



REVIEW

of the dissertation work of LYUBOMIRA SVILENOVA GRIGOROVA

on the topic «**Molecular-virological analysis of the transmission clusters of the introduced and widespread HIV-1 subtypes in Bulgaria**»

for awarding the educational and scientific degree "Doctor" in the scientific specialty Virology in the Field of Higher Education: 4. Natural Sciences, Mathematics and Informatics; Professional Direction: 4.3. Biological Sciences

Scientific supervisor: Assoc. Prof. IVAYLO ALEKSIEV IVANOV, PhD

Reviewer: Prof. Neli Stoyanova Korsun, MD, DSc,
member of the scientific jury, determined by order No 196/30.06.2025 of the Director of the
National Centre of Infectious and Parasitic Diseases (NCIPD)

Biography

Lyubomira Grigorova graduated from high school in 2014 at the "Geo Milev" Mathematical High School - Pleven. From 2014 to 2018, she was a student at the Faculty of Biology at Sofia University "St. Kliment Ohridski", where she obtained a bachelor's degree in "Molecular Biology". At the same university, she received a master's degree in "Food Quality and Safety" in 2020. Since August 2018, she has been working as a biologist at the National HIV Confirmation and Reference Laboratory (NCRL "HIV") of the NCIPD. From October 2020 to September 2023, she was a full-time doctoral student in this laboratory, supervised by Assoc. Prof. Ivaylo Alexiev. In May 2023, she underwent training for one week as a participant in the "Molecular Epidemiology and Cluster Analysis" course at Università Campus Bio-Medico di Roma (UCBM), Rome, Italy. During the COVID-19 pandemic, L. Grigorova has been actively involved in PCR diagnostics and sequencing of SARS-CoV-2 and is a co-author of 8 scientific publications dedicated to this infection. She has a total of 20 scientific publications, all with an impact factor or impact rank. Her H index (in Scopus) is 5.

Relevance of the topic of the dissertation

The HIV/AIDS pandemic has caused a global crisis, which in terms of the number of victims and damage caused to human society is comparable to the consequences of the two World Wars. According to UNAIDS data, from the start of the epidemic in 1981 to 2023, 88.4 million people worldwide were infected with HIV, and 42.3 million died from AIDS-related diseases. As of 2023, 39.9 million people globally were living with HIV. In Bulgaria, at the end of 2024, 4,569 people were infected with HIV.

The enormous scale of the HIV/AIDS pandemic, the wide variety of genetic forms of the virus circulating the world, and the lack of specific prophylaxis require molecular-epidemiological

studies of the transmission routes of the causative virus, the affected population groups, and the relationships between them to limit the spread of the virus. The need for a better understanding of the epidemiology of HIV infection in the country and for developing effective strategies for its prevention and control determines the relevance and significance of the topic of the dissertation.

General characteristics of the dissertation work

The dissertation work is structured according to the requirements of the Regulations for the Implementation of the Law for the development of the academic staff in the Republic of Bulgaria (LDASRB). It has a volume of 203 pages. It contains: title page; table of contents (3 pages); list of abbreviations used (3 pages); list of figures (2 pages); list of tables (1 page); introduction (2 pages); literature review (53 pages); aim and objectives (1 page); materials and methods (53 pages); results (50 pages); discussion (9 pages); conclusions (2 pages); contributions (2 pages); literature (14 pages, including 198 literary sources); acknowledgements (1 page); publications and participations related to the dissertation work (5 pages). The ratio between the individual sections of the dissertation work meets the accepted requirements. The presentation is illustrated by 19 tables and 42 figures.

Review and evaluation of the dissertation work

In the *introduction* to the dissertation work, L. Grigorova describes the history of the discovery of HIV, the scale of the HIV/AIDS pandemic, the genetic diversity of the causative virus, and the routes of its transmission. She justifies the need to conduct cluster analysis to track the spread of HIV and analyze the connections between the different transmission groups.

The *literature review* is detailed, well-structured, and contains up-to-date information on the classification, composition, and structure of retroviruses and, in particular, lentiviruses. The structure of HIV-1, its genome, viral proteins (structural, regulatory, and accessory), and the stages of the viral replicative cycle are presented in great detail. An important part of the review is the section dedicated to the diagnosis of HIV infection and virological monitoring. The various serological (screening - rapid, ELISA, and confirmatory) and molecular diagnostic methods are described. The doctoral candidate demonstrates excellent knowledge of the most modern methods in molecular virology (methods for determining viral load, sequencing, phylogenetic analysis). Data on the origin and evolution of HIV-1/HIV-2 and the nomenclature of HIV-1 are presented, as well as characteristics of the individual groups, subtypes, sub-subtypes, and CRF and their distribution around the world. The relationship between HIV-1 subtype and transmission type is examined, which is directly related to the topic of the dissertation.

The Literature review is competently written and testifies to the excellent literary awareness of the doctoral candidate, her ability to systematize, summarize, and interpret the existing scientific information on the problem. In the review, L. Grigorova follows the approach of citing the earliest research in a given direction, which explains the older literary sources included in this section.

The L.Grigorova's research begins with Chapter 2. ***Aim and objectives***. The dissertation aims to analyze the transmission phylogenetic clusters of the different HIV-1 subtypes introduced and distributed in Bulgaria, through sequencing and phylogenetic analysis of a fragment of the *pol* gene of HIV-1. Five tasks are precisely formulated, which include the main directions in the research process.

The ***Materials and methods*** used to achieve the goal and objectives of the study are described in great detail and clearly. Plasma samples from patients diagnosed in the National HIV Confirmation and Reference Laboratory in the period from 1986 to 2020 are examined. A wide range of serological and molecular biological studies are conducted during the diagnostic process and virological monitoring of patients with HIV. Demographic and epidemiological data on patients are obtained from questionnaires that patients completed during the diagnostic process. In these questionnaires, patients self-identified as belonging to vulnerable groups and risky practices. A total of 1466 sequences with a length of 1.2 kb from a fragment of the *pol* gene of HIV-1, which encodes protease and reverse transcriptase - the main target enzymes for three main classes of antiretroviral drugs: nucleoside and non-nucleoside reverse transcriptase inhibitors and protease inhibitors, are analyzed.

The detailed description of the sequencing method and software programs used makes an excellent impression, which indicates that the doctoral candidate is well acquainted with them and uses them in her research. She consistently follows all stages in the HIV-1 sequencing process using the Viroseq kit. For HIV-1 genotyping, she uses special software (ViroSeq® HIV-1 Genotyping System), which guarantees the precision and reliability of the results obtained. She conducts phylogenetic analysis, subtyping, and identification of recombinant forms of the viral genomes using a wide range of internet-based tools. For tracking transmission clusters, she uses the MicrobeTrace program, which combines genetic and epidemiological data and shows how HIV-1 infection has spread among different vulnerable groups of the population. The doctoral candidate not only consistently describes each step of the protocol of the methods used, but also explains the meaning of the given action. The unusually large volume of this section of the dissertation is also explained by the wide range of bioinformatics programs used, a competent description of which can serve as a valuable guide for other researchers in this field.

The ***"Results"*** section includes 3 major research areas. The first group of studies covers 663 patients diagnosed with HIV-1 subtype B during the period 1986-2018. The patients are distributed by gender, age, country of origin and country of infection, region of residence, and transmission group. Among those infected with HIV-1 subtype B, L. Grigorova found a predominance of the group of men who have sex with men (MSM), followed by the group of heterosexual persons (HET). The doctoral candidate presents important information about the spread of HIV-1 subtype B in Bulgaria over the years. The MicrobeTrace program used presents a clear visualization of transmission clusters by connecting nodes with lines, with each node representing a separate patient sequence. In total, 52 transmission clusters are identified, of which 24 clusters are composed of 3 or more sequences, 28 clusters include pairs of sequences, and 233 single sequences. The doctoral candidate presents a detailed description of the identified clusters,

indicating which transmission groups are included in the given cluster, the direction of transmission of the infection between them, the country of origin, and the site of infection in the infected. In the 3 large clusters, containing 56, 59, and 91 sequences, respectively, the main transmission group is MSM.

Similar studies are performed on the second group of patients, 270 in number, infected with the circulating recombinant form CRF01_AE of HIV-1 during the period 1995-2019. This group is dominated by intravenous drug users (IDUs), followed by HETs. A large cluster of 154 sequences is identified, mainly including the IDU group, as well as other smaller clusters with 7, 5, and 2 sequences, and 98 single sequences.

The third group of studies includes 1053 naive patients diagnosed during the period 2012-2020. Patients without previous treatment with antiretroviral drugs are defined as naive. L. Grigorova presents in detail the demographic and epidemiological data of the infected patients and analyzes them by gender, age, region in the country, country of infection, and transmission group. MSM again predominates in this group, followed by HET. The subtyping of the sequences reveals a diverse range of genetic groups, predominantly subtype B, followed by CRF01_AE, F1, and CRF02_AG. L. Grigorova analyzes the distribution of transmission categories in the individual HIV-1 subtypes and recombinant forms. She observes changes in the ratio between transmission groups by year (from 2012 to 2020). The doctoral candidate performs an extremely complex phylogenetic analysis, including 1053 sequences. For greater clarity, she also presents 2 separate phylogenetic trees, including sequences of HIV-1 subtype B (595 sequences) and HIV-1 non-B subtypes (458 sequences). Using the Cluster Picker program, she identifies multiple clusters within the individual subtypes and recombinant forms. She presents characteristics of the patients included in the individual clusters, by gender, age, region in the country, and country of infection, transmission group, presence of concomitant sexually transmitted infections, etc.

The results of the conducted studies are summarized and analyzed in detail in the "**Discussion**" section. In this section, the doctoral candidate presents an overview of the demographic and epidemiological characteristics of the studied patients, summarizing the data obtained in terms of age, gender, region of the country, transmission category, and subtype of the viruses included in the individual clusters. Particular attention is paid to the possible routes of spread of the virus between the individual vulnerable populations. The importance of the phylogenetic and transmission analysis of HIV-1 in Bulgaria for understanding the dynamics and complexity of the epidemic process and for developing preventive measures aimed at risk groups and the routes of transmission of the virus is emphasized.

At the end of the dissertation, 6 **conclusions** are presented, which are well formulated, correspond to the tasks set, and reflect the results of the studies.

I accept the 8 **contributions** presented by the doctoral candidate: 6 of an original nature and 2 of an applied nature.

The *bibliography* contains 198 literary sources, of which 2 are in Bulgarian, the rest in English. A total of 30 (15%) sources are from the last 10 years (2014-2024).

Evaluation of the abstract

The abstract is 82 pages long. Its content presents the main results of the dissertation systematically.

Publication activity

In connection with the topic of the dissertation, L. Grigorova has published 2 scientific articles in journals with Q1 and 1 article in a journal without SJR. Thus, she collects 50 points and significantly exceeds the required 30 points in the group of indicators D of the *Minimum National Requirements specified in the Regulations for the Implementation of the LDASRB - Section II. Conditions and Procedure for Acquiring the Educational and Scientific Degree "Doctor"* (amended and supplemented, State Gazette, issue 15 of 19.02.2019; Field of higher education 4. Natural Sciences, Mathematics and Informatics; Professional Direction 4.3. Biological Sciences). She has participated in 2 scientific forums held abroad and in 18 national scientific events.

CONCLUSION

The dissertation work of Lyubomira Grigorova is up-to-date, excellently executed at a high methodological level, and written in very good scientific language. In her study, she offers a comprehensive overview of the HIV epidemic in the country over a prolonged period, integrating the molecular and genetic characteristics of the detected viruses with the demographic and epidemiological data of the infected individuals. The doctoral candidate and her scientific supervisor effectively utilize modern molecular-genetic and bioinformatics tools to characterize HIV, the causative agent of this significant infection affecting individual and public health. The dissertation work meets the mandatory and specific conditions and scientometric criteria in the LDASRB (amended and supplemented by State Gazette No. 15 of February 19, 2019) and the Regulations for its implementation. I am pleased to provide a positive assessment and recommend to the esteemed scientific jury that Lyubomira Svilenova Grigorova be awarded the educational and scientific degree of "Doctor" in the field of higher education 4. Natural Sciences, Mathematics, and Informatics, Professional Direction 4.3. Biological Sciences, Scientific Specialty: Virology.

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/Prof. Neli Korsun, MD, DSc/