analysis, were used. Web-based tools such as COMET, REGA, HMM-based jpHMM, RIP, and BLAST Search, using different methods and algorithms for phylogenetic and recombination analysis, were used for preliminary evaluation of the studied sequences. Statistical analysis, which is key to uncovering the complex interactions between the genetic aspects of pathogens and their epidemiological manifestations, includes a carefully selected set of epidemiological characteristics to capture a wide range of potential influences and interactions. At the end of this chapter, an analysis of selected publications on the topic of the dissertation is attached - here the author offers the origin and spread of the sub-epidemics of HIV-1 CRF01_AE, subtype B, C, etc. I am not convinced that the analysis from section 3.5 is correctly situated in the "Material and Methods" section, but the significance of the conclusions from it is important because it is a prerequisite for the conclusions in the entire dissertation, and the author is always right. I especially emphasize that this chapter is of extreme interest.

Results. This is the most interesting part of the dissertation. In this review, it is possible to analyze only the most important and significant, not all, results. Based on previous results (initiated, implemented and published by the team led by the candidate), an integrated analysis of the spatiotemporal dynamics and global phylogenetic relationships of the main HIV-1 subtypes in Bulgaria has been carried out for the first time. This analysis combines complex migration patterns, multiple independent introductions of HIV-1 variants from different geographical regions "arriving" in Bulgaria via different routes. Already within the country, an uneven regional distribution is revealed, with a concentration in large urban centers, even with the characteristic of localized epidemic outbreaks, which highlights the importance of this approach for strategic epidemiological planning and control. This logically leads to a molecular-virological analysis of the transmission clusters of the introduced and widespread HIV-1 subtypes in Bulgaria, which is the crown and unifying link of all previous studies. Clusters are groups of viral isolates that show genetic proximity, suggesting that they belong to patients infected most likely through common routes. The main goal of phylogenetic analysis

is to establish the genetic relationship between viral isolates from different patients and to identify the routes of virus transmission within certain risk groups. This task has been successfully completed, and even more – it is the "data from the analysis of selected earlier publications" and used here that allow for a number of comparisons to be made about the course of the epidemic over time – e.g., about the path of entry (introduction) of the virus into the country. The data show that at the beginning of the epidemic, the sources of the virus were mainly within the country, and not imported from abroad - 88.4% of individuals from the first group declared infection in the country. This conclusion contradicts claims from the beginning of the epidemic that HIV was mainly imported to our country from outside. The characteristics of the individual clusters and the spread of the virus within them are comprehensive, informative and confirmatory. The main conclusion from these rich studies is that we must seriously engage in promotional, preventive, informational, and educational contemporary activities in the new generation of MSM and IDU communities. In this sense, the dissertation work has a real contribution to public health. To this I would add the "detective" discovery of active HIV-1 transmission networks with a predominance of MSM-MSM connections and significant inter-population MSM-HET connections, which requires specific targeting of epidemiological surveillance.

Regarding antiretroviral resistance - a particularly interesting conclusion is that a controlled epidemic of transmission resistance has been documented with a frequency of 5.7% in 1053 untreated patients, corresponding to the WHO criteria for successful epidemiological control. This is a very good attestation for both the laboratory and the treatment unit at the Infectious Diseases Hospital. My question here is why this result is described as a "controlled epidemic." Is the search for transmissible resistance continuing in every newly infected HIV-1 carrier and is there data on the dynamics of this resistance over time? Of particular importance is the assessment of the clinical significance of identified mutations associated with drug resistance. This assessment has two meanings: it directly influences the choice (when possible) of antiretroviral regimens, allowing the avoidance of medications with reduced effectiveness. The latter greatly contributes to the implementation of a personalized therapeutic approach based on genotypic analysis, with the aim of increasing therapeutic success and limiting the further development of resistance in clinical practice in people with HIV. Here we should also note the specialized monograph of the candidate titled HIV ANTIRETROVIRAL DRUGS AND RESISTANT MUTATIONS, 2024, which describes in great detail the experience of the author and the HIV Lab. on antiretroviral resistance. It discusses issues such as resistance thresholds and the emergence of multiple mutations in 84 patients treated with NRTIs. The author draws attention to a “striking” finding in the area of subtype-specific resistance—the exceptionally high level of resistance in subtype F1, where 94.5% of all isolates contained at least one resistance mutation. This is a unique phenomenon in the global context of HIV resistance and requires a thorough analysis of the possible mechanisms. The most recent study by the candidate [Alexiev et al., 2025] confirms the high levels of resistance in non-B subtypes in Bulgaria, indicating that different subtypes have different mutational profiles.

The discussion of the results is multifaceted, complex and once again emphasizes the unique diversity of the Bulgarian HIV epidemic, closely related to the geographical crossroads at which the country is located, as well as the serious contribution that the "small" epidemic can be an input to the global spread and evolution of the virus. The discussion, as well as the literature review, reveals a virologist - a molecular biologist and a specialist in bioinformatics with a high professional scientific culture, which the author replenishes daily, but also contributes to

its dissemination through his own experience. The dissertation is unique in its richness, with its analysis and contributions and conclusions, with which I fully agree. Again, I agree with the author and his unusually structured conclusions, fully consistent with the tasks set and the main goal. The work is written in a fascinating way, with a clear effort to use the Bulgarian language, beautifully illustrated and can serve as an example for both research work and the formulation of a dissertation.

Over the years, Ivaylo Aleksiev has developed an unusual and uncommon triad - experimenter, analyst, and organizer - with a clearly outlined following in the goals of his research, specific conclusions of a fundamental scientific nature, leading to subsequent experiments. In Alexiev's intense scientific life and activity, each publication gives rise to a new perspective, his works are complete in nature, but the new challenge lurking behind each of them predetermines his and the laboratory's activities as if they were endless and ever more profound. The promising nature of the research presented in this work is undeniable. And one more thing – his research always shows potential for possible practical application and practical benefit for the individual and for public health.

Ivaylo Aleksiev's **publication activity** is remarkable. For the purposes of acquiring the scientific degree "Doctor of Sciences", Ivaylo Aleksiev applied 8 scientific articles in peer-reviewed international journals with a total impact factor of 29.97 and one monograph, published in 2024. The publications have been published in 7 journals with a quartile of Q1 and one with a quartile of Q2. For the period 2020-2025, 363 citations were found, and for his entire scientific career so far - a total of 590 citations. His metric data meet and exceed the requirements for obtaining the scientific degree "Doctor of Sciences" – 498 points against the required 350 points according to the National Law and the Regulations of the National Center. Separately, 970 Bulgarian sequences isolated from the pol gene of HIV-1 in the HIV Lab have been published in GenBank and are available for the global scientific community.

Assoc. Prof. Ivaylo Alexiev has established lasting scientific international contacts with leading scientific institutes and organizations, which are often partners and co-authors in his research activities - CDC, Atlanta, USA, University of Florida, USA, Campus Bio-Medico University, Rome, Italy, Luxembourg Institute of Health, etc. His research was funded by the National Center of Infectious and Parasitic Diseases, the Ministry of Health of the Republic of Bulgaria (project DN03/2 of 16.12.2016, Scientific Research Fund), as well as under project BG05M2OP001-1.002-0001 under the Operational Program "Science and Education for Smart Growth 2014–2020", co-financed by the European Regional Development Fund. All of this shows complete confidence and reliability on the part of donors regarding the research being conducted and the results obtained.

I would like to separately emphasize the pedagogical abilities of Ivaylo Aleksiev, who has already trained about 10 people in bioinformatics on HIV model, as well as his teaching activities, which are not the subject of this review. He is the organizer of annual HIV Conferences, where scientific and practical experience in diagnostics, treatment, and all innovations in this rapidly developing field of medicine, biology, and evolution are exchanged.

In conclusion, the evaluation of Assoc. Prof. Ivaylo Aleksiev's dissertation presents the candidate as a highly erudite scientific researcher in the field of fundamental virology, molecular epidemiology and bioinformatics in the field of HIV infection in the context of the Bulgarian HIV epidemic, with unique characteristics of this epidemic, published in the world's

most prestigious scientific journals and databases, which is an excellent reason for me to vote categorically "FOR" the acquisition of the scientific degree "Doctor of Science" to Ivaylo Aleksiev - the author of the dissertation. I openly call on the other members of the scientific jury to support my vote and assessment.

Sofia, Nov.11th,2025

Reviewer:

(Prof. DSc Radka Argirova)