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PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 49, NUMBER 3/2021

ISSN 0204-9155

1504 Sofia; 26, Yanko Sakazov Blvd.
Tel.: +359 2/ 846 83 07, Fax: +359 2/ 943 30 75
e-mail: pipd@ncipd.org

PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 49, NUMBER 3/2021

Preprinting and Printing by Boris Zl. Pavlov & Alliance print

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SARS-COV-2: INSIGHT INTO THE EMERGING GENETIC VARIANTS

Neli Korsun[1], Ivelina Trifonova[1], Veselin Dobrinov[1], Iliyana Grigorova[1], Svetla Angelova[1], Ivaylo Alexiev[2]

[1]National Laboratory "Influenza and ARD", [2]NCL "HIV", Department of Virology, National Center of Infectious and Parasitic Diseases, Sofia

ABSTRACT:

SARS-CoV-2 is a highly contagious virus, which appeared in China in late 2019, spread rapidly and caused the largest pandemic in the last 100 years. Despite the intensive research, there is no specific antiviral drug currently. Effective vaccines have been developed in a short time and they are already widely used. As a RNA virus, SARS-CoV-2 mutates constantly, and several thousand genetic variants have emerged in the course of the pandemic, some of which are associated with increased infectivity, reinfection risk, reduced activity of therapeutic antibodies and reduced effectiveness of vaccines. This review highlights the features in SARS-CoV-2 structure and replication cycle that would help to understand the significance of individual mutations contained in the emerging genetic variants and to predict the impact of mutations on viral transmissibility, disease severity, diagnostics, therapeutics or immune escape. The main characteristics of the variants of concern are presented.

Keywords: *SARS-CoV-2, mutation, genetic variant*

Category of the manuscript: mini review

INTRODUCTION

At the end of December 2019, the World health Organization (WHO) reported cases of severe pneumonia caused by an unknown agent in Wuhan, Hubei Province, China (1). The causative agent was identified as a new, hitherto unknown coronavirus, which received the name SARS-CoV-2 from the International Committee on Taxonomy of Viruses, and the disease it caused was named COVID-19. In a short time, SARS-CoV-2 spread globally, causing an unprecedented health and economic crisis. On January 30th, 2020, the WHO declared the SARS-CoV-2 epidemic a public health emergency, and a global pandemic was declared on March 11th. Since December 31 of 2019 and as of week 22/2021, 174 032 728 confirmed cases of COVID-19 have been reported worldwide, including 3 738 030 deaths (https://who.int). The long-term circulation of SARS-CoV-2, affecting millions of individuals, has been accompanied by appearance of several thousand mutations in the viral genome, most of which do not affect the biological properties and behavior of virus. Mutations that are associated with increased virus infectivity; increased risk of severe disease, hospitalization, and death; increased frequency of reinfection; failure of some diagnostic tests or reduced efficacy of therapeutic agents and vaccines are of concern. Detailed understanding of the structure, key genomic elements, and replication cycle of the virus is needed to assess the significance of the particular mutations identified in circulating viruses and to predict changes in the behavior of viruses as a result of these mutations.

ADDRESS FOR CORRESPONDENCE:
Neli Korsun,
44A Stoletov Blvd, 1233 Sofia;
e-mail: neli_korsun@abv.bg;
mobile: 0885413620

Classification and origin of SARS-CoV-2

SARS-CoV-2 belongs to the subfamily *Coronavirinae*, family *Coronaviridae* of the order Nidovirales. This subfamily includes four genera: α, β, γ and δ. Among them, α and β coronaviruses (CoVs) only infect mammals, while γ and δ mainly infect birds (2). To this day, seven types of CoVs able to infect humans are identified: four endemic, low pathogenic CoVs (229E, NL63, OC43, and HKU1) causing 15-30% of common colds, and three highly pathogenic CoVs: Middle East Respiratory

Syndrome Coronavirus (MERS-CoV), Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) capable to cause severe respiratory illnesses such as pneumonia and acute respiratory distress syndrome (ARDS), leading to death (3). The CoVs 229E and NL63 belong to genus α coronavirus and the remaining are members of genus β coronavirus. SARS-CoV and SARS-CoV-2 belong to the subgenus: Sarbecovirus. The three highly pathogenic CoVs originate from bat CoVs. Palm civets and racoon dogs have been recognized as intermediate hosts for SARS-CoV and dromedary camels for MERS-CoV. Pangolin was found to be a potential intermediate host for SARS-CoV-2. During the 2002-2003 epidemic, SARS-CoV caused 8 096 disease cases in 26 countries including 774 deaths (mortality rate of 9.6%) then disappeared from circulation. MERS-CoV emerged in the Arabian Peninsula in September 2012, causing 2566 confirmed cases of infection in 27 countries and 882 deaths (mortality rate of 34%). SARS-CoV-2 is responsible for the current pandemic that caused enormous human casualties, serious consequences in many areas of human society and changed the world, as we know it.

Structure of SARS-CoV-2 and key viral factors

SARS-CoV-2 is an enveloped virus with nonsegmented, single-stranded positive-sense RNA genome, whose structure resembles that of other CoVs. Among known RNA viruses, CoVs have one of the largest genomes, ranging between 26-32 kilobases (kb) in size. The coronaviral virions are spherical or moderately pleomorphic particles with a diameter of approximately 60 to 140 nm with crown-shaped 9-12 nm-long spikes on their surface formed by the S glycoprotein. The envelope is a lipid bilayer derived from the host cell membrane in which virus proteins S, M and E are embedded. The membrane (M) protein, the most abundant structural protein, is responsible for shaping the virions. It plays an important role in viral assembly along with E and N proteins. The envelope (E) protein, the smallest structural protein, is present in small quantities in the virion. The fourth structural protein N binds the viral RNA forming a helical nucleocapsid, located in the core of the viral particle. It is required for packaging viral RNA into the viral particle during viral assembly and acts as an interferon (IFN) inhibitor.

The genome of SARS-CoV-2 is approximately 29.9 kb of size and comprises of a 5'-cap structure and a 3'-poly-A tail. It includes 5' and 3'-untranslating regions (UTR) consisting of 265 and 229 nt, respectively, and 15 open reading frames (ORFs) encoding at least 29 proteins. The first ORFs (ORF1a/b), located at the 5'end of the genome, cover about two-thirds of the entire genome length and encode 16 nonstructural proteins (nsp1-16), involved in viral replication and transcription. Remaining ORFs encode the four major structural proteins: S, M, E, and N and nine accessory proteins (3a, 3b, 6, 7a, 7b, 8b, 9a, 9b, and ORF14) participating in the assembly of viral particles (2,4,5).

The SARS-CoV-2 S protein plays a crucial role in the initial steps of viral infection - it mediates receptor recognition, cell attachment, and entry into host cells. It is the major viral antigen used as a key target for vaccines and therapeutic antibodies, therefore its structure and functions have been intensively studied (6). It exists as a homotrimer, each monomer of which is about 180 kDa and contains 1273 amino acids. The spike protein is comprised of three segments: large ectodomain, transmembrane anchor and a short intracellular tail. It is a heavily glycosylated protein with 22 host derived N-linked glycans, which serve as a shield reducing access of antibodies to specific epitopes. In the native state, the S protein exists as an inactive precursor consisting of two subunits: a distal S1 subunit (14–685 amino acid residues), which is responsible for receptor recognition and binding, and a proximal S2 subunit (a.a. 686–1273), which mediates membrane fusion and entry of virus

into host cell (7). The S protein contains several functional regions (domains): a signal peptide (SP) (a.a. 1–13) located at the N-terminus, an N-terminal domain (NTD) (a.a. 14–305) and a receptor-binding domain (RBD) (a.a. 319–541) in the S1 subunit; a fusion peptide (FP) (a.a. 788–806), heptapeptide repeat sequence 1 (HR1) (a.a. 912–984), HR2 (a.a. 1163–1213), transmembrane domain (TM) (a.a. 1213–1237), and cytoplasm tail (CT) (a.a. 1237–1273) in the S2 subunit (Figure 1) (2,8). The S protein binds to the receptors of sensitive cells via RBD, which is a key target for the most potent neutralizing antibodies (nAbs) of host. Due to its surface location, NTD is the least conserved region and is another potential target for nAbs. FP consists of 15-20 conserved amino acids. HR1 and HR2 are composed of a repetitive heptapeptide HPPHCPC. They form a six-helical bundle (6-HB), which plays an assential role in viral fusion and entry [7,9]. Unlike SARS-CoV and MERS-CoV, SARS-CoV-2 has a unique polybasic "RRAR" (Arg-Arg-Ala-Arg) cleavage site at the junction of S1 and S2 subunits (a.a. 682–685), which enables effective cleavage by furin and furin-like proteases (10). The presence of this furin recognition region enhances cellular tropism and transmissibility of SARS-CoV-2 due to the broad cellular expression of furin proteases (6). Such furin cleavage site is also present in highly pathogenic avian influenza viruses and is associated with their pathogenicity.

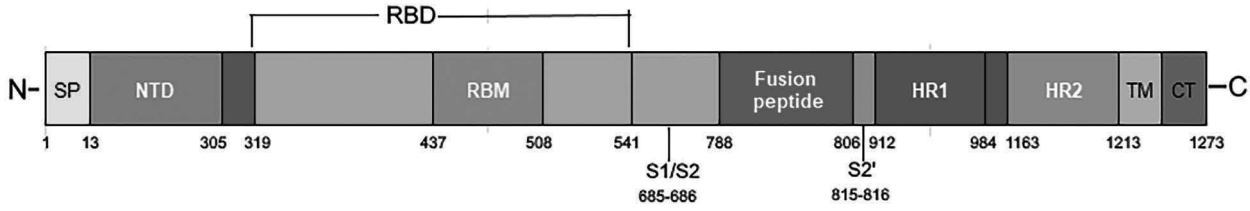

Figure 1. Schematic of SARS-CoV-2 S protein

SARS-CoV-2 replication

The first step in SARS-CoV-2 infection is binding of the S protein to the cell surface receptor, mediated by the RBD. SARS-CoV and SARS-CoV-2 use the angiotensin-converting enzyme 2 (ACE-2) as a cellular receptor, which is distributed in the lung, intestine, heart, and kidney. MERS-CoV recognizes the dipeptidyl peptidase 4 receptor. The RBDs of S1 subunits undergo hinge-like movements between two states ("up" or "down"), in which the residues that bind ACE2 receptor are transiently exposed or hidden. Based on the hinge-like movements of RBDs, S protein monomers are displayed in "open" or "closed" orientations: "open", receptor-accessible conformation (RBD up) for receptor binding and "closed", receptor-inaccessible conformation, (RBD down) for immune evasion (11). Within the RBD, there is a receptor binding motif (RBM) (a.a. 437-508), which makes direct contacts with the peptidase domain of ACE2. 17 amino acid residues in RBM are in contact with 20 amino acids in ACE2, six of these 17 residues - L455, F486, Q493, S494, N501 and Y505, are crucial for efficient binding to the ACE-2 receptor (4). RBD of SARS-CoV-2 recognizes and binds to the ACE2 with more than 10-fold higher affinity than the RBD of SARS-CoV (6). This may explain the higher contagiousness and transmissibility of SARS-CoV-2 as compared to SARS-CoV. Binding of RBD with the ACE-2 receptor is followed by a proteolytic cleavage of the S protein in two consecutive steps: in the S1/S2 junction (a.a. 685-686) ("priming" cleavage) and in the so-called S2' site located immediately after the FP (a.a. 815-816) ("activation" cleavage) (5,6). The first cleavage results in the separation of RBD from FP; the other cleavage leads to the exposure of FP. SARS-CoV-2 S protein is cleaved

by human cell proteases such as furin, trypsin, cathepsins and transmembrane protease serine 2 (TMPRSS2) (7). After the cleavages, the S1 subunit is dissociated and the S2 subunit undergoes dramatic conformational changes, in which the FP is exposed and triggers the fusion of the viral membrane with the host cell membrane. SARS-CoV-2 enters susceptible cells through endocytosis, in which fusion of the viral and endosome membranes leads to the release of the viral nucleocapsid into the cell cytoplasm. After entering the cellular cytosol, 5'-proximal ORF1a and ORF1b of the genomic RNA are translated to produce two large polyproteins (pp1a and pp1ab), where pp1ab is produced via a ribosomal frameshift mechanism. These polyproteins are processed by virally encoded proteases into 16 non-structural proteins, which form a replication-transcription complex in double-membrane vesicles (4,5). During the replication, full-length (-) RNA copies of the genome are produced, which are used as templates for full-length (+) RNA genomes. A characteristic feature of the CoV family is the synthesis of multiple negative-sense subgenomic RNA intermediates, which serve as templates for the production of subgenomic mRNAs. They are subsequently translated to produce virus-specific structural and accessory proteins (12). The subgenomic mRNAs share the same leader sequence of 70–90 nucleotides at their 5' ends and the same 3' ends. Subgenomic N mRNA is the most abundantly generated transcript in SARS-CoV-2 infected cells, which makes the N gene one of the most suitable targets for detection of SARS-CoV-2. Following translation, structural M, E and S proteins migrate along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). N proteins bind to the daughter's genomic RNAs and form helical nucleocapsids that interact with the other structural proteins to form mature viral particles. Following assembly and budding into the lumen of the ERGIC, virions are realesed from the infected cells through exocytosis.

Genetic variants of SARS-CoV-2

As a RNA virus, SARS-CoV-2 undergoes frequent mutations, in spite of some proof-reading capacity of its RNA polymerase complex (nsp14 protein acts as 3'-5'exoribonuclease) (3). Mutations in the circulating viruses are evaluated compared to the reference strain Wuhan-Hu-1 (GenBank accession MN908947), which comprises a 29,903-bp–long RNA and is the first virus with sequenced complete genome. Due to the important role of S protein in the early phase of coronaviral infection and the fact that it is the major target of most vaccines and therapeutic agents, mutations in this protein are of particular concern. *A group of SARS-CoV-2 that share the same inherited set of distinctive mutations compared to the reference strain but do not show significant changes in their phenotypic characteristics (such as antigenic properties, virulence, features in the epidemiology of infections) is defined as a genetic variant.* Over the course of the pandemic, several thousands of SARS-CoV-2 variants have arisen. The variants are grouped into larger groups called clades. Several nomenclatures of the clades have been proposed. Rambaut et al. identify five major lineages: A, B, B.1, B.1.1, and B.1.177 (https://cov-lineages.org). The Global Initiative on Sharing All Influenza Data (GISAID) distinguishes 7 clades: O, S, L, V, G, GH, and GR (www.gisaid.org). Nextstrain identifies five clades - 19A, 19B, 20A, 20B, and 20C (https://nextstrain.org/ncov). Genetic variants, which are associated with one or more of the following changes: increase in transmissibility or detrimental change in COVID epidemiology; and/or increase in virulence or change in clinical disease presentation; and/or decrease in effectiveness of available diagnostics, vaccines and therapeutics, have been accepted by the WHO as *variants of concern* (VOCs). VOCs include multiple mutations in S protein and at least one mutation in the RBD. The following

variants have been designated as VOCs: Alpha (B.1.1.7, GRY or 20I/S:501Y.V1); Beta (B.1.351, GH/501Y.V2 or 20H/S:501Y.V2); Gamma (P1, GR/501Y.V3 or 20J/S:501Y.V3) and Delta (B.1.617.2, G/452R.V3 or 21A/S:478K) (https://who.int).

B.1.1.7 variant was earliest documented in September 2020 in the UK. After detection of the first cases, it surged at an exponential rate and quickly spread in other countries. This variant harbors 23 mutations leading to 17 amino acid changes among which 8 are located in the S protein: deletion 69-70, deletion 144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H. The role of the following mutations has been established:

— The N501Y is one of the six key amino acid residues in the RBM determining the tight interaction of SARS-CoV-2 with the ACE2 receptor. This mutation is associated with increased infectivity and is also presented in the B.1.351 and P.1 variants.
— The P681H is located immediately adjacent to the furin cleavage site (a.a. 682–685). It has been speculated that this mutation increases transmissibility by facilitating cleavage of the S precursor.
— The H69–V70 and Y144/145 deletions are located in the NTD, which contains important antigenic sites, targets of many neutralizing antibodies. Mutations in this region increase the ability of the virus to escape immune recognition. The H69–V70 deletion is responsible for a negative result in some RT-PCR assays targeting the S-gene (S-gene drop-out) (13).
— The D614G mutation emerged in eastern China early in the pandemic and then quickly spread around the world, displacing other CoVs that did not have this mutation. The D614G mutation makes the SARS-CoV-2 more infectious, but it does not associate with increased disease severity or escape from host immunity. It increases the ability of the virus to bind to the ACE2 receptor and stabilizes the interaction between the S1 and S2 subunits of the spike, leading to increased transmissibility (14).

The B.1.1.7 variant is ~50% more infectious than other variants in circulation (15). Based on studies in the UK, it is associated with increased risk of hospitalizations and increased case fatality rate (16). There is evidence of minimal impact on the neutralization activity of convalescent and post-vaccination sera (17).

B.1.351 variant was initially detected in in South Africa in September 2020, from where it spread to 94 countries. It contains 23 mutations leading to 17 amino acid changes, including 9 changes in the S protein: L18F, D80A, D215G, 242–244 del, R246I, K417N, E484K, N501Y, D614G, and A701V. Mutations near the tip of the S protein include:

— N501Y, which helps the virus to bind more tightly to the ACE2 receptor.
— K417N, which is located in the RBD on the tip of the spike and helps the virus interact more tightly with human cells.
— E484K, which is located in the RBM and is potentially associated with antigenic change and immune escape. This mutation creates resistance to neutralizing antibodies contained in convalescent plasma and reduces the activity of some neutralizing monoclonal antibodies. It also increases the affinity of the S protein for the ACE2 receptor.
— Combination of E484K, K417N, and N501Y mutations leads to the most significant changes in the structure of the RBD allowing the virus escape antibody neutralization (18).
— L18F, D80A, D215G, 242-244 del, and R246I are located in the NTD, which is a preferential target of antibodies (14).

P1 variant was first detected in Japan in four travelers who arrived in Tokyo from Amazonas, Brazil. This lineage emerged in November 2020 in Manaus, the largest city in Brazil's Amazon region, where it has become the dominant circulating virus. As of June 4, 2021 it has been detected in 53 countries. P.1 variant contains approximately 35 mutations leading to 17 amino

acid changes including 11 changes in S protein: L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, H655Y, T1027I, and V1176F. Some of these mutations: L18F, K417T, E484K, N501Y, and D614G, are located in the same positions of S protein as in the B.1.351 variant.

Both variants, B.1.351 and P1 are associated with increased infectivity, significantly reduced susceptibility to some monoclonal antibodies and reduced neutralization ability of antibodies generated by a previous natural infection or vaccination (18,19). With regard to P1, there is an evidence of an increased risk of reinfections. At the end of 2020, this variant has widespread in Manaus, Brazil, where more than 75% of residents have been infected with SARS-CoV-2 earlier (20).

B.1.617.2 variant first appeared in India in October 2020 and is one of the variants responsible for the high morbidity of COVID-19 in this country. It carries the following mutations in S protein: T19R, 156del, 157del, R158G, L452R, T478K, D614G, P681R, and D950N (21).

- The L452R is also found in the B.1.427/429 variants that are widespread in California. This change in the RBD increases the affinity of the spike protein for the ACE-2 receptor and decreases recognition by antibodies that present in covalescent plasma as well as by some therapeutic monoclonal antibodies.
- The P681R is similar to P681H and is located immediately adjacent to furin-cleavage site.
- T19R, 156del, 157del and R158G are located in the NTD that is extensively mutated and is a target of many neutralizing antibodies.

Variants of interest (VOIs) represent groups of genetically changed viruses in which genetic changes are associated with established or suspected phenotypic implications; and they have been identified to cause multiple SARS-CoV-2 cases or they have been detected in multiple countries (https://who.int). Such VOIs are Eta/Iota (B.1.525/B.1.526) - spread in Nigeria and New York; Epsilon (B.1.427/429) - in California; Kappa (B.1.617.1/B.1.617.3) - in India, and others.

DIAGNOSTIC TESTING

For diagnosing current or recent SARS-CoV-2 infection, viral tests, including nucleic acid amplification tests (NAATs) and antigen tests, are used. NAATs detect one or more viral genes and are characterized by high sensitivity and specificity. Antigen tests have high specificity but are less sensitive than NAATs. To confirm infection with a specific genetic variant, whole genome sequencing (WGS) or sequencing of selected parts of the viral genome should be performed. WGS allows the identification of mutations in various viral genes and the detection of VOCs. Full or partial S-gene sequencing is a cheaper and faster method than WGS. To distinguish the circulating variants, sequenced region must cover at least the entire N-terminal and RBD (amino acid 1-541, 1623 bp), at best the entire S gene. For early detection of known VOCs, diagnostic screening PCR-based assays have been developed. A negative or significantly weaker positive S-gene result with positive results for the other gene targets can be used as an indicator of potential circulation of B.1.1.7 (Alpha) variant. The S-gene target failure is not exclusive to B.1.1.7 - it can be observed in other non-VOC-variants but does not occur for Beta, Gamma and Delta variants. Specific RT-PCR assays identifying VOC specific amino acid substitutions (e.g. spike N501Y, K417N, E484K, L452R) have been developed. Appropriate positive controls should be used.

CONCLUSIONS

Novel genetic variants of SARS-CoV-2 will emerge in the future as a result of the adaptation of the virus to the human population with selection of mutations that improve viral replication and transmissibility or permit the virus to escape from adaptive immune responses. As the proportion of the vaccinated and previously infected individuals worldwide increases, the evolution

of SARS-CoV-2 will be increasingly driven by the immune pressure of the human population. The course of the pandemic will depend on the effectiveness of the vaccines against emerging genetic variants, on the strength and duration of the immunity they create.

ACKNOWLEDGMENTS

The study was supported by grants from the Ministry of Education and Science, Bulgaria: contracts КП-06-Н43/5 /30.11.2020/ and КП-06-Н43/1 /27.11.2020/; by the European Regional Development Fund through Operational Program Science and Education for Smart Growth 2014–2020, Grant BG05M2OP001-1.002-0001-C04 "Fundamental Translational and Clinical Investigations on Infections and Immunity".

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DRUG RESISTANCE MUTATIONS AND TRANSMISSION CLUSTERS OF THE HIV-1 CRF01_AE SUB-EPIDEMIC IN BULGARIA

Lyubomira Grigorova[1*], Reneta Dimitrova[1], Aleksandra Partsuneva[1], Anna Gancheva[1], Asya Kostadinova[1], Ivaylo Elenkov[2], Nina Yancheva[2], Mariyana Stoycheva[3], Tsetsa Doychinova[4], Liliya Pekova[5], Minas Kosmidis[6] and Ivailo Alexiev[1]

[1] National Reference Confirmatory Laboratory of HIV, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.
[2] Specialized Hospital for Active Treatment of Infectious & Parasitic Diseases, Sofia, Bulgaria.
[3] Department of Infectious Diseases, Medical University, Plovdiv, Bulgaria.
[4] Department of Infectious Diseases, Epidemiology, Parasitology and Tropical Medicine Medical University – Pleven, Bulgaria.
[5] Clinic of Infectious diseases, University Hospital, Stara Zagora, Bulgaria.
[6] HIV/AIDS Sector Varna, St. Marina Hospital, Varna, Bulgaria.

ABSTRACT

Background. In Bulgaria the second most predominant HIV-1 strain after subtype B is CRF01_AE.

Material and Methods. 270 HIV-1 polymerase (pol) CRF01_AE sequences collected between 1995–2019 were analyzed with the phylogenetic approach. To identify transmission clusters, we used two different genetic distance thresholds (d), i. e. 1.5% and 0.5%.

Results. Genetic distance d=1.5% defined more distant infections in a huge cluster of 154 sequences composed mostly of people, who inject drugs (PWID), whereas when (d) was reduced to 0.5%, we determined more recent transmissions and the large cluster disintegrated into smaller ones. Different drug resistance mutations (DRM) were detected in patient's HIV-1 pol sequences and were most common in male heterosexual (HET) single sequences.

Conclusions. Our data showed repeated introduction of CRF01_AE in Bulgaria and rapid spread of the infection among PWID groups. Molecular monitoring of the epidemic among PWID communities could help reduce the spread of HIV-1 infection.

Keywords: *HIV-1, CRF01_AE, drug resistance, transmission clusters*

ADDRESS FOR CORRESPONDENCE:
Lyubomira Grigorova,
44A Gen. Stoletov Blvd., Sofia 1233,
lyubomiragrigorova@gmail.com,
tel.: +359 2 9318071

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by two lentiviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (1). HIV-1 is more prevalent in the world. The strains of HIV-1 can be classified into four groups: M (major), N (new), O (outlier) and P (2). Group "M" is the main group that is responsible for the global HIV-1 pandemic and contains at least 10 different subtypes (A-F, G, H, J, K and L), multiple circulating recombinant forms (CRFs) and various unique recombinant forms (URFs). HIV-1 subtype CRF01_AE was first isolated in Thailand and now predominates in South Asia and has spread throughout the world (3). The global prevalence of CRF01_AE is 5%, and it is responsible for up to 4% of the HIV-1 infections in Europe according to the SPREAD study (4).

In previous studies, CRF01_AE was found to be one of the most disseminated clades in Bulgaria with around 20%, which is likely the highest percentage across Europe (5, 6, 7). Within the country, CRF01_AE is unevenly distributed and mainly affects the vulnerable population of PWID in the region of the capital of Bulgaria, Sofia, with 35% of all HIV-1 infections (8). With this study, we aim to determine the dynamics of distribution and transmission of CRF01_AE in Bulgaria. We intend to use epidemiological data and to link it

to the data obtained by sequencing the samples. Thus, we aim to clarify the events that have led to the transmission and production of resistance mutations. In addition, we will analyze HIV-resistant mutations in this strain. We hope that our study results will help to develop better strategies for public health prevention in Bulgaria.

MATERIALS AND METHODS

- **Patient samples, research ethics and consent**

Plasma samples were collected in the National Reference Confirmatory Laboratory of HIV (NRCL of HIV) of the National Center of Infectious and Parasitic Diseases (NCIPD) in the period 1995-2019. Demographic and epidemiological information was collected during the diagnostic process in accordance with national regulations. Through anonymous codes, blood samples, demographic and clinical information was linked following the ethical standards of Bulgaria (6). The Ethics Committee of NCIPD (NCIPD IRB 00006384) approved the study.

- **Sequence generation and analysis**

Viral RNA extraction was performed following the protocol of Abbott ViroSeqTM HIV-1 Genotyping Test v2.0 from serum or plasma. A fragment of the HIV-1 pol gene was generated using a ViroSeq HIV-1 genotyping test using an Applied Biosystems 3130xl genetic analyzer (8). HIV-1 subtypes were determined using Internet-based tools REGA HIV-1 subtyping tool version 3.0 (9) and COMET v2.3 (10). We used the Genotypic Resistance Interpretation Algorithm of the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/hivdb/by-sequences/) to determine HIV-1 resistance mutations (DRMs) and their type. By using MUSCLE algorithm implemented in AliView version 1.23 (11, 12) and MAFFT version 7 (13, 14) sequences were aligned and some of the sequences were additionally manually aligned. The bioinformation program MicrobeTrace (15) was used to identify transmission clusters. Phylogenetic analysis was conducted with the Tamura-Nei algorithm (d) and clusters were determined at a genetic distance of 0.5% and 1.5%. Each pol sequence was represented graphically as a separate node. When two or more sequences were connected by certain threshold, they were labeled as a cluster, while nodes that did not connect to others were entitled as single sequences.

RESULTS

- **Characteristics of the CRF01_AE sub-epidemic in Bulgaria**

The first case of HIV-1 CRF01_AE infection in Bulgaria was detected in 1995 in a heterosexual man. The first mother-to-child transmission (MTC) was found 4 years later. The next important event in this sub-epidemic was the introduction of the CRF01_AE in the group of PWID in 2002. In 2009 in Sofia, 26 individuals were diagnosed with CRF01_AE, 18 (69.2%) of whom were PWID, indicating ongoing outbreak among PWIDs. In 2011, 31 cases were identified, 17 of whom PWID, 12 HET and 2 MSM.

The total number of people diagnosed with HIV-1 subtype CRF01_AE by the end of 2019 was 270 or 16% of all patients with HIV. Analysis of gender distribution showed higher number of men than women. In CRF01_AE, men were 187 (69.3%) and women 83 (30.7%) with a ratio of 2.25:1, while the ratio found in other subtypes was with a higher weight of men 4:1. The age at diagnosis varied, youngest diagnosed was a newborn (0 years), the oldest was 63 years old. The largest share of those diagnosed was between 20-29 (40%), followed by 30-39 (33.3%) years of age, while the infected young (≤19) and older individuals (≥50) represented a smaller share. Patients indicating that the infection was acquired in Bulgaria were 251 (93%) and those infected abroad (mainly Western European countries) were 19 (17%). Analysis of the geographic distribution demonstrated that most of the infections were found in individuals from Sofia, 164 (60,7%), and the rest were dispersed across the country. When comparing transmission groups with CRF01_AE, we found that PWID were 141 (52.2%), followed by HET – 101 (37.4) and MSM – 13 (4.8) (Table 1).

Table 1. Characteristics of individuals infected with HIV-1 CRF01_AE compared to infections with other HIV-1 subtypes and CRFs in Bulgaria.

Characteristic	Subtype CRF01_AE	Other subtypes
	number (%)	number (%)
Total	270	1413
Gender		
Men	187 (69.3)	1195 (84.6)
Women	83 (30.7)	218 (15.4)
Age (years)		
≤19	28 (10.4)	68 (4.8)
20-29	108 (40.0)	538 (38.1)
30-39	90 (33.3)	493 (34.9)
40-49	35 (13.0)	204 (14.4)
≥50	9 (3.3)	110 (7.8)
Country of Origin		
Bulgaria	269 (99.6)	1361 (96.3)
Other country	1 (0.4)	52 (3.7)
Likely Country of Infection		
Bulgaria	251(93.0)	1176 (83.2)
Other country	19 (7.0)	237 (16.8)
Region in Bulgaria		
Sofia	164 (60.7)	625 (44.2)
Other regions	106 (39.3)	788 (55.8)
Transmission category		
HET	101 (37.4)	592 (41.9)
MSM	13 (4.8)	630 (44.6)
PWID	141 (52.2)	158 (11.2)
Other	15 (5.6)	33 (2.3)

- **Analysis of CRF01_AE transmission clusters**

In order to characterize transmission clusters among the sub-epidemic of HIV-1 CRF01_AE in Bulgaria we used MicrobeTrace program applying two thresholds of genetic distance, i.e. 0.5% delimited more recent infections and 1.5% for more distant infections (Fig. 1). Analysis of genetic distance of 1.5% revealed the presence of 6 clusters and 98 single sequences. One of the clusters contained 154 sequences, 75.3% were men and 24.7% women. Most of them were PWID – 70.1%, followed by HET – 19.5%. The dissemination between genders was more evenly distributed in the group of single sequences, where HET were 65.3%. DRMs were 12.3% and 4.55% of them were NRTI and 7.8% NNRTI. DRMs were 23.3%. 2% of all single sequences had PR DRM, 20.4 % NRTI and 19.4% NNRTI.

With genetic distance of 0.5% 10 transmission clusters and 218 single sequences were found. The largest cluster consisted of 18 sequences, all of which were isolated from PWID, 55.6% men and 44.4% women. With exception of one NRTI DRM falling into a dyad, none of the sequences of the newly formed clusters had DRM. At 0.5% cutoff male single sequences were twice as many as women and the number of HET individuals and PWID were relatively equal. All DRMs were found among single sequences, 1% PR DRM, 12.8% NRTI and 14.7% NNRTI.

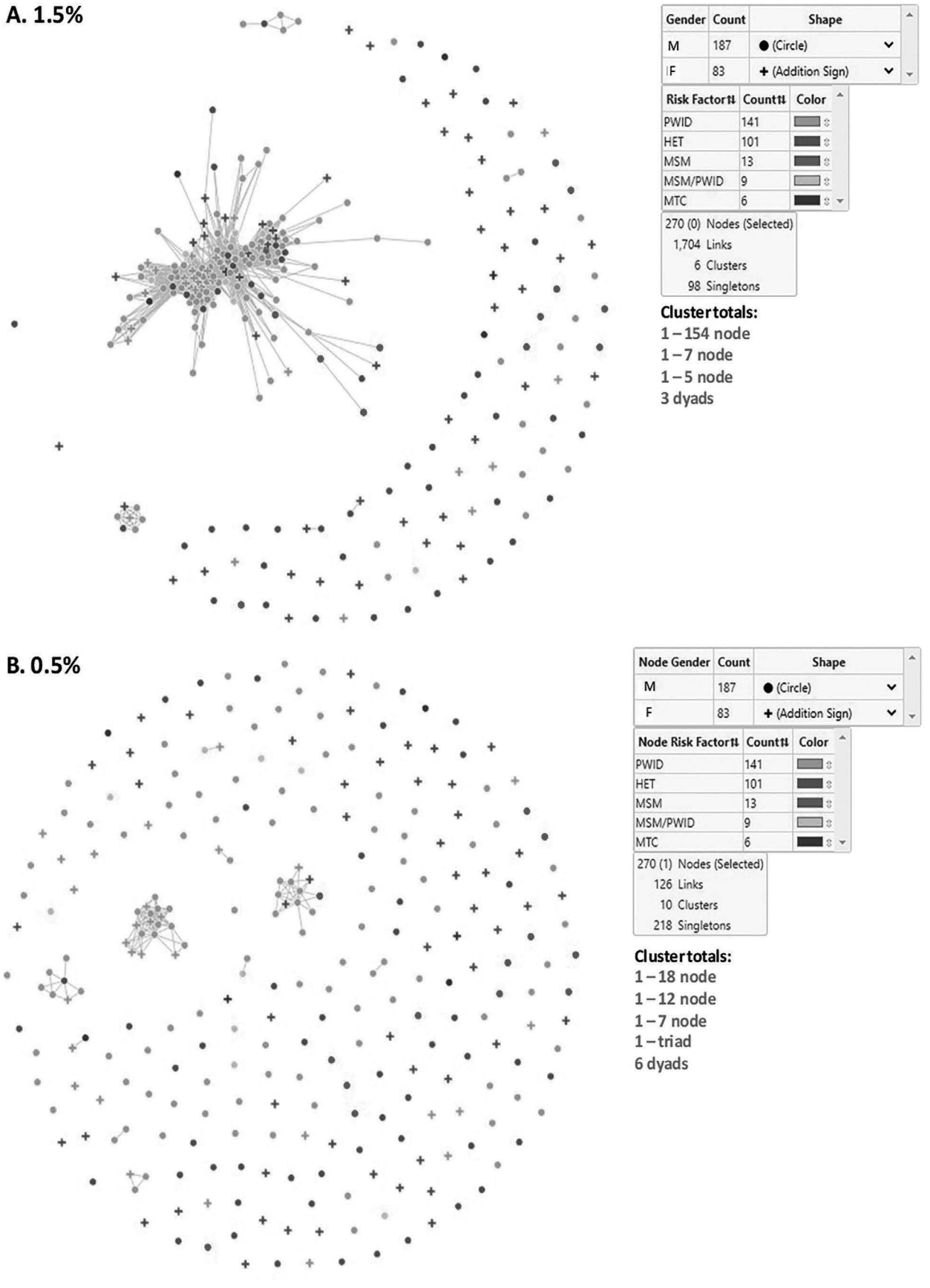

Figure 1. Graphical representation of CRF01_AE clusters in Bulgaria using MicrobeTrace.
A. genetic distance 1.5% **B.** genetic distance 0.5%. Gender is marked with circles (male) and plus sign (female). Color indicates transmission category: green PWID, blue HET, red MSM, yellow MSM/PWID and purple MTC. Cluster totals is provided.

- **Identification of drug resistance mutations in CRF01_AE infections in Bulgaria**

We identified 51 (18.9%) patients with DRMs, including 2 persons (1 HET and 1 MTC) with multiple protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI), and non-nucleoside reverse transcriptase inhibitor (NNRTI) DRMs (Table 3). Eight additional persons had both NRTI and NNRTI DRMs, including 4 HET, 3 PWID and 1 MTC transmission. The most prevalent NRTI and NNRTIs DRMs were M184V (17/57, 31.6%), and E138A (8/42, 19.0%), respectively (Table 2). Twenty-nine patients had NRTI DRMs, including 2 patients with 5 mutations, 3 with 4 mutations, 2 with 3 mutations, 7 with 2 mutations, and 15 patients with a single NRTI DRM. Thirty-two persons had mutations to NNRTIs, including 2 with 3 mutations, 6 with 2 mutations, and 24 had single mutations.

Overall, the highest prevalence of DRMs were found in MTC (3/6, 50%), followed by HET (29/81, 35.8%), MSM (3/13, 23.1%), MSM/PWID (1/9, 11.1%), and much less in PWID (15/141, 1.1%). For HIV-1-infected HET, 10.9% and 12.9% had NRTI and NNRTI DRMs, respectively. For HIV-1-infected MSM, 7.7% and 15.4% had NRTI and NNRTI DRMs, respectively, while 2.0% of PWID had both NRTI and NNRTI DRMs. All patients with PI or NRTI DRMs reported acquiring HIV-1 infection in Bulgaria, while 3/32 (9.4%) cases (2 HET and 1 MSM) with NNRTI DRMs specified that they acquired their infections abroad in either Germany, Turkey, or Spain. Although 60.7% of our study population resided in the capital Sofia, 52.6% and 54.5% of those with NRTI and NNRTI DRMs, respectively, were from other regions in Bulgaria.

Table 2. Drug Resistance Mutations (PR/NRTI/NNRTI) of HIV-1 subtype CRF01_AE persons in Bulgaria.

PR Major Mutation	Number of Patients with Mutation	NRTI Mutations	Number of Patients with Mutation	NNRTI Mutations	Number of Patients with Mutation
M46I	1	K65R	1	K101E	1
I54V	1	D67G	1	K103KN	2
V82A	1	D67N	3	K103N	5
I84V	1	K70N	1	V106I	3
		K70KT	1	V106VI	4
		K70R	4	V108VI	1
		L74LI	1	E138A	8
		L74V	5	E138G	1
		V75M	4	E138EG	1
		F77L	1	E138K	1
		Y115F	1	V179VD	3
		Q151M	1	Y181C	1
		M184MV	17	Y188L	3
		M184V	1	G190Q	1
		L210W	1	G190GA	1
		T215Y	1	G190GE	1
		T215I	1	G190A	1
		T215A	1	H221Y	1
		T215TA	2	P225PH	1
		T215TS	1	M230L	1
		T215F	2	K238RT	1
		K219E	3		
		K219KQ	1		
		K219Q	2		

Table 3. Multiple drug resistance mutations of HIV-1 subtype CRF01_AE persons in Bulgaria.

Sequence Name	PR Major	NRTI	NNRTI
2380208TG08	M46I, I84V	F77L, M184V	E138K
2580305TG05	I54V, V82A	D67N, K70R, T215I, K219Q	V106I
3820505TG05		D67G, L74V, V75M, Q151M, M184V	G190Q, H221Y
15930517VS17		L74V, M184V, K219E	E138G, M230L
5790415VS15		L74LI, M184MV	K103N, P225PH
8410209TG09		L74V, M184V	K103KN, Y181C, G190GA
12010914TG14		M184V, K219E	K103N, Y188L
6520112TG12		M184V	K103N, E138A, Y188L
8100313TG14		M184V	K101E, G190A
9800619VS19		K219KQ	E138A

DISCUSSION

In 1995 CRF01_AE was identified, approximately 10 years after the first detected HIV-1 infection in Bulgaria. CRF01_AE was first introduced in HET and in 2002 the strain was transferred into the PWID community. CRF01_AE was rapidly disseminated among this population leading to a local outbreak in 2009 (6, 8, 7). Thereafter CRF01_AE became the second predominant subtype in Bulgaria and nowadays it is the most prevalent among PWID (52%) and least in MSM (4.8%). In addition, we found that a significantly higher number of women were infected with CRF01_AE (30.7%) than those infected with other HIV-1 subtypes (18, 19).

In our study, we used two different thresholds of genetic distance to determine recent and more distant infections of CRF01_AE in Bulgaria. Six clusters were identified at 1.5% genetic distance, featuring a large outbreak cluster containing 154 sequences, the majority of participants, almost 41%, were young male PWIDs. The data collected in our study confirmed the understanding that CRF01_AE was distributed mainly among PWID and PWID/MSM. The dominance of a large cluster indicates the rapid spread of CRF01_AE infection in a group that does not follow safety measures, such as PWIDs. The main participants in the clusters were young male PWIDs. Very small fraction of PWIDs did not cluster, unlike HETs, which were mostly single sequences. This indicates that CRF01_AE does not spread massively among other transmission groups or leads to likely dead-end transmission. However, CRF01_AE is also spreading among other groups indicating the presence of transmission networks between risk groups and the general population. To find more recent HIV-1 CRF01_AE transmissions we lowered the genetic distance threshold to 0.5% and the large cluster collapsed. All clusters at 0.5% contained sequences mainly from PWIDs diagnosed in 2009-2019 matching the period of expansion in HIV-1 CRF01_AE infections in Sofia (Table 1). The local sub-epidemic of HIV-1 CRF01_AE in Bulgaria was maintained through the spread in PWIDs although it was probably introduced into the country through HET transmission. We identified a variety of antiretroviral DRMs in HIVs from persons with CRF01_AE infection. Most prevalent NRTIs and NNRTIs DRMs were M184V (17/57, 31.6%), and E138A (7/41, 17.1%), respectively. The NNRTI E138A DRM confers resistance to rilpivirine (RPV) and is present in 1.2% and 0.1% of treated and untreated people with CF01_AE infection, respectively, and is a polymorphic mutation that can survive for several years (16, 20).

Fortunately, as in our previous HIV-1 studies in Bulgaria, most DRMs were found in cases that did not cluster or were in transmission pairs (7, 17) and were much less prevalent in PWIDs (1.1%) compared to HET (28.7%) and MSM (23.7%).

The increased numbers of HIV-1 CRF01_AE infections in PWIDs in Bulgaria has led to a strong public response and measures by the Bulgarian Ministry of Health and NGOs (21). Campaigns have been put in motion to distribute free needles, syringes and condoms, free testing for HIV and hepatitis, and various educational initiatives. The study included only patients whose HIV-1 pol sequences were successfully obtained, thus excluding patients who had underwent a successful therapy and their viral load was low, as well as people who had not yet been diagnosed. These limitations may affect our analyzes and conclusions. Our study also included individuals who are likely infected abroad, this suggesting a more extensive study of transmission networks.

To analyze the spread and transmission clusters of HIV-1 subtype CRF01_AE we conducted a phylogenetic analysis of the available HIV-1 pol gene sequences. Our study found transmission clusters indicating local outbreak among PWID. Resistance mutations were found mostly in HET and non-clustering singleton sequences. To better understand and control HIV-1 epidemic, continuous monitoring of this and other subtypes of HIV-1 in Bulgaria is further needed.

Funding: This work was supported in part by a grant from the Ministry of Education and Science, Bulgaria (contract: DN03/2-16.12.2016); by the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria; by the National Program for the Prevention and Control of HIV and Sexually Transmitted Infections, Bulgaria; by the European Regional Development Fund through Operational Program Science and Education for Smart Growth 2014-2020, Grant BG05M2OP001-1.002-0001-C04 “Fundamental Translational and Clinical Investigations on Infections and Immunity”.

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NOROVIRUSES - A HIDDEN THREAT

Asya Stoyanova

National Reference Laboratory „Enteroviruses“,
Department of Virology, NCIPD

(Mini review)

ABSTRACT

Acute nonbacterial gastroenteritis is ubiquitous, and noroviruses are considered to be among the most common etiological agents. Noroviruses affect people of all ages. As a rule, infections caused by them are mild and self-limiting within 1-3 days but there is always a risk of a more severe course, especially in infants and the elderly. Being considered as mild and fast transient, noroviruses receive less attention than other infectious pathogens. The clinical and economic burden of norovirus gastroenteritis is often underestimated. Detection of the etiological role of noroviruses is essential not only from the clinical point of view, but also from the economic one because of the damage that norovirus infections cause to tourism and food industry. At present, prevention and counter disinfection measures are the only weapon against norovirus infections.

In this narrative review, results from a non-systematic search on the recent literature on noroviruses are presented. The review describes the basic biological characteristics of noroviruses, their genetic diversity and current classification, as well as the epidemiological aspects of the norovirus infection, its clinical manifestation, the diagnostic approaches, prevention and control measures and current state-of-the art for norovirus vaccine development.

ADDRESS FOR CORRESPONDENCE:
National Reference Laboratory “Enteroviruses”
National Center of Infectious and Parasitic Diseases
44A Gen. Nikolay Stoletov Blvd, 1233 Sofia, Bulgaria
tel: +(359-2) 831 00 42
e-mail: asya.s@ncipd.org

Keywords: *noroviruses, acute gastroenteritis, diarrhea, vomiting*

INTRODUCTION

Diarrheal diseases are among top 10 causes of death (1). Acute nonbacterial gastroenteritis is the second most important infection of all infectious diseases, and human noroviruses (HuNoVs), especially after the introduction of the rotavirus vaccine, are now arguably considered the leading etiological factor among all age groups (2). Unlike rotaviruses, which are the leading causes of acute intestinal disorders in children under 5 years of age, HuNoVs attack people of all ages. However, following the introduction of the rotavirus vaccine in some countries, HuNoVs tend to be the dominant etiologic agent in pediatric cases of acute gastroenteritis (3).

It has been found that one in every five cases of acute nonbacterial infectious gastroenteritis is caused by HuNoVs, which accounts for almost 700 million clinically manifested infections per year, one third of which are in children under 5 years of age (4). And although mortality, especially among children, is observed mainly in developing countries, HuNoVs are the same problem for both these countries and the countries with the highest gross domestic product per capita. Globally, norovirus infections cost humanity more than $ 60 billion a year: $ 4.2 billion in direct health care costs (including hospitalizations) and $ 56.2 billion in lost economic benefits - lost working and school days, emergency disinfection, quarantine measures, etc. (5).

Despite these startling numbers, HuNoVs remain relatively unknown compared to other infectious pathogens. They are almost ignored by the media, do not receive attention from politicians, and programs that systematically fund research on them, are almost non-existent.

BASIC BIOLOGICAL CHARACTERISTICS

Virions of HuNoVs are small (approximately 27 nm in diameter), non-enveloped with

icosahedral geometry, and the virus particle morphology is spherical without any protruding spikes. The capsid is composed of 90 dimers of viral protein VP1 arranged in such a way that cup-like hollows are seen under electron microscopy (6). The capsid encloses the virus genome which consists of a relatively small, single-stranded, non-segmented, positive-sense, linear RNA, approximately 7500 nucleotides in length. The genome is organized into three overlapping open reading frames (ORF1–ORF3). ORF1 encodes a large polyprotein which is further cleaved into six smaller non-structural proteins (NS1/2 to NS7) by the virus specific protease (NS6). ORF2 and ORF3 encode for the major (VP1) and minor (VP2) viral capsid proteins, respectively. The 5' end of the genome is covalently linked to VPg, and the 3' end contains a polyadenylated tail (7).

RESISTANCE IN THE EXTERNAL ENVIRONMENT

HuNoVs are extremely resistant in the environment. Outside the human host they remain viable for weeks and even months on surfaces and in water (8). The lack of a lipid envelope makes norovirus virions resistant to organic solvents such as ethanol, ether and chloroform. Capsids remain stable at neutral and acidic pH and remain intact at temperature up to 55^{o}C (9). These characteristics of HuNoVs greatly contribute to the high transmission rate of the infection, especially in closed groups and premises.

CLASSIFICATION AND GENETIC DIVERSITY

HuNoVs belong to the *Norovirus* genus in the family *Caliciviridae.* The genus is currently subdivided into 7 genogroups based on the complete nucleotide sequence encoding the major capsid protein VP1 (10). Genogroups are denoted by the capital Latin letter G and the corresponding Roman numeral. Viruses from genogroups I, II and IV (GI, GII, GIV) are human pathogens, three genotypes of GII are detected in feces from swine and one genotype from GIV is detected in cats and dogs. The rest genogroups include viruses that infect only animals and humans are not affected by them. Genogroups are further divided into genotypes denoted by an Arabic numeral, and genotypes are subdivided into variants. In general, genogroups differ by about 40–60 percent of their amino acid sequence and genotypes by about 20–40 percent (11). Since the mid-1990s, GII.4 has been the most prevalent genotype worldwide and it is responsible for 70–80% of all norovirus-associated gastroenteritis outbreaks worldwide (12). Chhabra et al., 2019, propose an update of the classification of noroviruses and have expanded the number of genogroups to 10 (13). The abundance of a variety of different norovirus strains is due to their extremely high variability. There is a continuous process of genetic and antigenic diversification and a new variant is generated every 2-3 years due to the accumulation of point mutations or recombination events. Thus, in the winter of 2014/2015, the new variant GII.17 Kawasaki emerged in Japan, which quickly replaced the dominant GII.4 Sidney (14). In the same season, the new variant was detected in Europe, in Italy (15), and in the summer of 2015 it was already established in Bulgaria (16). The GII.17 genotype has become the predominant strain in some parts of Asia (17).

EPIDEMIOLOGICAL ASPECTS OF NOROVIRUS INFECTION

In the absence of a suitable and accessible laboratory model for studying HuNoVs and the infection caused by them, their epidemiology has been elucidated thanks to healthy volunteers experimentally infected with HuNoVs. Thus in fact, the virus itself was discovered, the fecal-oral mechanism of transmission and the duration of virus shedding in the environment were proven (18).

Data from human challenge studies have shown that the virus is found in the feces of the infected shortly before the illness, and is present for up to several weeks after patient's full recovery.

There may be considerable heterogeneity in the number of viruses that are shed (19). However, the titers of shed virus are the highest during the clinical manifestation and a few days after. Vomiting appears to facilitate transmission due to the formation of fine infectious aerosols during fountain vomiting. It has been found that about 30 million virus particles are released in a single vomiting session. (20). Virus deposited on surfaces can also cause outbreaks (21) and virus can be transferred by hand contact followed by ingestion (22).

HuNoVs are highly contagious (23). Routes of transmission (food-borne, water-borne and contact-to contact) facilitate transmission within closed or narrow spaces. That is the reason norovirus infection to be often referred as 'the cruise disease' (24). In the enclosed space of a cruise ship, along with the presence of a significant number of people, literally anyone can be infected in a short period of time. However, the same rule also applies to closed and semi-closed institutions such as boarding houses, hospices, places of imprisonment, etc.

Different genotypes and variants show relatively different "behaviors" regarding mode of transmission, characteristics of the outbreak, and clinical picture (25). For example, GII.4 variants are more often associated with vomiting and prolonged diarrhea, and with a higher number of diarrheal bowel movements. GII.4 is much more often associated with transmission via the fecal-oral mechanism from direct human-to-human contact than with other known factors of transmission (contaminated food, water, surfaces).

Norovirus genotypes other than GII.4, such as GI.3, GI.6, GI.7, GII.3, GII.6 and GII.12, are more commonly transmitted through contaminated food. It has also been found that GI strains cause waterborne infections much more often than GII strains (26). Foodborne route of transmission is very important for the global spread of HuNoVs (27). This can happen when food is prepared by an infected staff member or at even earlier stages - when growing, transporting or storing food. Numerous food outbreaks, as well as sporadic cases, are caused by viruses that contaminate products that are usually consumed fresh or with minimal processing such as leafy vegetables, strawberries, raspberries, sun-dried tomatoes, and seafood. Cases of norovirus infections due to oyster consumption have been reported in many countries (28,29,30). Such outbreaks may simultaneously involve more than one norovirus strain, which in turn can lead to viral recombination events (26). Shellfish can be infected when grown on farms contaminated with wastewater, as well as ground fruits watered with contaminated water (31).

With regard to the risks of norovirus contamination during irrigation, it has been found that, on the one hand, possible fecal contamination in irrigation facilities can lead to virus "sticking" to the surface of fruits and vegetables, and on the other hand, an infectious virus can reach the fruit, entering through the root system of the plant and spreading by its vascular tissue (32).

CLINICAL MANIFESTATION AND COMPLICATIONS

In healthy adults, HuNoVs cause a self-limiting disease lasting one to three days, but in the elderly, young children and immunocompromised patients, these viruses can cause a long-lasting infection (33,34,35). Clinical course is usually characterized by a sudden onset of nausea and several debilitating and almost fountain vomiting, quickly followed by repeated diarrhea without pathological impurities. In children, vomiting is a more common symptom than diarrhea, while in adults, it is the opposite – diarrhea is the leading symptom. Body temperature may be slightly elevated. There are complaints of abdominal cramps, malaise, severe fatigue. As both vomiting and diarrhea are profuse, patients quickly lose a relatively large amount of fluid and dehydration-related complaints come to the fore. The patient feels so bad that he is unable

visit the doctor. However, the complaints are short-lived, severe clinical manifestation lasts no longer than a day, rarely two days. Average duration of the disease is 12-60 hours. A very quick recovery comes and according to the patient, the need to visit a doctor disappears. That is the main reason for not knowing the real frequency and burden of disease, as well as the economic impact, especially of sporadic norovirus infections. Despite rapid and complete recovery, however, shedding of viable HuNoVs through the feces continues for several days and even up to two weeks imposing a real threat to an epidemic outbreak.

No matter how mild the course of a norovirus infection is, it poses dangers especially for the vulnerable age groups - infants, young children, and the elderly. HuNoVs are an important risk factor for complications and increased mortality in immunocompromised individuals, including transplant patients and those receiving immunosuppressive therapy (36). People suffering chronic diseases, especially of the gastrointestinal tract, are also at a relative risk. In young children, body temperature can be greatly elevated, the general condition can deteriorate very quickly and lead to febrile seizures (37,38). Cases of necrotizing enterocolitis in children have also been reported (39). In adult patients, on the other hand, underlying inflammatory bowel disease may worsen (40). There is evidence that severity of the disease is increased if the patient takes statins to regulate cholesterol levels (41).

Extraintestinal manifestations of norovirus infections are considered a rare phenomenon, and their mechanisms are still unclear. Upon a detailed review of the available literature, Ho et al. (2020) found rare reports of norovirus-induced hepatitis (42).

DIAGNOSTICS

Noroviruses were discovered in 1972 by electron microscope analysis of stool samples from an outbreak of acute non-bacterial gastroenteritis with unknown aetiology in an elementary school in Norwalk, Ohio, USA (18). Electron microscopy is a relatively insensitive method with a detection limit of approximately 10^6 norovirus particles per gram of feces and it requires skilled personnel and sophisticated equipment (43).

At present, reverse transcriptase polymerase chain reaction (RT-PCR) is the most frequently used technique for detection of norovirus RNA in clinical specimens (feces or vomitus), contaminated food, water, or fomites, and it is considered as “the gold standard” in norovirus diagnostics. Due to the high sequence variability among norovirus strains, most RT-PCR assays use primers that target a conserved region in ORF1 (coding for the viral RNA-dependent RNA-polymerase) or a conserved region in the ORF1-ORF2 junction region (44). While RT-PCR assays provide only qualitative results, real-time quantitative qRT-PCR assays allow rapid detection as well as comparison of viral RNA levels. A major concern regarding the PCR techniques is the inability to discriminate between inactivated and potentially infectious virus particles (45). Infectious viruses can be detected by cell-culture based techniques but in the case for HuNoVs these are available for limited laboratories (46).

Immunochromatographic and enzyme immunoassays (ELISA) are also available to detect norovirus antigen in stool samples. The advantage of the latter assays compared to RT-PCR based methods, is the simplicity and rapidity of the assay. No specialized equipment is required and results can be ready within few hours.

The sensitivity of rapid immunochromatographic tests varies considerably and they may be useful for the early diagnosis of outbreaks, for which a greater number of stool samples is tested and only a few positive results are enough for etiologic confirmation. However negative samples cannot be considered true negatives and must be controlled by RT-PCR (47).

Comparative studies with commercial ELISA assays show a wide range of sensitivity and specificity values (48). When ELISA is used,

the probability of detecting positive samples is lower if small numbers of samples are tested. Using ELISA instead of RT-PCR for the detection of norovirus in stool samples will result in a considerable number of false negative outbreaks unless a minimum of 6 samples per outbreak are tested. This precludes use of ELISA assays for individual patients, but diagnosis of outbreaks may be possible (49). Nevertheless, due to the high genetic and antigenic diversity of norovirus strains, certain genotypes can be missed with these assays and therefore they should preferably be used in combination with a confirmation of negative samples by RT-PCR (50).

PREVENTION AND TREATMENT

Outbreak management focuses on preventing further spread of the virus by containment of infected individuals and hygienic measures. Hand washing is the key hygienic action and has demonstrated to prevent further spread of health-care associated infections (51). Because of their extreme stability in the environment, HuNoVs require chemical disinfection with high concentration of hypochlorite, detergents based on hydrogen peroxide or phenolic-based cleaning solutions (52).

Historically, the development of a norovirus vaccine has been hampered by the lack of a small-animal model and a cell culture system, both of which have been described only recently, and licensed vaccines are not yet commercially available. Nevertheless, there are currently three types of norovirus vaccines in different phases of testing – non-replicating virus-like particles (VLPs), P particles, and recombinant adenoviruses. All these vaccine platforms have challenges and limitations but the real problem is which genotypes should be included in the vaccine. A vaccine including the prototype GI.1 strain has been developed but its low cross reactogenicity with the dominant GII.4 genotype imposes rather a combined approach (53). Some norovirus vaccines have now completed Phase I and Phase II clinical trials (54).

There are several types of experimental antiviral drugs that are in the process of their very early development. The only antinorovirus agent that has almost completed a clinical phase of testing is nitazoxanide (NTZ) (55). There is controversial evidence for the efficacy of NTZ, some studies prove its effectiveness, others reject it. However, NTZ is one of the therapeutic options (excluding ribavirin, immunoglobulins and maintenance care) for patients with persistent infections (56).

CONCLUSION

Each discovery is followed by a series of new questions: what is the role of the human microbiome in norovirus infection, what is the role of the immune system or the blood type and tissue compatibility. New discoveries and future scientific advances surely await us, providing tools for assessing potential antiviral strategies and candidate vaccines against noroviruses. But before that, a more active surveillance on norovirus infections is needed.

ACKNOWLEDGEMENT

This work was supported by Operative Program “Science and Education of Intelligent Growth”, project BG05M2OP001-1.002-0001-C04**.**

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STUDIES ON THE EPIDEMIOLOGICAL AND MOLECULAR CHARACTERISTICS OF THE HEPATITIS E VIRUS IN BULGARIA: A COMPREHENSIVE REVIEW

Elitsa Golkocheva-Markova[1*], Chiydem Ismailova[1], Tencho Tenev[1], Lubomira Nikolaeva-Glomb[2]

[1] NRL "Hepatitis viruses", Virology department, NCIPD, Sofia
[2] NRL "Enteroviruses", Virology department, NCIPD, Sofia

ABSTRACT:

Hepatitis E virus (HEV) is a RNA virus that belongs to the family *Hepeviridae*. The virus causes self-limited acute hepatitis in immunocompetent individuals, but can become chronic or present with extrahepatic manifestations in immunosuppressed patients. In recent years, due to the increased scientific interest in HEV infection, the number of laboratory-confirmed cases have also increased. The first study of HEV infection in Bulgaria was carried out in mid-90s of the last century by Teoharov et al. Ten years later, more in-depth studies of HEV infection began. The main focus was on the evaluation of HEV seroprevalence among different target populations. Attention was also paid to the zoonotic potential of the infection. The aim of the present review is to summarize studies on HEV conducted by Bulgarian authors in regards to HEV seroprevalence among humans and animals, clinical and epidemiological characteristics of HEV infection, and molecular-characteristics of HEV.

Key words: *Hepatitis E virus, prevalence, haemodialysis patients, domestic pigs.*

ADDRESS FOR CORRESPONDENCE:
Elitsa Golkocheva-Markova
NRL "Hepatitis viruses",
Departament of Virology, NCIPD,
44A Stoletov BLVD.
1233 Sofia, Bulgaria
Tel: +359 2 8329 118;
e-mail: elmarkova2007@gmail.com

INTRODUCTION

Hepatitis E is a quasi-enveloped single-stranded RNA virus with icosahedral symmetry, measuring 27-30 nm in diameter. The genome has positive polarity (1). Hepatitis E virus (HEV) classification is based on morphology of the infectious particles, mode of transmission and clinical features. The virus has been initially classified in the *Caliciviridae* family. The latest genetic analysis reveals limited genetic similarities with amino acid sequences of the replicative enzymes in rubella virus and alphaviruses of *Togaviridae* family and plants' *Furoviruses* (2). As a result of these discoveries made in 2009, HEV was classified as the only member of the newly formed family *Hepeviridae*, genus *Hepevirus*. In 2019, the family *Hepeviridae* has been divided into two genera: the genus *Orthohepevirus*, which includes viruses isolated from mammals and birds, and the genus *Piscihepevirus*, which consists only of the species *Piscihepevirus A,* isolated from fish. There are four species in *Orthohepevirus* genus, e.g. *Orthohepevirus A*, *Orthohepevirus B, Orthohepevirus C, Orthohepevirus D*, and each species has different hosts (3). At least, 9 viral genotypes belong to *Orthohepevirus A*. The latter species includes four genotypes of HEV – genotypes 1, 2, 3, and 4, which are important for human pathology (3). Usually HEV infection is asymptomatic. According to the World Health Organization, about 20 million new HEV infections occur in the human population worldwide each year, of which only 3.3 million are symptomatic (4). The most common clinical presentation of HEV infection is acute viral hepatitis, and symptoms may vary from mild to fulminant acute hepatitis in immunocompetent individuals, or progress to chronic hepatitis in immunosuppressed patients. The icteric form of infection can be observed

in 5% to 30% of the infected individuals. The mortality rate is below 0.5% up to 4% during an outbreak, but can hit up to 25% in pregnant women (5).

HEV was identified in 1983 by Balayan et al (6). From that moment on, HEV studies began worldwide. For the first time, HEV infection in Bulgaria was reported in 1995 by Teoharov and co-authors (7). In 2008, a new case report of HEV infection was published by the latter team (8). From this moment on, a number of studies have been conducted for a better understanding of HEV and the infection caused by it.

The aim of the present review is to summarize studies on HEV conducted by Bulgarian authors and to compile data concerning: 1) HEV seroprevalence among humans (general population, patients with acute viral hepatitis and specific populations) and among animals; 2) clinical and epidemiological characteristics of HEV infection; and 3) molecular characteristics of HEV.

MATERIAL AND METHODS

To achieve the aim of the present review an electronic keyword literature search in PubMed and the Central Medical Library at the Medical Univerity (MU) in Sofia, was performed. To define the research question of the present review the terms "hepatitis E virus infection", "acute hepatitis E virus" "hepatitis E virus prevalence", "hepatitis E virus seroprevalence", "hepatitis E virus genotype" AND "Bulgaria" were applied during search in PubMed database. Only publications in English were selected. For the databases of the Central Medical Library, the terms applied for the search, were "hepatitis" AND 'E". Articles published between 1990 and 2021 year were screened. Reviewing was conducted independently by two of the authors. Initially, 146 articles of potential interest were found (Figure 1): 16 from PubMed and 130 from the database of the Central Medical Library at MU-Sofia. Following screening titles and abstracts and applying the following exclusion criteria: 1) to be related with methods development, 2) to be a review, and 3) to have restricted free on-line access to full text, finally, 23 online free full-text papers had met exclusion and inclusion criteria and were used for further data analysis.

The review is focused on six different research questions (Table 1), that are important for a better understanding of HEV infection.

STUDIES EVALUATING THE SEROLOGICAL PREVALENCE OF HEPATITIS E AMONG PATIENTS WITH ACUTE VIRAL HEPATITIS (AVH) OR THE GENERAL POPULATION.

In Bulgaria, the first study on HEV prevalence was conducted in 1995 by the National Reference Laboratory "Hepatitis viruses", National Center of Infectious and Parasitic Diseases (NCIPD), Sofia. The aim was to evaluate the presence of antibodies to HEV (anti-HEV) among 53 patients with non-A non-B AVH (Table 2). The study revealed that 7,55% (4/53) of the patients were anti-HEV positive. Among all positive patients, 5.67% were HEV mono-infected, in four patients anti-HAV IgG was detected, and one patient was hepatitis B virus (HBV) and cytomegalovirus (CMV) co-infected (7). In a later study, conducted in 2010-2012, serum samples from 32 patients with clinical and laboratory data for AVH, were tested for anti-HEV IgM and/or IgG and HEV RNA. Out of the 32 samples tested, 44% (14/32) were anti-HEV positive with detected HEV RNA, of them, 12 were simultaneously positive for both antibody classes – IgM and IgG. Two of the sera were positive for specific IgM (1/14) or IgG antibodies (1/14), respectively (9).

In another study, 806 patients with AVH were retrospectively evaluated. Among them, 16,13% had laboratory confirmed HAV infection, 12,03% – HBV infection, 2,23% –HCV infection, 5,33% – Epstein-Barr virus (EBV) infection, 1,74% – CMV infection, and 60,05% (484/806) were with undifferentiated AVH. Of all AVH, 2,48% patients (20/484) were HEV positive, who comprised 0.29% of all hospitalized patients within the study period (10). HEV seroprevalence among

Table 1. Research questions evaluated within the review.

Research question	*Reported results*
Seroprevalence of hepatitis E among AVH patients and general population/blood donors	Anti-HEV seroprevalence varies between 2% up to 44 %
Distribution of HEV in different age groups	Age-dependent tendency of anti-HEV IgG prevalence
Molecular/genetic characteristic of HEV	Circulation of HEV genotype 3, subgenotypes e, f and c
Serological prevalence of hepatitis E among swine	Persistance of HEV infection among industrial pigs
Evaluation of clinical and epidemiological characteristics of HEV infection	HEV infection is more severe in co-infected patients with pre-existing liver pathology
Hemodialysis as a risk factor for HEV infection	The established HEV seroprevalence is close to that in general population

Legend: anti-HEV = antibodies against hepatitis E virus; AVH = acute viral hepatitis

Table 2. Characteristics of the studies reporting serological prevalence of hepatitis E among AVH patients and general population / blood donors

Total number of studied samples (N)	Anti-HEV IgM / IgG positive samples (%)	Co-infection/ co-morbidities	Reference
53	7,55 / NS	CMV; ch HBV	(7)
32	44*	NS	(9)
806	2,48 / NS	HBV	(10)
325	13,2 / 20,9	NS	(11).
741	ND / 9,04	NS	(12)
896	11,5 / 16,1	NS	(13)
555	NS / 21,7 to 28,8	NS	(14)

Legend: NS = not studied; CMV = cytomegalovirus; ch HBV = chronic HBV infection; HBV = hepatitis B virus; ND = not detected; * = anti-HEV positive.

AVH in North-Eastern Bulgaria was investigated, too. In still another study, 325 patients (287 hospitalized patients with clinical data of AVH and 38 outpatients with laboratory data of liver dysfunction) were tested for anti-HEV IgG and Ig M. Four of the patients had travelled to HEV endemic areas. The data indicated that 13.2% patients (43/325) were with acute HEV infection. In tested patients, 20.9% were anti-HEV IgG positive (68/325), which is a marker for a past HEV infection (11).

In accordance to the general population, overall anti-HEV IgG prevalence of 9,04% was found by Theoharov et al. (Table 2). Serum samples from 741 individuals were tested for the presence of anti-HEV IgG and IgM. Anti-HEV IgG, as a marker for a past infection, were detected in 9,04% (67/741) of individuals, and in 1,48% (11/741) a concomitant appearance of both classes IgG and IgM antibodies was established. The presence of anti-HEV IgM as a single marker was not detected (12). Another study of the general population revealed anti-HEV IgM and anti-HEV IgG seroprevalence of 11,5% and 16,1%, respectively

(13). Extremely high HEV seroprevalence among Bulgarian blood donors from different districts was established, the percent values varying between 21,7% and 28,8% (23/80) for Shumen district, 23.2% (22/95) for Pleven district, 27.1% (38/140) for Stara Zagora district, 27.5% (44/160) for Plovdiv district, and 21.3% (17/80) for Sofia. The authors evaluated the seroprevalence in connection with hunting activities and detected values of 48,7% to 51,6% (14).

Summarised studies on HEV prevalence suggest spread of the infection among Bulgarian population should not be neglected as HEV is detected from 2% up to 44 % among AVH, in 11,5% of general population and up to 28,8% among blood donors.

STUDIES ON DISTRIBUTION OF HEV IN DIFFERENT AGE GROUPS

Clinical presentation of HEV infection is age dependent (15). The rate of anti-HEV IgG positivity increases with age, which could be explained with the increased risk of exposure to HEV by age (16) The same trend is established in a study of 741 individuals from Plovdiv region, conducted between 2012 and 2013 by Teoharov et al. (12). The age of the population studied varied from 1 year to more than 82 years old. A clear association between the proportion of anti-HEV-IgG positive individuals and the age was detected – an increase in anti-HEV-IgG positivity from 3,53% in individuals aged 1-9 years to 19,23% in the group over 60 years. The authors established discrepancy to 8,27% for age group 50-59, which is a prerequisite for future studies. No differences were found in the distribution of antibodies against HEV between males and females. Therefore, the age is one of the host factors, which defines the correlation of antibodies against HEV virus.

STUDIES ASSESSING MOLECULAR-GENETIC CHARACTERISTICS OF HEV

Following successful genomic sequencing of isolates from around the world nine HEV genotypes have been established (1 to 9), and their genetic sequences differ from one another by up to 25%, and they infect both humans and animals (17, 18). The genetic characteristics of the virus are relevant to the clinical course of the infection – severity and outcome, and its geographical spread. Several molecular-genetic investigations of HEV strains, isolated from Bulgarian patients, have been conducted. The genotyping of 14 samples by amplification of the 337 nt fragment (4228 nt-4565 nt) of HEV open reading frame 1 (ORF1), revealed the presence of HEV genotype 3 subtypes f and e (12) (Table 3).

Table 3. Characteristics of the studies on HEV genotype distribution.

Total number of studied samples (N)	Amplified genome region	Genotype	Sub-genotype	Reference
14	ORF1	3	e, f	(12)
103	ORF2	3; 1*	e, f, c	(19)

Legend: ORF – open reading frame; *HEV genotype 1 was imported for Bulgaria

In another study, 103 anti-HEV IgM positive serum samples from AVH cases (epidemic outbreaks between 2013 and 2015) from all over Bulgaria were analysed for HEV RNA, and viremia was detected in 90 of the samples. Out of these 90 samples, 64 were sequenced by the 355 nt fragment in ORF2 region of the HEV genome. The applied post-sequencing analysis revealed distribution of HEV genotype 3, subtypes e, f and c. The authors established different circulation of 3e comparing with 3f and 3c, with 3e restricted to South-West Bulgaria while 3f and 3c diffused over the country. During this study an imported HEV genotype 1 was detected (19). Subsequently, after applying a deep Bayesian

phylogenetic analysis, information on the genetic diversity and the spread of HEV genotypes in Bulgaria was obtained. Three different data sets of the HEV viruses were built - for demographic history investigation, and for selective pressure analysis. The evolutionary rate of 351×10^{-3} substitution/site/year for genotype 3e was established. The root of the time to the most recent common ancestor of the Bayesian maximum clade credibility tree of HEV 3e genotype corresponded to year 1965. The demographic history showed a slight growth from 1995 to 2000, followed by a sort of a bottleneck in 2010s, a peak in 2011 and a new growth to 2015. Selection pressure analysis did not reveal positive pressure sites but detected 64 statistically significant sites under negative selection (20).

The factors underlying the observed molecular and geographical differences in HEV genotype distribution, remain to be investigated. The application of molecular epidemiological surveillance by Bayesian phylogeny of HEV virus is a suitable scientific approach to public health control.

STUDIES EVALUATING THE SEROLOGICAL PREVALENCE OF HEPATITIS E AMONG SWINE AND WILD BOAR.

HEV genotypes 3 and 4 are zoonotic ones and have been isolated from a large variety of animals (wild boar, swine, rabbits and deer). These genotypes are transmitted to humans by consumption of undercooked infected meat and by contact with an infected animal and its products, such as offal, and sewage (21). Therefore, veterinarians and animal keepers are in a greater risk to be infected with HEV. The first study on HEV seroprevalence among domestic pigs in Bulgaria was conducted by Pishmisheva and co-authors (22). HEV infection among pigs from five industrial farms was assessed (Table 4). For the purpose of that study, 85 serum samples from healthy pigs (1–6 months) were tested and the result showed that 50% of piglets were positive for anti-HEV antibodies. In the group of fattening pigs, 29,2% were seropositive. The established overall HEV seroprevalence among pigs was 40%. Even higher results were reported in another study that investigated HEV seroprevalence among 171 East Balkan swine (EBS) from North-Eastern and South-Eastern Bulgaria. The established overall HEV seroprevalence was 82,5% (141/171), as for weaner animals it was 77,2% (44/57), for fattening pigs 79% (45/57), and for adult animals 91,2% (52/57) (23).

Table 4. Characteristics of the studies reporting HEV seroprevalence among domestic and wild animals.

Animal species (age in month)	Total number of studied samples	Number of covered pig farms/ slaughterhouses	Percent anti-HEV antibodies positive samples	Reference
Pig (1-6)	85	5	40	(22)
EBS	171	NA	82,5	(23)
Pig, Wild boar	433 32	19 NA	60 12,5	(24)

Legend: NA = not applicable; EBS = East Balkan swine.

Another study established 60% anti-HEV IgG seroprevalence in domestic pigs and 12,5% in wild boar. The detected seroprevalence in wild boar was significantly lower in comparison with domestic pigs (24). The established high HEV prevalence among domestic pigs and wild boar suggests that these animals might be the reason for the increased HEV transmission across Bulgaria.

It is important to know the prevalence of HEV infection among domestic and wild animals in order to reduce the risk of human infection with zoonotic strains. Data demonstrate the existence

of HEV infection between industrial pigs and wild boar in the country. These findings are a prerequisite for more in deep studies on the risk of occupational exposure.

STUDIES EVALUATING THE CLINICAL AND EPIDEMIOLOGICAL CHARACTERISTICS OF HEV INFECTION

In the European countries, in most cases HEV infection is an autochthonous one, mild and self-limited. Chronic infection is common for immunocompromised patients, such as transplant patients and hematological patients on chemotherapy, HIV infected patients and those under treatment with corticosteroids and immunosuppressive agents (5). Testing for HEV should be recommended as part of the diagnostic algorithm for all patients diagnosed with hepatitis or with liver biochemical pathology (25). A few years ago data on the clinical presentation of HEV infection were limited due to unawareness and low number of laboratory confirmed cases. More in depth study of the clinically acquired HEV infection in Bulgaria began after 2008 (26). Laboratory confirmation of suspected HEV infections among patients with AVH, was done by enzyme immunoassay detection of anti_HEV IgM and/or IgG antibodies. The main clinical symptoms associated with acute HEV infection were nausea, loss of appetite, dark urine and jaundice. In both reports the patients in admission presented with extremely elevated liver enzymes: alanine aminotransferase (ALAT) up to 4264, aspartate aminotransferase (ASAT) – to 3752 and total bilirubin – up to 344,5 (Table 5). Co-infection with HBV and HCV, as well as past HBV infection were detected (27; 10). In another study, 78 patients with AVH were retrospectively evaluated. In addition to liver pathology, prolonged anti-HEV IgM seropositivity up to two years was established in 12% (5/78) of the patients after resolving HEV infection. In 28% of the anti-HEV positive patients concomitant liver disorders were reported (28).

Table 5. Characteristics of the studies evaluating the clinical characteristics of HEV infection.

Clinical diagnosis	Number studied cases (N)	Median age	Liver enzymes maximal value			Co-infection/ co-morbidities	Reference
			ALAT [IU/L]	ASAT [IU/L]	Total bilirubin [mmol/L]		
AVH	2	43,5	4264	3752	344,5	HBV	(27)
AVH	20	51	1851,8	1324,9	123,6	HBV HCV	(10)
AVH	78	57	4000	3000	NS	LD	(28)
ALF	1	42	2940	4844	1088	RhA; SpA	(29)

Legend: AVH – acute viral hepatitis; ALF-acute liver failure; NS-not studied; LD = liver disease; RhA = rheumatoid arthritis; SpA= spondylo-arthritis

Acute HEV infection is reported with clinical presentation of acute liver failure (29), thrombocytopenia (30), holestatic (31) and hemophagocytic syndrome (32). Thereby, the differentiation of acute HEV infection is a complex process that requires combining epidemiological, clinical and serological data. In accordance with Ordinance No. 21 on the Procedure for Registration, Reporting and Control of Infectious Diseases, since 2019 the acute form of viral hepatitis E is a subject to mandatory registration and reporting in Bulgaria.

HEMODIALYSIS AS A RISK FACTOR FOR HEPATITIS E VIRUS INFECTION

In addition to the main route, transmission

through contaminated blood products has also been described for HEV infection (33). As a result, hemodialysis patients are in a greater risk to be infected with HEV. In a study from 2020, Pishmisheva and co-authors investigated the distribution of HEV infection in hemodialysis patients from Pazardzhik region. Among 102 patients on dialysis treatment 14,7% (15/102) were positive for antibodies against HEV (34). In another study, among 80 haemodialysis patients from Plovdiv, 8,75% of the tested samples were anti-HEV IgM and/or IgG positive. All serologically positive samples were negative for HEV RNA (35). In both studies the authors concluded that the established HEV seroprevalence is close to that in the general population. However, because of the possible transmission of HEV by blood products, a periodic serological testing for Hepatitis E in patients on hemodialysis is needed.

CONCLUSION

Overall, this review comprehensively addresses the current knowledge on HEV in Bulgaria. The first studies on HEV infection in Bulgaria began in the mid-90s of the last century and they were conducted by the team of the National Reference Laboratory (NRL) for Hepatitis Viruses. Studies have been focused on the prevalence of serological markers among patients with acute viral hepatitis and among the general population. Worldwide, increased scientific interest in HEV infection is observed after the establishment of locally circulating viral strains in Europe and the zoonotic nature of the infection. The team of NRL for Hepatitis Viruses carries out studies on HEV viral strains circulating in Bulgaria. Research on the spread of HEV among animals has also begun. After the inclusion in 2019 of HEV infection in the ordinance on reports of AVH, the medical and scientific interest in the etiological cause of the infection in Bulgaria has increased. Identification and isolation of HEV from patients with different extrahepatic manifestation suggests expanded study of different immunosuppressed patients' populations. Seroepidemiological surveys will help to identify changes in epidemiology of HEV infection in Bulgaria. In addition, more in deep studies are needed to understand HEV mechanisms for crossing the inter-species barrier, and to fill in the missing data on Bulgarian HEV phylogenetics.

ACKNOWLEDGMENT:

This review is supported by the scientific Grant No KP-06-N33/2 from the National Science Fund of Bulgaria and by Operative Program "Science and Education of Intelligent Growth", project BG05M2OP001-1.002-0001-C04.

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MOLECULAR AND MICROBIOLOGICAL APPROACHES FOR RAPID ETIOLOGICAL DIAGNOSIS OF SYSTEMIC MYCOSES

Review

Lyubomira Boyanova

National Reference Laboratory of Mycosis and Sexually Transmitted Infections
National Center of Infectious and Parasitic Diseases

ABSTRACT

The review presents common laboratory methods for the diagnosis of the majority of invasive fungal diseases, i. e. candidosis, aspergillosis and cryptococcosis. Some studies reveal an increasing range of the infectious agents such as Trichosporon and endemic fungal pathogens like Histoplasma, Blastomyces and Coccidioides.

The most common serological tests for antigen and antibody-detection in body fluids (serum, plasma, bronchoalveolar lavage) are: indirect immunofluorescense, ELISA – Platelia (enzyme linked immunosorbent assay), latex-agglutination, immunodiffusion and molecular techniques like PCR and Real Time PCR.

Not only current data on invasive fungal disease diagnostic methods are reviewed, but also studies on new biomarkers and recent discoveries in molecular diagnostics.

New molecular approaches are needed to provide faster results.

Key words: *systemic fungal disease; Candida, Aspergillus, Cryptococcus, Real Time PCR*

INTRODUCTION

It is estimated that around 1.9 million patients worldwide develop invasive fungal disease (IFD) each year. These infections are associated with high mortality and economic burden due to hospitalizations. Therefore, a fast and timely diagnosis is needed (1).

ADDRESS FOR CORRESPONDENCE:
National Center of Infectious and Parasitic Diseases
26, Yanko Sakazov blvd.
1504 Sofia, Bulgaria
e-mail: miraanbo@abv.bg

Systemic fungal diseases may cause mortality in immunocompromised individuals (oncology, chemotherapy, transplantation, catheterized or heart prosthetic and AIDS patients, patients in intensive care units (ICU), etc.) (fig.1).

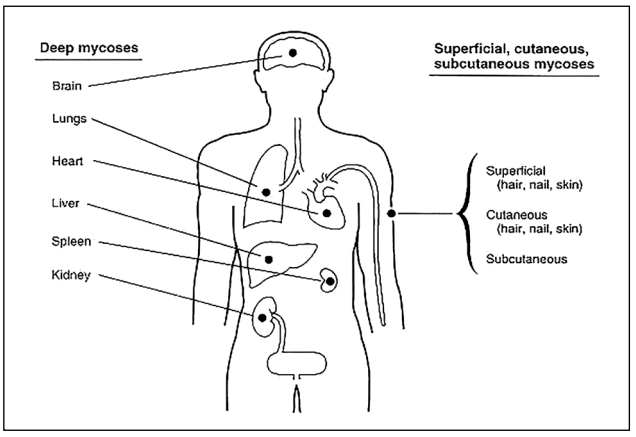

Fig.1 Schematic representation of the types of fungal infections (https://microdok.com/)

Routine microscopic and culture based methods are the classical ones, but diagnosis is provided in 48-72 hours (2, 3) (fig.2, 3). For this reason, faster and reliable at the same time screening methods for early detection of pathogenic fungi are needed. (4, 5).

The majority of invasive fungal diseases are still caused by *Candida* (6) and *Aspergillus* species, but recent studies indicate an increasing incidence of other species, such as *Cryptococcus* and *Trichosporon* (7). There are endemic fungi in some areas, for example in the USA *Histoplasma, Blastomyces* and *Coccidioides* are reported (8).

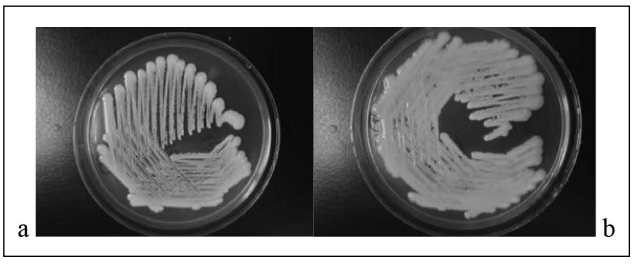

Fig.2. Macroscopic view of Candida albicans (a) and Cryptococcus neoformans (b)-

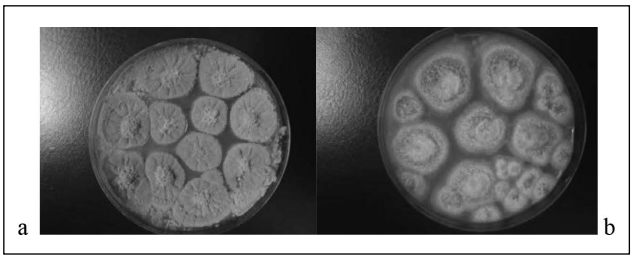

Fig.3. Macroscopic view of Aspergillus flavus (a) and Aspergillus fumigatus (b)

Microbiological laboratory diagnostics most often includes:

- Microscopy - directly from the clinical specimen or after an appropriate staining, by Gram, for example;
- Histology - evidence of fungal elements from biopsy;
- Culture examination - on culture media, including blood cultures, followed by identification and test for sensitivity to antifungals (antimycogram);
- Serology - detects antigens or specific antibodies;
- Molecular genetic methods, i.e. PCR;

MICROSCOPIC EXAMINATION

Microscopy of fungi can be done following appropriate staining - by Gram, or by fluorescent dyes, cotton blue, etc.

The formation of germ tubes in *C. albicans* is a factor of virulence, which can be detected by the so-called Germination test (Fig 4).

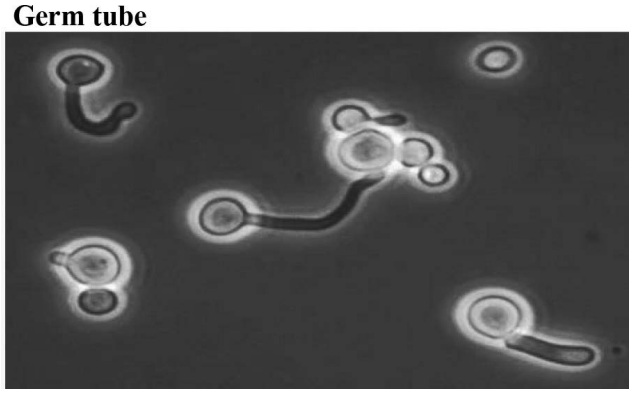

Figure 4. Candida albicans and a central true germ tube (www.mycology.adelaide.edu.au)

Microscopy and identification of *Aspergillus* is not easy and requires a guidance. *Aspergillus* spores are carried by a long conidiophore, and it originates from the so-called „foot cell" (Fig.5). On its top it ends with the so-called vesicle, which can vary in shape and size. From the surface of the vesicle conidia-forming cells called mettuli and phialides, begin, which can partially or completely cover the vesicle.

HISTOLOGY

Histological examination of biopsy materials for the presence of fungal elements is considered one of the main criteria for a proven fungal infection (Fig 6). Biopsy or surgical resection of pathological tissue are often definite signs for the diagnosis of an IFD). Timeliness and expedience are essential for early diagnosis.

CULTURE EXAMINATION

Sabouraud dextrose agar or chromogenic medium with color reaction during growth for 48-72 hours at 30°C, the so called Chrom agar, is the most often used solid nutrient medium for the culture study. Following isolation of a fungal strain as a pure culture, biochemical identification follows. This can be achieved with the help of non-automated (commercial identification kits) or automated identification systems (VITEK2, Merlin, Phoenix, MALDI-Toff, Microscan, etc.). There are various biochemical identification kits, the most commonly used of which are Api 20 CAux and Auxacolor.

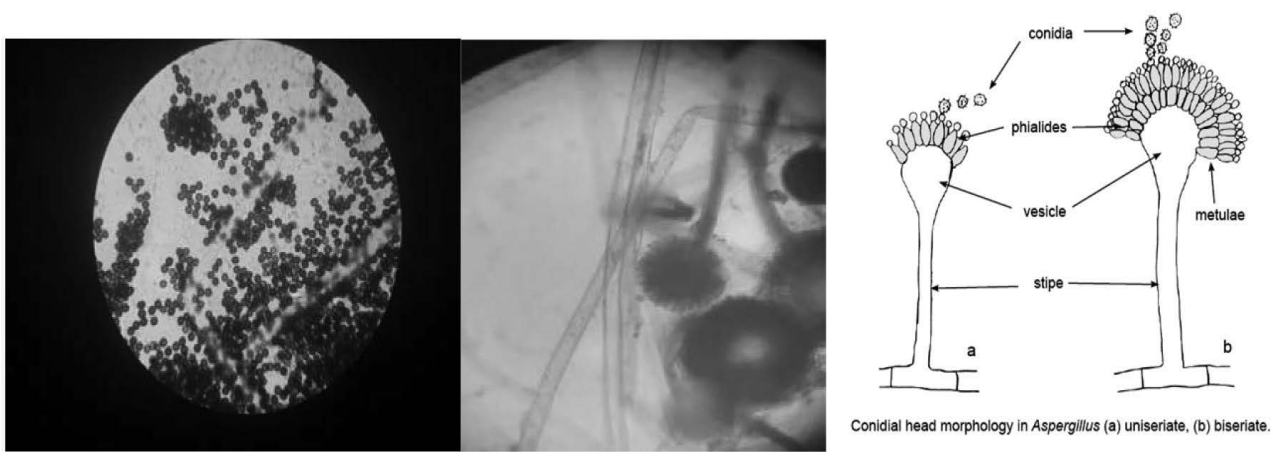

Fig.5. Microscopic view and schematic representation of the microscopic characteristics in Aspergillus (www.mycology.adelaide.edu.au)

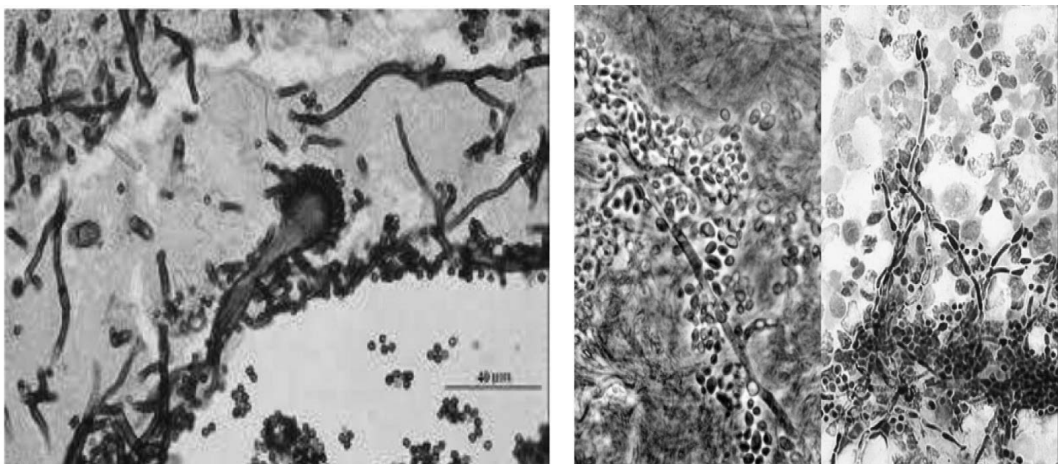

Fig .6. Visualization of fungal elements in biopsy tissue (www.mycology.adelaide.edu.au)

ANTIBODY DETECTION

The method that we use for determination of specific antibodies against *Candida, Aspergillus* and *Cryptococcus* is the indirect immunofluorescence (IIF). For performing it, it is necessary to prepare a microscopic slide from a culture suspension (10^6-10^7 CFU/mL) of Candida strain (CIP 628), Aspergillus (*A. fumigatus*) and *Cryptococcus neoformans*. The slides with the fixed suspension should be stored at – 20°C (9).

The principle of the method includes 2 steps: in the first step, cover glasses should be coated with diluted serum samples (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 for Candida and Aspergillus or 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 – for *Cryptococcus neoformans*) for 30 minutes.

In the second step, after washing the slides, the attached antibodies are stained with fluorescein-labeled anti-human immunoglobulin antibodies and visualized under the fluorescent microscope (fig.7) (10;11; 12).

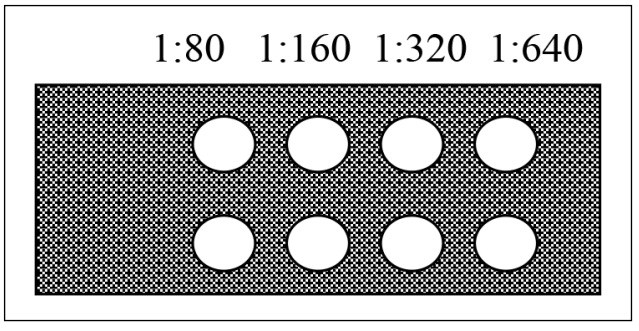

Fig.7. IIF for the detection of serum antibodies.

ANTIGEN DETECTION

In our laboratory we have experience about serology antigen detection with enzyme linked immunosorbent assay (ELISA).

ELISA – Platelia (BioRad) is an immunoenzyme sandwich microplate assay for detection of the circulating antigen (mannan in *Candida* or galactomannan in *Aspergillus*) in human serum and in bronchoalveolar lavage (BAL) fluid (fig.8). Mannan and galactomannan antigen are polysaccharides non-covalently bound to the yeast cell wall and they appear to be the main biomarkers for the diagnosis of IFD.

Fig.8 Elisa - Platelia commercial kits (BioRad, France) and schematic presentation of the sandwich Elisa method (www.microbionotes.com)

Another serological test for the detection of circulating antigens using a sample technique, is the latex agglutination (LA) test (BioRad). It is a qualitative test detecting the polysaccharide antigen, mannan (for *Candida*) and glucoronoxylomannan (*Cryptococcus neoformans*) in biological fluids (serum, BAL, cerebro-spinal fluid (CSF)). The procedure uses latex particles coated with monoclonal rat antibody directed against the fungal antigen (13). The particles react with the fungal antigen resulting in agglutination visible to the naked

eye (fig.9) (14). Detection of Candida antigen by LA in body fluids is a reliable criterion for a systemic infection. LA tests for antigen detection in immunocompromised patients with cryptococcosis of the central nervous system (CNS) are even more reliable. The test has a very high sensitivity and specificity.

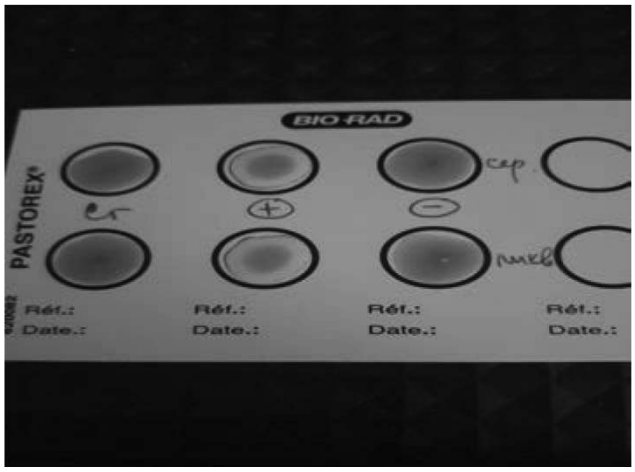

Fig. *9.* LA test for yeast

A third method for antibody detection is immunodiffusion (ID). In our laboratory we have experience for searching antibodies against *Aspergillus* and endemic fungal pathogens such as *Histoplasma, Blastomyces* and *Coccidioides* – the spill phenol agar for ID in a few petri plates. Immediately after spilling agar, the petri plate is placed in the refrigerator at 4°C. After making wells with smooth edges, we drip:

- Control serum (commercial kit)
- Antigen (commercial kit)
- Patient's serum

Precipitation lines are sought following incubation in a wet camera at 25°C for a few days (9).

MOLECULAR GENETIC METHODS FOR DIAGNOSIS

The non-culture based method is PCR – DNA-detection for the rapid diagnosis of infections – amplification of a specific DNA-fragment by means of short, artificially synthesized oligonucleotides (primers) (15;16). They hybridize specifically with a particular region of the target DNA, with participation of Taq-polymerase (9).

The following are the three reaction steps in one PCR cycle:

1. Denaturation of double-stranded DNA (dsDNA) to two single-stranded chains at 95°C (also called DNA melting)
2. Binding of primers to their complementary regions in the single-stranded ssDNA chains (also called annealing of primers)
3. Amplification of new DNA at each end in the 5'-3' direction, using DNA Taq polymerase in the presence of deoxynucleotides (dATP, dTTP, dCTP and dGTP) to build new daughter DNA frangments.

REAL-TIME PCR

Real-time PCR allows tracking of DNA amplification at the time it occurs, not at the end of the reaction, as in conventional PCR (17).

In most eukaryotes, coding rDNA genes and spacers are found in tandem repeats separated by non-transcribable DNA, called "no"-transcribed spacer (NTS). The coding regions of 18S, 5.8S and 28S nuclear ribosomal DNA (rDNA genes) are relatively conserved among fungi and provide a molecular basis for establishing phylogenetic links. Between the coding regions are: the internal transcribed spacer 1 and 2 - regions, respectively ITS1 and ITS 2. The ITS region is perhaps the most widely sequenced DNA region in fungi. Universal fungal primers are used to amplify the entire internal transcribable ITS spacer. The ITS region, located between 18 S and 28S ribosomal DNA, is divided into two (ITS 1 and ITS2), and between them the preserved region 5.8S is situated (Fig.10)

Amplification techniques offer increased sensitivity over traditional staining and culture methods but may report positive results in asymptomatic individuals because of colonization (18; 19).

Real time PCR has advantages as speed, enhanced sensitivity and wider range of detectable organisms, which cannot be cultured (20).

Double-labeled hybridization probes can be used. The probe is an oligonucleotide that is labeled with a fluorescent dye (reporter) at one end and a fluorescence quencher at the other end. The proximity of the extinguisher to the fluorescent dye suppresses the emission of the natural fluorescence by the fluorescent dye. In

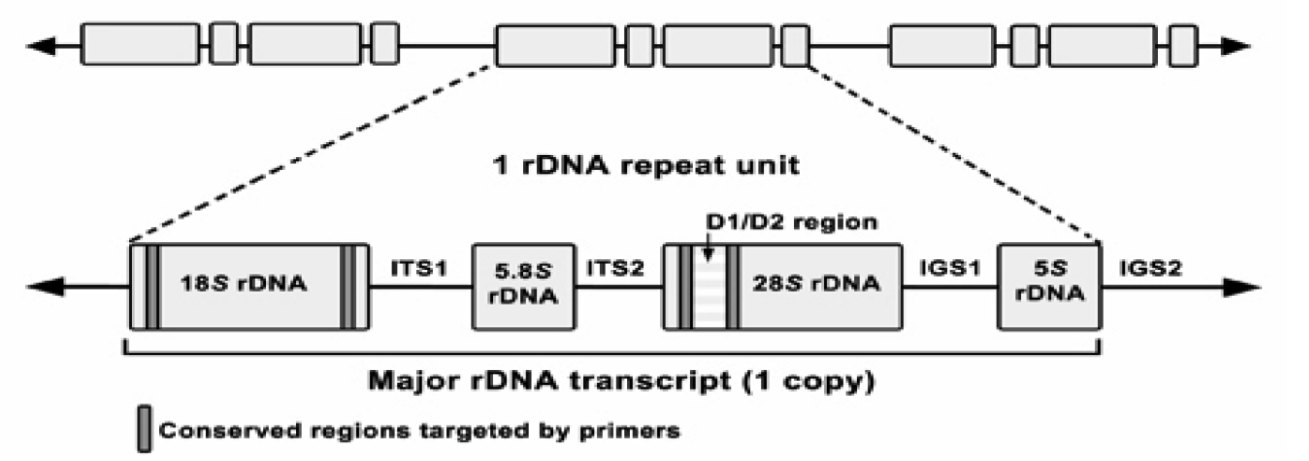

Fig.10 Schematic representation of the organization of an rDNA-transcribed region comprising 18S, 5.8S and 28S-genes in fungi. Between the genes for 18 S and 5.8S rRNA is ITS1, and between the genes for 5.8 S and 28 S rRNA is ITS 2 (www.research.gate, www.mycology.adelaide.edu.au)

PCR, the hybridization probe binds to a specific region of the template DNA. Thus, there is a directly proportional correlation between the increased fluorescent signal and the amount of the PCR product (Fig.11)

Real-Time PCR can also be used for quantitative analysis - determination of the number of DNA molecules in the sample in real time - to distinguish colonization from infection, because some medically important fungi are opportunistic pathogens. They are part of the normal human microflora.

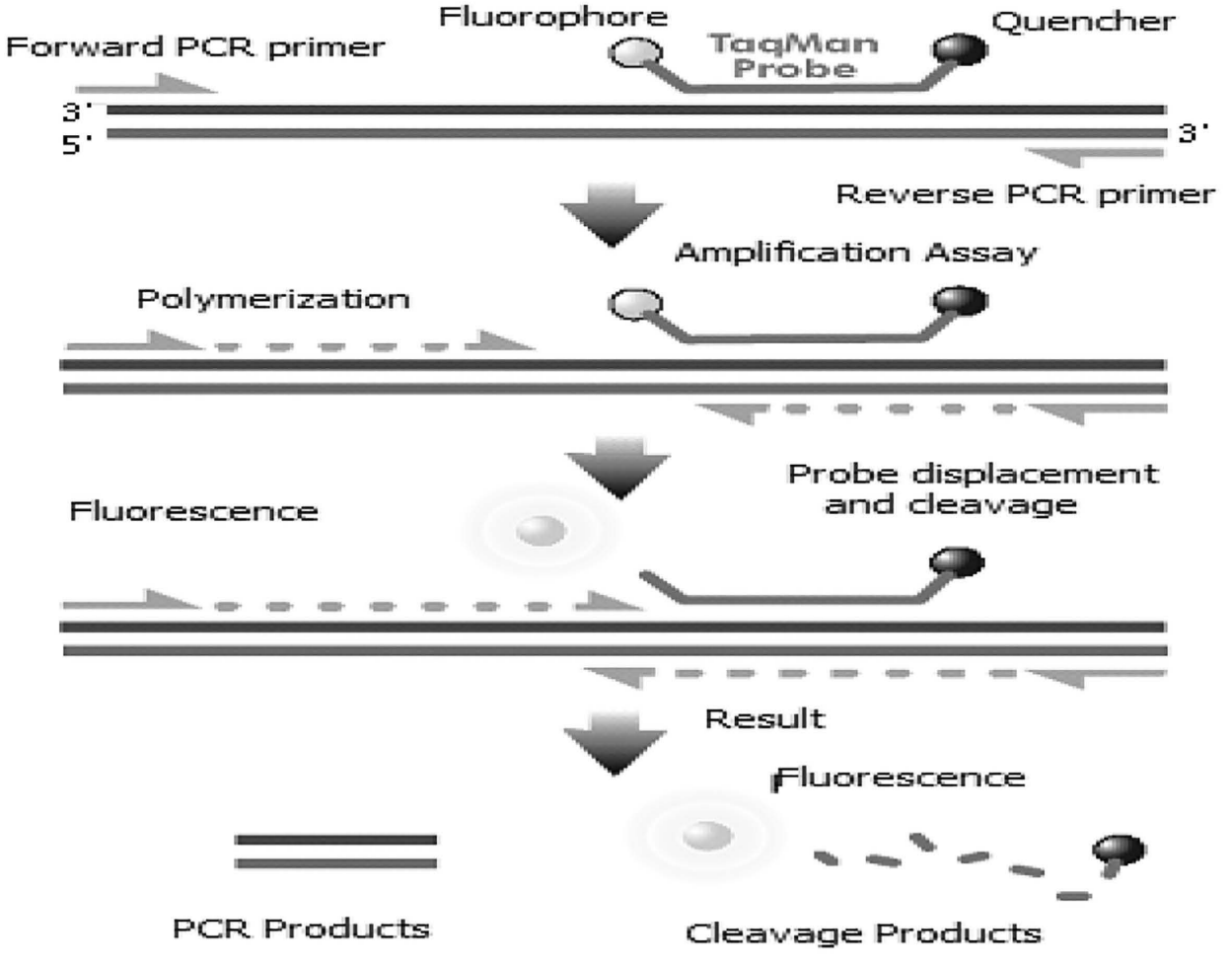

Fig. 11. Real-Time PCR (https://www.wikiwand.com)

The treatment options for invasive fungal infections are presented in a table 1.

Table 1. Recommendations for therapy of SANFORD guide, USA

<table>
<tr><th>SANFORD</th><th colspan="2">Primary therapy</th><th colspan="2">An alternative</th></tr>
<tr><td>Invasive aspergilosis</td><td colspan="2">-Voriconazole 6 mg/kg iv/po q 12h on day1, then 4 mg/kg iv/po q12h or
-Isavuconazole 372 mg po/iv q 8hx6 doses, then 372 mg po/iv daily</td><td colspan="2">-Liposomal Amphotericin B(L-AmpB) 3-5 mg/kg/day iv or
-Amphotericin B lipid complex(ABLC) 5 mg/kg/day/iv or
-Posaconazole
-300 mg po 2 bid x2 doses, then 300mg po/day or
-suspension 200 mg qid, then 400mg po bid or
- iv 300mg over 90 min bid/day 1, после 300mg iv/day</td></tr>
<tr><td rowspan="2">Invasive Candidosis</td><td>Empirical therapy</td><td>Target therapy</td><td>Patients without neutropenia</td><td>Patients with neutropenia</td></tr>
<tr><td>-Caspofungin 70mg iv, then 50 mg iv qd or
-Micafungin 100 mg iv qd or
-Anidulafungin 200 mg iv, then 100 mg iv qd</td><td>-for C.albicans, C.parapsilosis, C.tropicalis: Fluconazole 800 mg(12 mg/kg), then 400 mg iv/po qd o pure blood culture and clinical stability
-for C.glabtara: echinocandins or Voriconazole
-for C.krusei: echinocandins, then Voriconazole 6mg/kg bidx 2 doses, then 4 mg/kg bid o pure blood culture and clinical stability</td><td>-Lipid-based Amphotericin B 3-5 mg/kg iv qd
- Amphotericin B 0.7mg/kg iv qd
-Voriconazole 6mg/kg bidx2 doses,then 4 mg/kg bid</td><td>- Lipid-based Amphotericin B 3-5 mg/kg iv qd
- Amphotericin B 0.7mg/kg iv qd
- Voriconazole 6mg/kg bidx2 doses, then 4 mg/kg bid
-Fluconazole 800mg(12mg/kg), then 400 mg iv/po qd</td></tr>
<tr><td>Cryptococcosis (meningitis)</td><td colspan="2">- Liposomal Amphotericin B(L-AmpB) 3-4 mg/kg iv q24h or
- Amphotericin B lipid complex(ABLC) 5 mg/kg iv q 24h+Fluconazole 25 mg/kg po q6h
- can with
:Fluconazole 400-800 mg po/day/8 weeks</td><td colspan="2">- Liposomal Amphotericin B(L-AmpB) 3-4 mg/kg iv q24h or Amphotericin B lipid complex 5 mg/kg iv q 24h or Amphotericin B 0.7-1 mg/kg iv q24h+Fluconazole 800-1200mg/day iv/po/2weeks
- Liposomal Amphotericin B3-4 mg/kg iv q24h или Amphotericin B lipid complex 5 mg/kg iv q 24h or Amphotericin B 0.7-1 mg/kg iv q24h/4-6 weeks
-Fluconazole 800-1200/day iv/po+Flucytosine 25 mg/kg po q6h/4-6 weeks
-Fluconazole 1200-2000 mg po/day/10-12 weeks</td></tr>
</table>

Abbreviations: iv – intravenous; po – per os; qd (quaque dia) – once at the appointed time; bid – twice a day

GENERAL CONSIDERATIONS

The different methods for laboratory diagnosis of IFD vary in specificity and sensitivity (21).

For *Candida* spp, there are now direct tests to detect DNA. Recently, the Food and Drug Administration (FDA) approved a test with direct detection in blood samples of DNA of the most commonly isolated Candida species, with high sensitivity and specificity (95%) (T2 Candida; T2 Byosystems, Inc, Lexington, MA) (1).

New biomarkers for invasive aspergilosis (IA) – studies are being performed to detect volatile organic compounds in exhaled air as a result of *Aspergillus* metabolism (respiratory samples of patients with IA and those without IA are compared). Other studies have focused on the search for pan-fungal serum disaccharide (DS) as an additional marker in the diagnosis of fungi (1).

Antibody titers to antigens of the germ tubes of *Candida albicans* show high specificity and sensitivity. Many authors also confirm the ability of the method in the diagnosis of systemic mycoses because this is used to distinguish for colonization with *C. albicans* in a systemic infection. This is very important for empiric therapy in patients (22).

Detection of galactomannan in serum is a feasible approach in adult neutropenic patients for the early diagnosis of invasive aspergillosis.

ELISA – Platelia tests perform higher sensitivity and they are more appropriate for the analysis of a great number of serum samples. On the other hand, LA tests are easier to perform in laboratory conditions (23). We must not forget also the costs of antigen-detection tests.

Real time PCR is currently one of the fastest diagnostic methods for fungal species, that are the most frequent causative agents of systemic infections (24; 25).

Molecular methods are faster and more accurate than the biochemical ones for the needs of diagnosis and identification of medically significant fungi. These methods are new, relatively inexpensive and certainly take less time to refine.

Further studies are needed on the usefulness of PCR in routine practice, as well as the combination with other biomarkers as an optimal strategy. European Organisation for Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) includes PCR method for *Aspergillus* detection as a method for probable IFD, but PCR for *Candida* remains a research approach in most research centers, due to limited standardization (26).

CONCLUSIONS

Genetic methods perform higher specificity than serological ones. They can significantly reduce detection time, but there are still no fully standardized protocols for isolating fungal DNA and performing PCR (27;28). Standartization is necessary for their introduction in the routine practice in mycological laboratories. Problems with the avoidance of contamination of clinical materials and with the development of methodologies to distinguish infection from contamination are to be resolved (29). Meta-analysis confirms that if both PCR and Elisa are consistently negative, then this is sufficient to rule out invasive aspergillosis (30).

Clinical mycology, unlike bacteriology, has a slower pace of development. The use of serological tests for antigens and antibodies, as well as the introduction of molecular techniques make non-cultural methods essential for the prediction of IFD. None of these tests, however, can be used as a stand-alone definitive diagnostic test. Compliance with clinical data, imaging studies, etc is essential (1).

Laboratory diagnosis of invasive fungal disease has to be used in conjunction with other diagnostic procedures such as culture technique, histological examination of biopsy samples, computed tomography (CT) imaging (31,32). Further research is needed to establish the benefits of PCR for fungal detection in routine practice (26).

ACKNOWLEDGEMENT

This work was supported by Operative Program “Science and Education of Intelligent Growth”, project BG05M2OP001-1.002-0001-C04.

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