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REGULATORY MECHANISMS OF ANGIOTENSIN-CONVERTING ENZYME 2 AND THEIR SIGNIFICANCE IN THE DEVELOPMENT OF SEVERE COVID-19

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ABSTRACT

From 2019 to the present day, the coronavirus infection COVID-19 continues to be a serious health problem. Scientists and clinicians from all over the world have joined efforts in studying the molecular interaction mechanisms between SARS-CoV-2 and ACE2, including virus-induced changes in ACE2 transcription, expression, and functionalities leading to disruption of basic regulatory pathways for vascular homeostasis, and reprogramming of key proteases, co-receptors and adhesion molecules.

Here, we aimed to clarify the mechanisms and signals that would restore the virus-induced imbalance between destructive and protective effects of ACE2.

Understanding why only certain individuals are predisposed to infection with SARS-CoV-2, and development of severe pathology is at the center of scientific interest, and the strategies for prevention and therapy.

Keywords: ACE2, COVID-19, SARS-CoV-2

INTRODUCTION

Over the past 20 years, society has witnessed an increasing number of pandemics that have led to the hospitalization and death of millions of people worldwide, causing an unprecedented strain on the healthcare systems: the severe acute respiratory syndrome (SARS) in 2002, the Middle East respiratory syndrome (MERS) in 2012 and the COVID-19 infection in 2019, all caused by beta-coronaviruses, SARSCoV, MERS-CoV and SARS-CoV-2, respectively.[1] Despite the enormous efforts of the research community, the new SARS-CoV-2 virus has infected globally more than 771,679,618 people worldwide (as of 2 November 2023) and caused the death of more than 6,977,023.[2] A large percentage of those had multiorgan dysfunction including pulmonary, cardiovascular, neurological and endocrine symptoms with a powerful cytokine storm unfolding.[2] Understanding the mechanisms underlying the individual predisposition to infection with SARS-CoV-2, as well as the reasons for the development of severe pathology only among certain individuals is at the center of scientific interest, strategies for prevention and therapy.[3] Despite the vast amount of data illuminating the effects of SARS-CoV-2 on the human body, there are still numerous unanswered questions. The mechanisms and signals that would restore the virus-induced imbalance between destructive and protective effects of ACE2 are of interest, as well as the intimate interactions between the regulators of receptor-bound signaling cascades.

Specificity of SARS-COV-2

The mechanism underlying the attachment of SARS-CoV-2 to the cell membrane is well studied and described. The receptor-binding domain (RBD) of S1-subunit of the spike protein of SARS-CoC-2, exhibits a 10- to 20-fold greater affinity for ACE2 than SARS-CoV, which is explained by a greater number of matches between the N-terminus of ACE2 and SARS-CoV-2 (1204 Å) as compared to SARS-CoV (998 Å) (4). Kowever, in order to attach to the receptor and the cell membrane, the spike protein of SARS-CoV-2 needs to be previously "functionalized" by separating the S1 and S2 subunits: a process known as priming. Unlike SARS-CoV-1, the SARS-CoV-2 virus cannot self-attach. The attachment is realized by using the human transmembrane serine protease 2 (TMPRSS2) through proteolytic-mediated activation of the virus. The enzyme FURIN, a type 1 membrane-bound protease, also assists in the process of separating the

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two subunits of SARS-CoV-2 and plays an alternative role in the process of attachment of the virus to ACE2. In this way, SARS-CoV-2 is less dependent on TMPRSS2 and has a "backup plan" in its pathophysiological endocytosis program. This gives SARS-CoV-2 the advantage to interact with cells with low TMPRSS2 expression, and to adhere to membranes with high FURIN expression.[4] This is another distinguishing feature of SARS-CoV-2 as compared to SARS-CoV-1, which lacks a FURIN-binding domain. The cellular co-expression of ACE2, TMPRSS2 and FURIN and tissue localization of the proteases are important factors in the pathogenetic mechanism of cell binding (4).

Another key factor in this intermolecular play is ADAM17 (disinterring and metalloproteinase domain 17) popularly known as TNFα-converting enzyme as it releases proteolytically the precursor of TNFα (pro-TNFα) into the extracellular space when expressed on the cell surface membrane. There, TNFα exerts auto- and paracrine functions associated with activation of systemic pro-inflammatory cytokines (IL-1β and IL-6) as well as autonomous effects resulting in TNFαR activation. Thus a key signaling pathway is realized resulting in ADAM17 activation, proteolytical cleavage of the ectodomain of the membrane-bound long-chain ACE2 and its conversion to soluble ACE2 with a short half-life (5,6).

In this way, the first axis of influence on the pathologically low cell membrane expression of ACE2, suppressed hydrolysis of Ang II to Ang(1-7) and reduced activity of MasR is involved. The resulting excess of Ang II exerts its destructive general effects (oxidative stress through increased ROS production, hypertrophy, fibrosis, vasoconstriction and intestinal dysbiosis) (7).

On the other hand, excess AngII also causes local overactivation of AT1R, which is an essential modulator of ADAM17 overactivity through AT1R, thus realizing the second axis of suppressing membrane expression of ACE2 and deepening the effects of AngII in a vicious circle. A third ADAM17 over-activation signaling pathway related to endocytosis of the ACE2-SARS-CoV-2 complex is added to both axes (5).

The cumulative effects of suppressed ACE2 expression and function are associated with inability to metabolize AngII and, hence, increased AT1R/AngII-ADAM17/TNFα/IL-1β/IL-6 signaling with multiorgan dysfunction and cytokine storm-driven hyperinflammation (5).

Effect of SARS-COV-2 on ACE2 functions

In addition to an imbalance in the renin-angiotensin-aldosterone system (RAAS), the pathological program of SARS-CoV-2 includes adverse effects on the kallikrein-kinin system (KKS), the alpeinergic system (AS), th eamino acid transport in the gastro intestinal tract (GIT), and intestinal dysbiosis (ACE2-B0AT1). These add to and amplify the adverse effects of COVID-19. Although most studies have focused on the RAAS, due to the potent destructive effects of AngII and the wide tissue distribution of the AT1R, changes in the regulation of KKS and AS cannot be neglected. The involvement of ACE2 as a key protease makes them closely linked; they share common signaling pathways and receptor apparatus, interrelated effects and consequences in the course of the COVID-19 disease (2,8,9,10).

Hepatic angiotensinogen is hydrolyzed to AngI by the renal protease renin, then through the receptor with proteolytic function ACE, AngI is metabolized to AngII with a direct effect on AT1R and an alternative effect on AT2R. Negative regulation of AngII activity is realized by ACE2 at two points: directly on AngII to Ang(1-9) and on AngI to Ang(1-7), followed by MasR activation. An additional pathway to reduce the activity and effects of AngII is via APA to AngIII, again with effects on AT1R and AT2R. The balance between the protective and destructive effects of AngII is maintained through two signaling pathways: destructive AngI/AngII/AT1R; AngI/AngII/AT2R and protective AngI/Ang(1-9); AngII/Ang(1-7)/MasR. ACE2 plays a central role as a switch between the two opposite effects.[2,8,9,10] Accumulation of AngII and overactive AT1R lead to activation of three intracellular signaling pathways: 1) via NADPH/COX-2/ROS/cytochrome C/ to apoptosis; 2) through Caspase 3 to apoptosis; 3) via p38MAPK/JNK to hypertrophy. An alternative signaling is also discussed, which leads to overproduction of reactive oxygen species (ROS) under oxidative stress, and direct activation of transcription factor (NF)-Kb resulting in over-expression of IL-6, IL-1β and TNFα (2,10). Thus, the vicious cycle involving cell-autonomous overproduction of proinflammatory

cytokines, pathological over-activation of ADAM17, persistently suppressed ACE2 expression, and AngII accumulation is closed

The kallikrein-kinin system is also involved in the pathogenesis of COVID-19 via ACE2. Under physiological conditions, it regulates coagulation, inflammation and pain (11). The main activities are associated with the peptides bradykinin (BK), Lys-BK, [des-Arg9]-BK (DEABK) and Lys-[des-Arg9]-BK (LDEABK. After binding to the receptor pore PB2, BK and Lys-BK induce locally to increase the synthesis of nitric oxide and realize a vasodilatory effect.

It opposes the vasopressor effects of the RAAS by balancing them. Bradykinin regulates the secretion of tissue plasminogen (tPA) and plays an important role in thrombus formation (12). The peptides DEABK and LDEABK interact with βB1 and play an important role in inflammation (13,14). Unlike βB2, βB1 is weakly expressed in endothelial cells, but is induced after tissue injury and is overexpressed under the influence of proinflammatory cytokines IL-1B, TNFα, IL-2, and IFNγ (13). Upon activation, βB1 can exacerbate the inflammatory response by hypersecreting proinflammatory cytokines and promoting neutrophil infiltration (15). The receptor with proteolytic activity ACE2 cannot inactivate bradykinin, but is able to hydrolyze the terminal chains of DEABK and LDEABK, making them lose affinity for BPB1. Therefore, internalization of ACE2 upon SARS-CoV-2 infection will create an imbalance in the kallikrein-kinin system, causing overstimulation of the DEABK/LDEABK/BRP1 axis and subsequent severe inflammation, vascular effusion, and angioedema (16). Although KKS potentiates the pathology in COVID-19, its regulation has not been targeted by the main therapies applied to date. According to the available literature, there is only one treatment regimen that targets RAAS and KKS simultaneously (17).

The involvement of ACE2 in the apelinergic system (AS) is also at the fringes of worldwide research interest. Apelin peptides are a family of proteins that bind to the apelin receptor and mediate protective effects on the cardiovascular system (18). The apelin signaling pathway leads to increased ACE2 mRNA transcription and ACE2 expression (4). On the other hand, however, ACE2 hydrolyzes the C-terminus of phenylalanine in apelin and inactivates it. Thus, mutual regulation between apelin and ACE2 is realized. In addition, the pyr-apelin-13 peptide suppresses Ang II-mediated free radical (superoxide) production, myocardial hypertrophy, dysfunction, and fibrosis (19). Through its mono-carboxypeptidase activity, ACE2 cleaves and inactivates apelin-13 and apelin-36 peptides (20). The interrelationship between the apelin receptor and the AT1R responsible for the destructive effects of AngII is interesting. Active receptor leads to heterodimerization of the AT1R and sequesters it, thereby disrupting the AngII/AT1R axis (4).

The B0AT1 receptor is expressed in the intestinal epithelium and kidney and has a role in the absorption of neutral amino acids. The ACE2-B0AT1 complex occurs as a dimer or heterodimer (21). Thus, ACE2 performs a completely different function, regulating amino acid transport, and antimicrobial peptides expression, and interacting with the GIT microbiome (22). The regulation of ACE2-B0AT1 in SARS-CoV-2 infection remains unexplored.

ACE2 tropism: physiological conditions after SARS-COV-2 infection

The expression of ACE2 under physiological and pathological conditions (SARS-CoV-2 infection, chronic cardiovascular, pulmonary, hepatic, and endocrine diseases) in various human tissues has been the subject of scientific interest due to its key role as a gateway to COVID-19. The influence of the demographic, and geographic factors on the occurrence, progression and prognosis of the disease is interesting. High expression of ACE2 was found in the small intestine, testis, kidney, heart, thyroid gland, adipose tissue and salivary glands. Moderate expression is described in the pancreas, esophagus, lung, colon, liver, adrenal gland, while low expression of ACE2 is found in nervous tissue, stomach, uterus, blood vessels, bone marrow, and spleen (23). No gender- or age-related differences in receptor expression were found (23). Other similar studies have focused on ACE2 gene expression specifically targeting the respiratory system and found similar tissue tropism (24). High expression of ACE2 was found in type II epithelial cells of alveoli and nasal epithelium, in which very high co-expression of ACE2 and TMPRSS2 was detected. This would explain the predisposition of these cells as a gateway in SARS-CoV-2 pathogenesis (24).

An interesting finding in a study by Xu et al., who found ACE2 expression in oral mucosa, the epithelial cells of the dorsum of the tongue and in lymphocytes (25). This also provides a logical explanation for the serious lymphocytopenia especially in severe cases of COVID-19 (26). Despite local oral lesions associated with SARS-CoV-2 infection such as taste loss, xerostomia, mucosal ulcerations, enanthemas, and macules, the role of the oral mucosa in the pathogenesis of the disease is poorly understood. In a March 2021 publication in Nature Medicine, Huang and colleagues presented a detailed comparative multi-methodology study of ACE2 and the TMPRSS family (TMPRSS2, TMPRSS4, TMPRSS11D). They found that the investigated biomarkers are expressed in the gingiva, minor and major salivary glands, buccal mucosa, ventral and dorsal part of the tongue, soft and hard palate, palatine tonsils (27). The oral cavity is an important portal of entry for SARS-CoV-2, and saliva is a potential route for the virus to spread, including extraoral, oral-pulmonary, and oral-intestinal transmission (27).

Studies on the cellular expression of ACE2 are linked to the study of ACE2 gene polymorphism. More than 1,700 gene variations in ChinaMAP and 1KGP databases are annotated,[27,28] seeking to answer how do the structural and spatial variations of proteins in the binding domains of ACE2 affect (in the protective or destructive way) the adherence of SARS-CoV-2 (28,29).

Molecules modulating ACE2 expression and activity

Despite an intensive scientific research, there is still uncertainty about the pathogenetic mechanism of SARS-CoV-2 binding to the cell wall. An additional curious fact raising discussion is the low expression of ACE2 in the respiratory system on the one hand, and the severe lung pathology on the other. Trying to explain this lack of correlation, alternative modulators, co-receptors and adhesion factors were investigated: neurothelin's, heparan sulfate (HS), sialic acids (SA), CD147 and GRP78 (24,25).

The neurothelin receptor NRP1 (VEGF165R) may be a co-receptor for SARS-CoV-2 along with ACE2, potentiating virus binding (29). Heparin sulfate binds to the Spike protein of SARS-CoV-2 and induces a conformational change that stabilizes the open configuration of the S1 subunit. Since only in this form a binding to ACE2 occurs, HS promotes infection.[30] A similar role was described for SA (31). Hoffmann et al. found that ammonium chloride, an endosomal pH modulator, could block the activity and inhibit the entry of SARS-CoV-2 in TMPRSS2–293T cell lines (32). Another TMPRSS2 inhibitor, camostat mesylate, partially blocks the virus, and in combination with E-64d (inhibitor of cathepsin B and L), it can completely stop the attachment of SARS-CoV-2 to the cell membrane and realize a protective effect (32).

The alternative receptor CD147 was found to be highly expressed by IHC in alveolar epithelium, and a direct association with the spike protein was demonstrated (33,34). The chaperone BiP (HSPA5, GRP78), which is expressed upon cellular stress, can also bind SARS-CoV-2, although literature data are debatable (32).

Recently, due to the great interest in ACE2 and with the help of bioinformatics and RNA-sequencing, the whole human ACE2 genomic region was revised and a new isoform of ACE2, called deltaACE2 (δACE2), was discovered, which is different from the full-length ACE2 (full-length ACE2, flACE2) in the 356 aa N-terminal region (35,36). This renders deltaACE2 unable to bind to SARS-CoV-2 (36). Analysis of flACE2 and dACE2 found that IFNα, IFNγ, and IFN-l3 could induce δACE2 expression in human bronchial epithelial cells and prevent binding to SARS-CoV-2 (32). The interferon-dependent regulation of ACE2 mRNA remains to be elucidated, though low plasma levels of IFNα were found in critically ill patients with COVID-19 as compared to those with milder symptoms, and IFNα plasma levels remained stable for 17 days in mild cases (37,38).

CONCLUSION

The RASS system and the ACE2/angiotensin-(1–7)/MAS axis play important roles in various physiological and pathophysiological processes.

Since ACE2 is a major player in the SARS-CoV-2 binding and host cell entry, and is highly expressed in various organs and tissues, it is particularly important to trace the signaling pathways through which this connection may be affected.

Knowing the main mechanisms of action would allow the development of appropriate cytoprotective

substances blocking critical points or reducing the multiorgan dysfunction and hyperinflammation.

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LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION- WHAT IS DONE AND PROSPECTS FOR FUTURE STUDIES

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ABSTRACT

Background:

Lymphocytic choriomeningitis virus (LHMV) infection is a neglected rodent-borne zoonotic infection but it is found all over the world because of the cosmopolitan distribution of its reservoirs. The diagnostic of this disease is not widely applied that is why it has been underreported. The aim of this study is to investigate infection with LCMV in hospitalized patients in 2015-2022 in Bulgaria and to analyse the data compared to the worldwide data available in this field of research.

Materials/methods: A total of 66 serum samples and 25 cerebrospinal fluid (CSF) samples from 73 patients with suspected LCMV infection from different hospitals in Bulgaria were collected. All samples were tested with a commercial enzyme-linked immunosorbent assay (Human LCMV-Ab ELISA, SSBT, China), based on the principle of double-antibody sandwich technique to detect Human LCMV-Antibody.

Results: A total of 11/91 (12.09%) positive samples were found in 5 males and 6 females throughout the study period. The positive samples were from patients from the cities: Sofia, Stara Zagora, Montana. A total of 3/25 (12%) positive samples were from CSF samples and 8/66 positive samples (12.12%) were from serum samples.

Conclusions: It's found that this infection occurs in our country and should not be underestimated, due to the possible severe neurological course and the danger of fetal damage in pregnant women. The diagnosis of LCMV infection is based on previous experience, placed in the light of the continuous introduction of new more sensitive and specific approaches.

Key words: *Lymphocytic choriomeningitis virus*, diagnosis, serology

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INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) infection is an acute viral disease, zooanthroponosis, occurring in various forms in humans, and causing intrauterine infection with fetal damage in pregnant women. In the recent decades, LCMV has been prevalent among mice in the Americas, Africa, Asia, Europe, Australia; it circulates practically worldwide. It can cause aseptic meningitis, especially in patients who report contact with rodents, although clinical cases are rarely recorded. The diagnosis of this disease, mainly serological, is limited to a small number of laboratories and it is difficult to determine the incidence and seroprevalence rates [1, 2]. LCMV was the first isolated arenavirus, detected accidently in 1933 by Armstrong and Lillie during an encephalitis outbreak (St. Louis, Missouri, USA). In Bulgaria, LCMV was isolated for the first time in 1956 [3]. LCMV infection is detected in 20% of the cases of aseptic meningitis [3]. There are about 30 strains of LCMV that have been isolated from rodents and humans in the USA, Europe, Japan that show distinct patterns of tissue tropism. [4, 5]. Traub identified the common house mouse (*Mus musculus*) as the natural reservoir host of the virus in 1935 [6]. Mice infected in the intrauterine period cannot mount an immune response and develop asymptomatic life-long infection with large quantities of virus in nasal secretions, saliva, milk, semen, urine, and feces [7]. Hamsters and pet mice have been identified as sources of infection [8, 9]. In animals, horizontal and vertical transmission is possible and bites also play a role. Human infections occur after mucosal exposure to aerosols contaminated with rodent excreta, direct contact with rodents or through rodent bites [10]. Transmission from human to human has not been documented, except through organ transplantation

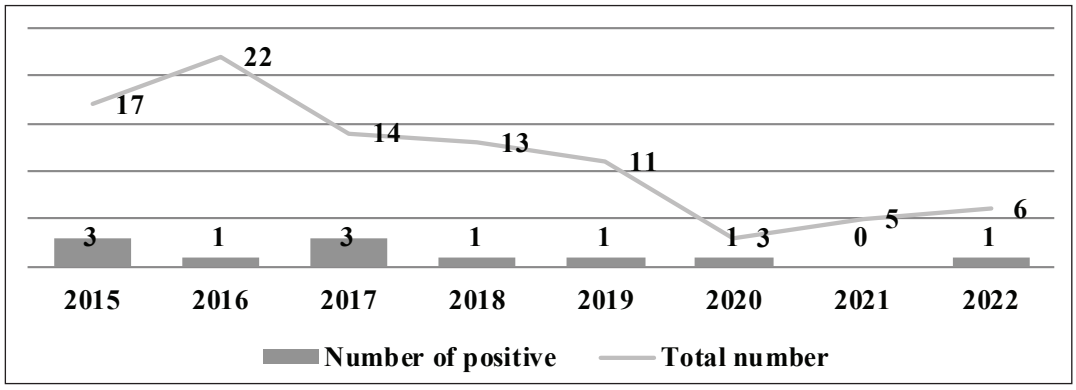

Figure 1. Distribution of number of positive samples over all samples tested.

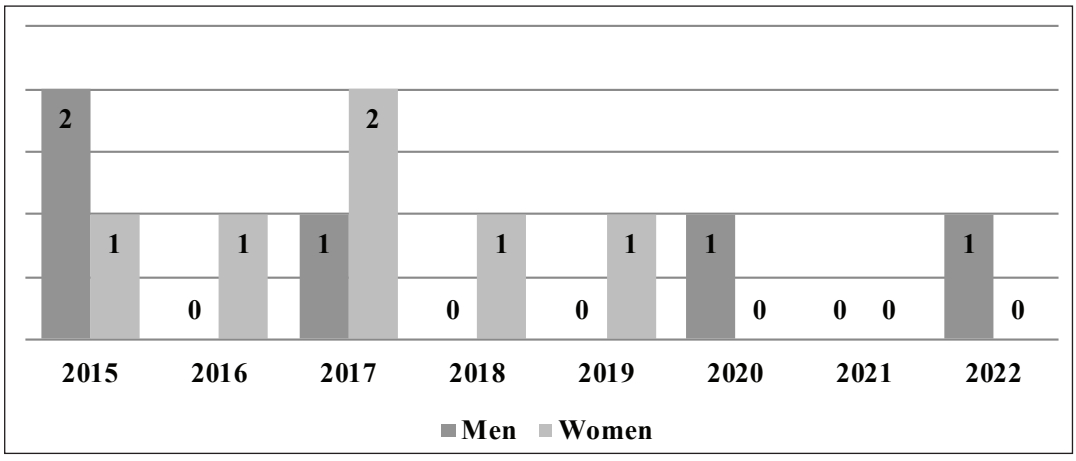

Figure 2. Distribution of positive samples of the patients by sex.

[11, 12] and vertical transmission from infected pregnant women to fetus [13]. The portal of entry is the mucosal lining of the digestive system or the respiratory tract. The viruses attach to cell surface receptors during the infection and are internalized by endocytosis. The incubation period is 3-13 days. The clinical forms of LCMV infection are: inapparent, flu-like, meningitis, meningoencephalitis, hemorrhagic, fetal infections. LCMV infection may be asymptomatic in immunocompetent persons (one third of infected or present as a non-specific, self-limiting febrile disease. However, the illness can progress to meningitis or meningoencephalitis. Most cases recover fully within one to three weeks [14]. In immunocompromised patients, such as organ transplant recipients, infection can resemble the Lassa hemorrhagic fever with a very high fatality rate [11,12]. Many reports from Germany, Lithuania, and France in the 1970s have shown the association of intrauterine LCMV infection with spontaneous abortion and a congenital LCMV infection that occurs with hydrocephalus, chorioretinitis and periventricular calcifications in 87.5% of the cases [15-17]. Mortality among newborns with congenital infection is about 35% and 70% of them show long-term neurologic consequences [18]. There are also asymptomatic forms of the infection, as evidenced by the presence of antibodies in non-diseased individuals. The prevalence of LCMV in patients with fever and neurologic manifestations and healthy persons was analyzed in southern Iraq (2012-2016). The seroprevalence was 12.2% in the healthy control group and 7% in the acute febrile patients [19]. The most typical classic form is meningitis. Fever lasts 7-14 days and often has a two-wave pattern. During the second phase, which develops after 8-10 days, neurological manifestations also appear. The disease has a long undulating course with periods of improvement and deterioration but the prognosis is favorable. The meningoencephalitis form is less

common but more severe, with more neurological symptoms and mental disorders. The CSF is often not clear, with elevated protein levels and pleocytosis, mainly lymphocytes (98-100%).

The aim of this study is to investigate infection with LCMV in hospitalized patients in 2015-2022 in Bulgaria and to analyse the data compared to the worldwide data available in this field of research.

MATERIALS AND METHODS

A total of 66 serum and 25 cerebrospinal fluid (CSF) samples from 73 patients with suspected LCMV infection from different hospitals in Bulgaria were collected as follows: 13 serum samples and 4 CSF samples in 2015, 16 serum samples and 6 CSF samples in 2016, 11 serum samples and 3 CSF samples in 2017, 8 serum samples and 5 CSF samples in 2018, 8 serum samples and 3 CSF samples in 2019, 3 serum samples in 2020, 3 serum samples and 2 CSF samples in 2021, 4 serum samples and 2 CSF samples in 2022. The samples were taken from 35 men and 38 women. The patients had the following diagnoses: encephalitis - 12, meningitis - 26, meningoencephalitis - 28, cerebral edema - 3. There were only 4 patients with flu-like symptoms and with epidemiological data of contact with rodents. Human LCMV-Ab ELISA kit (Shanghai Sunred Biological Technology Co., Ltd., China), based on the principle of double-antibody sandwich technique to detect Human LCMV-Antibody was used according to the manufacturer's instructions.

RESULTS AND DISCUSSION

A total of 11/91 (12.09%) positive samples were found in the patients throughout the study period in 5 males and 6 females (Fig.1, Fig.2). In 2015 three cases of LCMV infection (3/17, 17.65%) were detected in patient samples (two males and one female) using the ELISA method (Fig.1, Fig.2). The men had meningoencephalitis and cerebral edema, and the woman had meningitis (Fig.5). One of the men also had reported contact with rodents. In 2016 one positive sample (1/22, 4.55%) was found from a woman with meningoencephalitis (Fig.1, Fig.2, Fig.5). The number of laboratory positive samples in 2017 was three (3/14, 21.43%), from two women with meningitis and one man with encephalitis (Fig.1, Fig.2, Fig.5). In the period 2018-2020 infection was proven in three patients altogether – one in each year (1/13, 7.69%; 1/11, 9.09%; 1/3, 33.33%, respectively). Of the three, two were women with meningoencephalitis and the third was a man with meningitis (Fig.1, Fig.2, Fig.5). The small number of samples examined in 2020 is explained by the COVID-19 pandemic that started in 2020, when all other infections were neglected for a certain period of time. In 2021 only five samples from suspected patients were tested, but no positive samples were found among them. Of the six examined samples in 2022 one positive sample (1/6, 16.67%) was found from a man with meningitis (Fig.1, Fig.2, Fig.5). The four samples from patients with flu-like illnesses were tested but the results were negative.

High rates of positive samples were found in the years 2015, 2017, 2022, 17.65%, 21.43% and 16.67%, respectively. The highest percentage of positive samples was found in 2020, but too few samples were tested (1/3, 33.33%). The distribution of positive cases by age was as follows - two in each of the age groups 0 to 9 years, 40 to 49, 50 to 59 and 60 to 69 and one in the age groups: 10 to 19, 20 to 29, 30 to 39 years (Fig.3). The positive samples for LCMV infection were from patients from the cities: Sofia - 6, Stara Zagora - 4, Montana - 1.

A total of three (3/25, 12%) positive CSF samples and eight (8/66, 12.12%) positive serum samples were found (Fig.4). They were from patients with the following diagnoses: meningitis (5 cases), meningoencephalitis (4 cases), encephalitis (1 case), cerebral edema (1 case) (Fig.5). According to literature data, the most common form of infection is meningitis, which was also confirmed by us. A resent publication from 2021 for a study conducted in our country on the etiological structure of neuroinfections showed the presence of LCMV in 3% of the cases of viral and bacterial neuroinfections in the University hospital of Stara Zagora [20]. Several studies showed higher LCMV seropositivity in individuals exposed to rodents. LCMV was detected in 8% of hospitalized patients with neuroinvasive disease in 1950s in the United States, especially during winter when mice move indoors [21]. In the 1990s, the seroprevalence rates were found to be 2.3% in Argentina [22], 2.4% in Texas (USA) [23], 4%

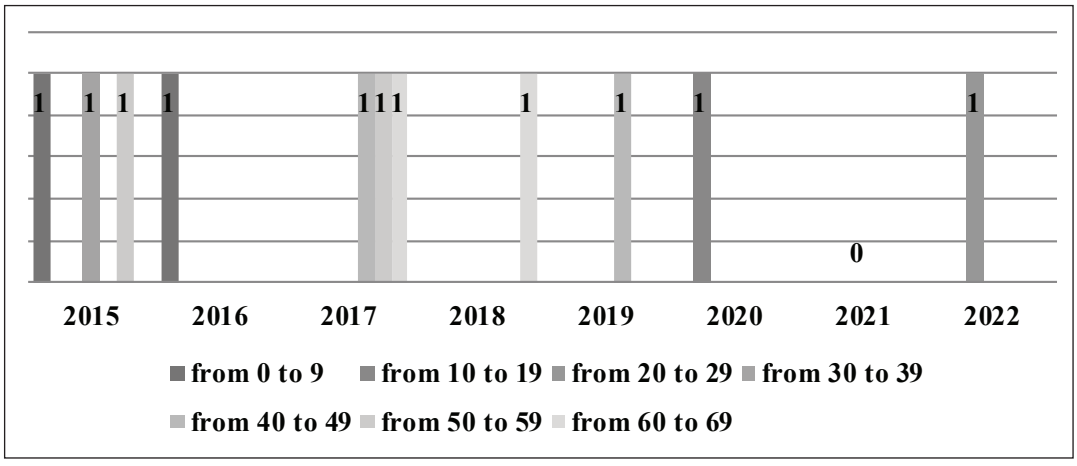

Figure 3. Distribution of positive samples of the patients by age.

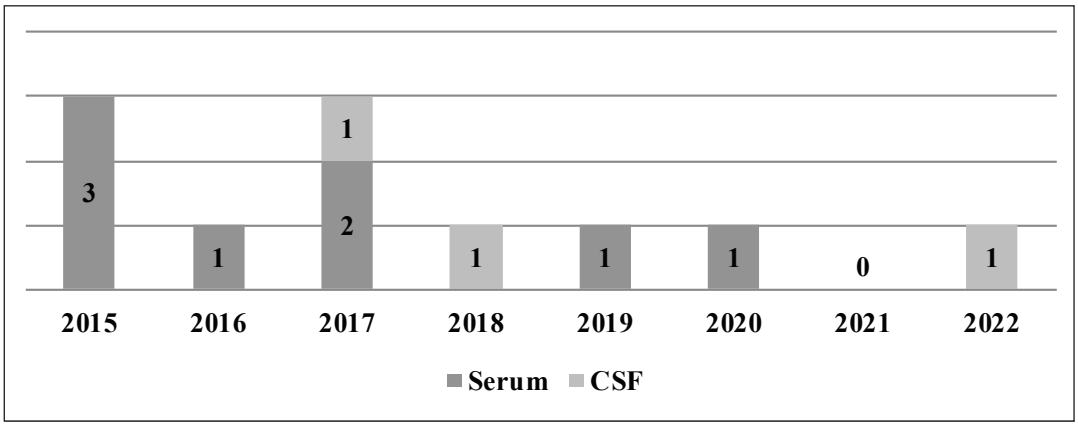

Figure 4. Distribution of positive samples of the patients by type of the sample.

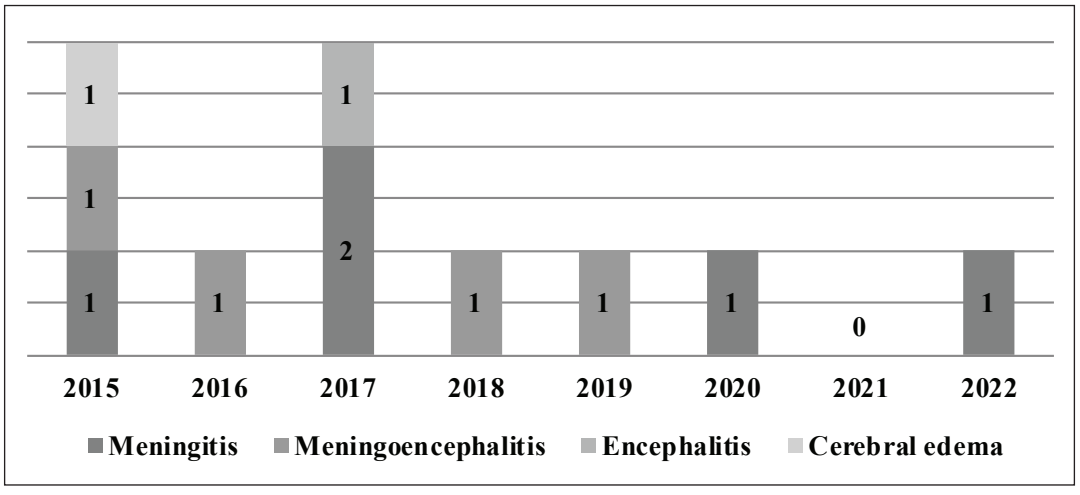

Figure 5. Distribution of positive samples of the patients by clinical manifestations.

in Canada [24], and 4.3-5.1% in Alabama (USA) [23]. Two studies from 2000s showed a seropositivity of 1.7% in Spain [25] and 3.3% in Argentina [26]. A study from Finland (2013-14) found that 5.0% of patients with neuroinvasive disease tested positive for LCMV IgG antibodies. Seropositivity was equally distributed between female and male patients [27], as our results also showed. A study in Gabon (2015-17) showed a high LCMV seropositivity of 21.5% [28]. Another study conducted in the continental Croatian regions (2017-18) showed an overall seroprevalence of 6.8%, ranging from 3.9% in non-exposed populations to 9.8% in professionally exposed individuals (forest workers, hunters, persons in frequent contacts with rodents) [29, 30]. Seroprevalence studies conducted among the general population have revealed that up to 15% seropositivity [31, 27, 25, 26, 32, 33]. This data correlates well with our results. An Austrian study found that 13% of employees of the Vienna zoo were LCMV seropositive [34]. A study from Italy conducted among forest workers showed an increase in the LCMV seroprevalence from 2.5% in 2002 [32] to 7% in 2015. Additionally, seroprevalence was high among hunters (12.9%) [35]. In 2012, 8 to 47% of the employees of rodent breeding facilities in the USA tested LCMV seropositive [36]. In our country, seroepidemiological studies conducted by Katsarov in the 80s showed the presence of antibodies among livestock breeders and forest workers between 6.8% and 24.6% [3].

Antiviral therapeutic options in human LCMV infection are currently exceptionally limited and the majority of research and future prospects are focused on re-purposing antiviral drugs approved for the treatment of other infectious diseases. The first antiviral drug repurposed for the treatment of transplantation-associated LCMV infection in humans was ribavirin, a guanosine analogue with complex mechanisms of action including direct inhibition of RNA-dependent RNA polymerase [37]. The antiviral drug favipravir (pyrazinecarboxamide derivative) inhibits the activity of RdRp of various RNA viruses. It is currently used and evaluated in clinical trials for the therapy of COVID-19 [38]. Umifenovir (arbidol) is an indolyc carboxylic acid that inhibits various stages of viral replication [39]. It is used for the prevention and treatment of influenza virus infection and is currently being evaluated in clinical trials as a COVID-19 antiviral drug. Kim et al. (2019) identified 10 anti-LCMV compounds inhibiting different steps in the replication cycle that represent promising candidates for further pre-clinical evaluation [40]. Bösch et al. (2020) showed that landornamide A inhibits LCMV infection in mouse cells in vitro. [41]. Encouraging data comes from a related field, the treatment of Lassa virus infection. Mire et al. (2017) reported that combinations of human monoclonal antibodies (huMAbs) specific for glycoproteins of Lassa virus provided a 100% rescue, even when treatment was initiated at advanced stages of the disease [42]. Several of these huMAbs cross-react with LCMV glycoprotein complex in vitro, suggesting that further studies specifically focusing on monoclonal-antibody based treatment of LCMV are warranted [43, 44]. The treatment of LCMV infection is pathogenetic and symptomatic. Immunity is humoral and cellular, and remains lifelong after recovery. There is no vaccine prophylaxis available. General epidemiological measures (control of rodents, protection of water sources and food, etc.) are carried out in the cases of infectious outbreak. Reverse genetically engineered recombinant LCMV (rLCMV) is an important candidate for the development of vector-based vaccines [45]. Schmidt et al. (2020) constructed a new vaccine TT1-E7E6 based on replicating attenuated LCMV [46]. An assay based on RT-PCR has been developed for the detection of LCMV RNA in blood and CSF. The highly sensitive assays target the envelope precursor glycoprotein (GPC) and nucleoprotein (NP) genes [47, 48]. Testing of serum and CSF by both serology and RT-PCR is recommended to improve diagnostic yield [49]. Additionally, next-generation sequencing has been used retrospectively for donor-derived LCMV infection [50]. The application of these new methods of diagnosis of LHMV infection and new drugs for its treatment, based on modern achievements in science are good future prospects for our country.

Many epidemiological studies showed that wild mice are infected with LCMV in some regions of the world. LCMV antibodies were detectedin 25% of Norway rats (*Rattus norvegicus*) from the UK [51], in 5.6% of wild rodents (6.1% *Apodemus flavicollis*, 3.3% *Clethrionomys glareolus* and 14.3% *Microtus arvalis*) in Trentino, Italy [32], in 2.9% of wild mice

(*Mus musculus*) in China [52], in 6.9- 20.1% of *M. musculus* in urban areas of Argentina [22]. Another data showed LCMV antibodiesin 2.4% of rodents trapped in Turkey [53], in 9% of mice (*M. musculus*, *M. spretus*) caught in Spain [25], in 10% among mice in Sincelejo, Colombia, [54]. The prevalence was highest in locations with high anthropogenic influence (53.33%), while it was significantly lower in rodents from natural habitats [55]. The seropositivity of trapped rodents from breeding facilities in the USA varied from 20.8% to 66% [36]. Only 0.4% of rats trapped in the Mekong Delta or purchased in wet markets in Vietnam (2012 - 2013) were LCMV seropositive [33]. There are currently no studies available on the presence of specific antibodies against LCMV in rodents in Bulgaria and this is a perspective field for future research.

CONCLUSIONS

Although the general clinical interest for the disease is low and LCMV is rarely considered, our study found 12.09% positive samples from hospitalized patients suspected for LCMV infection. Fatal LCMV infection in several cases of transplant recipients highlight the pathogenic potential and clinical significance of this neglected human pathogen. Even hough we have not conducted studies among pregnant women, other studies show that LCMV should be considered as a fetal teratogen [56, 57, 58] and obstetricians should be aware of an emerging role of LCMV in addition to TORCH panel. Timely diagnosis of LCMV infection using the most commo serological methods, as well as the introduction of modern molecular genetic methods will clarify the importance of this pathogen in our country and prevent possible complications in pregnant women.

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NONTUBERCULOUS MYCOBACTERIA - CURRENT RISK IN BULGARIA

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ABSTRACT

Background: Nontuberculous mycobacteria (NTM) are generally free-living and have a potential to cause opportunistic infection. More than 190 species of NTM have been identified (http://www.bacterio.net/mycobacterium.html), up to 60 species of them are pathogenic. The distribution is geographically specific for some species and others are cosmopolitan. Understanding their diversity has practical value for the treatment and management of NTM disease. Except for isolated reports, there is no accurate information about NTM spread among Bulgarian patients.

Material and Methods: We retrospectively analysed the data on the NTM isolated from patients throughout the country for the period from January 2010 to December 2017. Identification to the species level was done by Line Probe Assay (LPA).

Results: 586 NTM strains were identified. We calculated the rate of NTM isolation in Bulgaria and NTM disease incidence by applying microbiological criteria defined by the American Thoracic Society (ATS). As a result, the laboratory isolation rate amounted to 1/100 000 people for the period, and when applying only the ATS criteria, the prevalence of NTM disease was 0,23 per 100 000 people. In both cases, we reported that NTM disease incidence remains low as compared to tuberculosis, though with an increasing trend. The prevalence of NTM varied significantly between northern and southern Bulgaria, as well as between the capital and the rest of the country's regions.

Conclusions: Slow growing NTM species predominate in Bulgaria but rapidly growing ones have isolation levels higher than the average for Europe and closer to that of Asian countries. Given the demographic situation in Bulgaria and the changing climatic factors, NTM infections need special attention.

Keywords: Nontuberculous Mycobacteria, geographical diversity, LPA.

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INTRODUCTION

An increasing isolation of NTM has been reported worldwide and is most pronounced in countries with declining incidence of tuberculosis . The trend for Bulgaria is owards a permanent decrease in the incidence of tuberculosis - from 35/100,000 in 2011 to 19.1/100,000 in 2019, 13.4/100,000 in 2020, 9.9/100 000 in 2021 [1]. Except for isolated reports, there is no accurate information about NTM situation on the territory of Bulgaria [2, 3]. An assessment of NTM isolation rates, as well as the geographic variation in isolation and disease severity is lacking.

NTM diseases will continue to aggravate in the country as a result of aging population, environmental exposure and the impact of climate change, geographical region, and migration flows that occurred in the recent years.

METHODS

The study was retrospective, conducted in the National reference laboratory of tuberculosis (NRL of TB). It is the only laboratory in Bulgaria performing molecular identification of NTM (GenoType© CM/AS; Hain Lifescience, Germany), and supplemented with phenotypic, biochemical and immunochromatographic methods. We used data of patients suspected of tuberculosis with an isolated NTM strain from all the TB network in country (30 laboratories for culture diagnostics). For the period from January 2010 to December 2017 inclusive, 586 strains of NTM were isolated. To determine the frequency of NTM over the period, each isolate corresponded to one patient. Each clinical material/

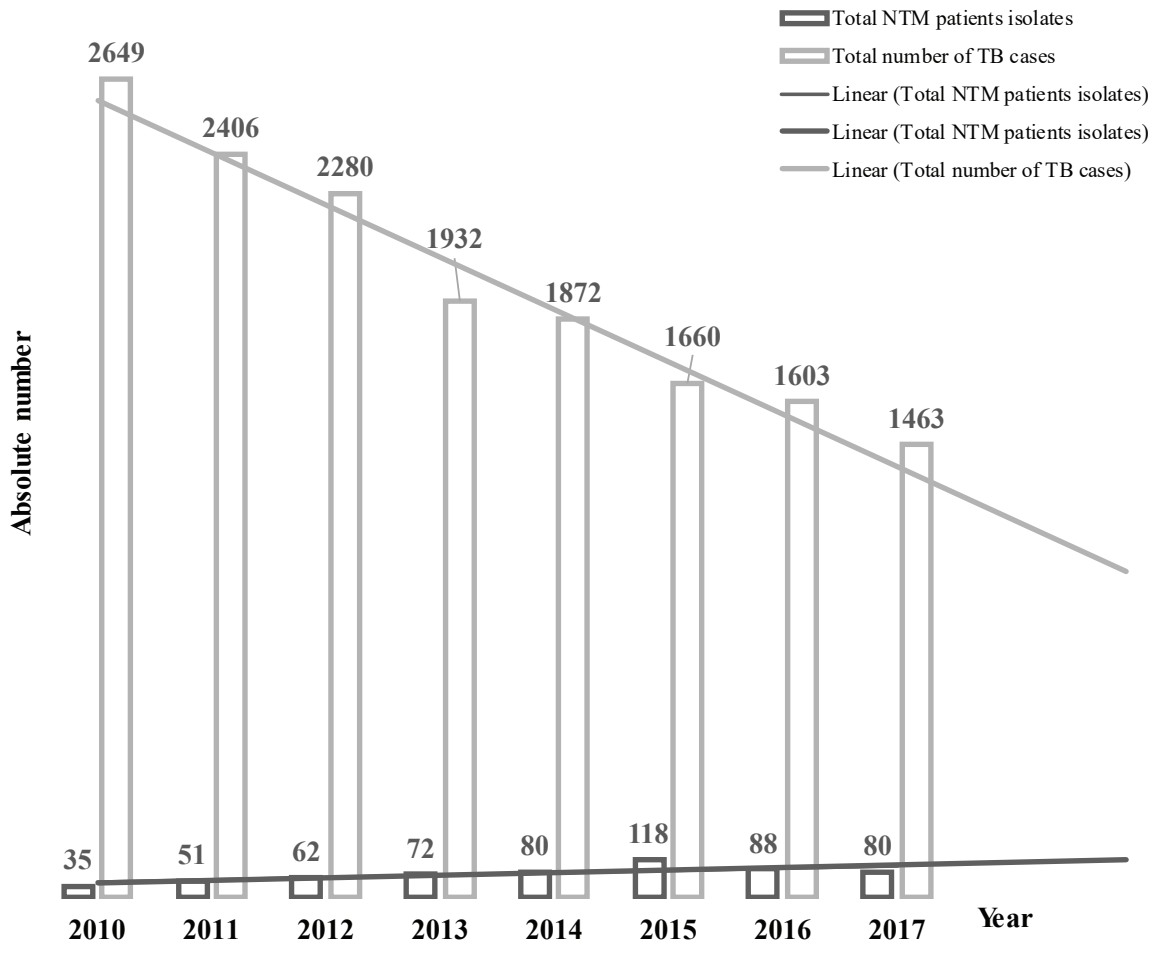

Figure 1. Distribution and trends for patients with isolated NTM strain and those registered with tuberculosis according to ECDC data for the study period.

strain was accompanied by information on the patient's age, sex, address, date and type of clinical material. We used demographic data reported by National Statistical Institute (NSI), which is a part of the Statistical Office of the European Communities (Eurostat).

Microsoft Excel (Microsoft, Redmond, Washington) was used to calculate prevalence rates, percentages, and mean ages.

RESULTS AND DISCUSSION

Patients and specimen data:

A total of 586 patients with identified NTM strain were confirmed in the NRL of TB. The trend was of increasing number of isolates over the eight-year period. There was a marked peak in 2015 (n=118, 20%). The reason was due to the accumulation of several factors including: introduction of the cycle for External Quality Assessment for cultural diagnostics of tuberculosis with differentiation between NTM and MBTC, modern laboratory equipment and regular supply of reagents and consumables, the professional qualification of the laboratory staff through periodic trainings.

According to our data, the global trend of increasing frequency of NTM isolation alongside with decreasing incidence of tuberculosis was observed in the country. (Fig 1).

Overall 97% (n=567) of the clinical materials were pulmonary (mostly sputum - 68% (n=392) and 11% BAL (n=67)). NTM strains from extrapulmonary sites (cerebrospinal fluid (CSF), urine, biopsy material) were 3% (n=19).

A positive result of smear microscopy had 8% (n=48) of all examined types of clinical material. Among them, smear positive sputum dominated - 85% (n=41). One of the two CSF included in the study was also positive.

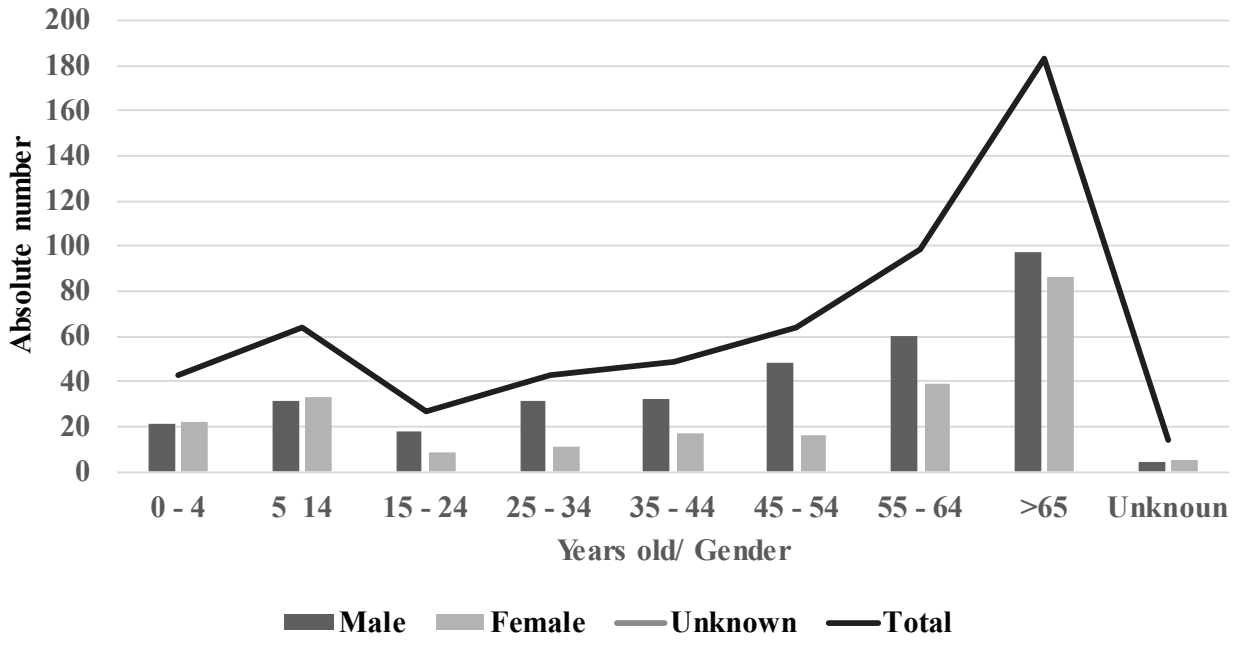

Figure 2. Gender and age distribution of patients with an isolated NTM strain, for the study period.

Demographic structure:

The gender ratio of patients with an isolated NTM strain followed the trend for tuberculosis patients in the country. NTM were isolated more often in males - 59% (n=343), 40% (n=237) were female and no gender information was available for six patients (1%). The biggest gender ratio difference was observed in the age group 45-54 years, where males were 75% (n=48) and females - 25% (n=16). Only in the age group 5 to 14 years old, a slight predominance of female gender was observed - 51,56% (n=33) (Fig. 2). This gender distribution, can be explained by the target group. We searched for and isolated NTM, mainly among suspected tuberculosis patients and those with pulmonary pathology as a differential diagnostic plan of TB. A more comprehensive national survey could change the situation.

The average age of the patients was 47 years.

Species structure of the isolated NTM in Bulgaria:

We performed species identification of isolated mycobacteria using conventional and molecular methods (LPA) and revealed valuable epidemiological information. The most frequently isolated in the country were the following species: *M. gordonae* (17%), *M lentiflavum* (15%), *M. forthuitum* (11%), *M. intracellulare* (9%), *M. chelonae* (8%), *M. avium* (7%). The total species diversity in the country, and their distribution according to the geographical regions and in the tree biggest cities: Sofia-city, Plovdiv and Varna are presented on Table 1. The species diversity of NTM was greater in Northern Bulgaria, but the frequency of their isolation was higher in Southern Bulgaria . In a few areas, only Slowly Growing Mycobacteria (SGM) were detected while Rapidly Growing Mycobacteria (RGM) were absent. That was valid in the regions of: Gabrovo, Smolyan, Targovishte, Shumen and Yambol. Montana and Haskovo were the regions dominated by RGM.

The influence of regional environmental factors could explain the situation, but this requires further investigation.

Slowly Growing Mycobacteria:

Slowly Growing Mycobacteria were dominant While *M. gordonae* prevailed all over the country, the reason for *M. lentiflavum* leading position was a peak in the children's ward of one hospital in 2015-2016. Possible contamination of gastric lavage at the stage of the clinical sampling procedure may explain this result.

As a total, the clinically significant isolates *M. avium - intracellulare* complex (MAC) were 16% (n=94) of NTM, and *M. kansasii* - 3% (n=17). While in EU *M. avium* is the dominant species in MAC, in Bulgaria *that* was *M. intracellulare*. It was more common in

Table 1. NTM isolation frequency in Bulgaria according to the geographic region.

NTM species	Total n (%)	Northern Bulgaria n (%)	Southern Bulgaria n (%)	Foreign residents n (%)	Sofia city n (%)	Plovdiv city n (%)	Varna city n (%)
M. abscessus	11 (1.9)	3 (27)	8 (73)		2 (18)	2 (18)	-
M. avium	42 (7.2)	11 (27)	30 (71)	1 (2)	22 (52)	-	4 (10)
M. celatum	1 (0.2)	1 (100)	-	-	-	0	1(100)
M. chelonae	47 (8)	12 (25)	35 (75)	-	6 (13)	21(45)	7(15)
M. forthuitum	62 (10.6)	15 (24)	47 (76)	-	19 (31)	17(24)	6 (10)
M. genavense/M. triplex	1 (0.2)	-	1 (100)	-	-	-	-
M. gordonae	102(17.4)	38 (37)	64 (63)	-	30 (29)	20 (20)	16 (16)
M. intracellulare	52 (8.9)	9 (17)	43 (83)	-	20 (38)	8 (15)	2 (4)
M. kansasii	17 (2.9)	5 (29)	12 (71)	-	7 (41)	2 (12)	4 (24)
M. lentiflavum	90 (15.2)	22 (25)	68 (75)	-	30 (33)	4 (4)	2 (2)
M. marinum	1 (0.2)	1 (100)	-	-	-	-	-
M. mucogenicum	10 (1.7)	6 (60)	4 (40)	-	1 (10)	2 (20)	3 (30)
M. peregrinum	19 (3.2)	8 (42)	11 (58)	-	6 (32)	2 (11)	3 (16)
M. scrofulaceum	2 (0.3)	1 (50)	-	1 (50)	-	-	1(50)
M. shimoidei	1 (0.2)	1 (100)	-	-	-	-	1 (100)
M. simae	1 (0.2)	1 (100)	-	-	-	-	-
M. szulgai	1 (0.2)	1 (100)	-	-	-	-	-
M. xenopi	2 (0.3)	2 (100)	-	-	-	-	-
genus *Mycobacrium*	123 (21)	43 (35)	80 (65)	-	34 (28)	9 (7)	19 (15)
mixed culture	2 (0.3)	1 (50)	1 (50)	-	-	-	1 (50)

males over 45 years old from Southern Bulgaria. *M. avium* was isolated with equal frequency in females and males over 25 years old, and predominated among the inhabitants of Southern Bulgaria. This was not surprising because *M. avium* is one of the species most frequently co-infecting the HIV-positive patients while in Bulgaria the rateof these patients is low. [4]. A possible risk factor for developing *M. intracellulare* lung infections is the exposure to domestic and natural water supplies [5]. This potential relationship may be the subject of a future study given the large volume of *M. intracellulare* lung infections (Table 1). At the same time, SGM (n=10) with single isolates in the country were identified: *M. celatum*, *M. genavense*, *M. marinum*, *M. shimoidei*, *M. simiae*, *M. szulgai* - 0.2% each. One of the most common NTM for European countries - *M. xenopi* was represented by only two isolates such as *M. scrofulaceum* (0.3%). One *M. scrofulaceum* was isolated from a foreigner - a citizen of Pakistan (Southeast Asia), where this species has a high isolation rate of 6.8% [6]. According to ATS we defined all species with single isolation as rare and geographically limited NTM species for our territory [7]. According to the address registration of those patients, predominated inhabitants of Northern Bulgaria& 90% (n=9). Notably, 40% (n=4) of them lived in the highly urbanized coastal region of Dobrich-Varna and only one (10%) lived in Southern Bulgaria, the district of St. Zagora. Some of the factors that could be considered include: the marine wetter and warm climate, karst rocks, characteristic of the area, as well as the incidence of tuberculosis [8]. It was shown that minerals widely found in clay soils such as kaolin facilitate the growth of NTM [9]. The frequency of tuberculosis for the region of Varna and Dobrich, is at the average level for the country (27.9/100 000), with a trend of increasing rate of MDR-TB [10].

When we compared our results to those of the other European countries, a unidirectional trend towards an increase of the overall NTM isolation rate was found. The most frequently reported species vary between the EU countries but always included: *M. avium*, *M. gordonae*, *M. intracellulare*, *M. fortuitum* and *M. xenopi* with an average of 7% unidentified isolates [11]. In the Netherlands, the increase was mainly due to isolation of *M. avium* and *M. gordonae* in pulmonary samples and was most pronounced in patients over 40 years of age [12]. In Italy, the most frequently isolated species were *M. avium* (29.2%), *M. intracellulare* (21.5%), *M. xenopi* (10.6%), *M. gordonae* (10.6%), *M. abscessus* (5.9%), *M. chimaera* (5.1%), and the most common patients - men over 75 [13].

***Rapidly Growing Mycobacteria*:** The isolation rate of 25% RGM in Bulgaria was closer to the Asian and higher than that for the EU countries. The dominant RGM - *M. fortuitum* (42%) prevailed in both Northern and Southern Bulgaria, followed by *M. chelonae* (32%), *M. peregrinum* (13%), *M. abscessus* (7%), and *M mucogenicum* (6%).

In the EU countries, the total of RGM strains represented between 10 and 20% of all NTM isolates. The most commonly identified were *M. abscessus* and *M. fortuitum* [7]. In Asia, RGM constituted 27% of NTM isolates compared to North America (17.9%), and South America (16%). Within Asia itself, there were differences in the levels of isolation between countries and regions. For example, in Tokyo (Japan) they were only 6,6% of all isolates, in contrast to South Korea (28,7%) and Taiwan (50%) [14]. In Taiwan, *M. fortuitum* and *M. abscessus* were the second and third most frequently isolated NTM species after MAC, while in South Korea *M. abscessus* was the second most frequently isolated NTM after MAC [14]. Serious problems due to the very high levels of antibiotic resistance of RGM were reported in the East Asian countries, including Japan, Korea and Taiwan [14]. The reason for the high rate of isolation of RGM in the Asian countries remains unclear, although geographical, climatic or racial ones have been discussed. Some studies focus on ecological niches and their subsequent transmission to humans [15]. Except that geographical reasons for distribution, RGM themselves (primarily *M. abscessus*) are often associated with patients with cystic fibrosis all over the world [16].

Without the possibility of more accurate identification we defined 21% of all NTM as genus *Mycobacterium*. The reason for this high percentage should be sought in the identification methods we used.

This was the first study that showed the frequency of isolation of NTM species from patient samples and estimated the nationwide NTM prevalence for Bulgaria.

We determined the rate of NTM isolation in Bulgaria as 1/100 000. The number of NTM patients' isolates (one NTM per patient if it duplicated during the years) was divided by the average annual population of the country, according to the National statistical institute data for the relevant year, and was multiplied by 100 000 [18]. Our results were comparable to EU countries with a population close to ours. Based on data from Croatia, Estonia, Greece, Luxembourg and Slovenia, NTM isolate rate were between 0.4-2% of samples [17]. Because of the often insufficient clinical and radiological data accompanying the samples, this approach was useful and predictive of disease. It had its limitations. In some cases, we would overestimate the severity of isolation (e.g., *M. gordonae*, *M. lentiflavum*) or underestimate it, if only one sample was sent for laboratory identification without additional conformation material or the patient was tested in a time interval ot in accordance with the study.

Only 23% (n=132) of the patients included in the study met the ATS criteria for diagnosis of nontuberculous mycobacterial disease [19]. Applying these, we obtained NTM disease incidence rates of 0,23/100 000 population.

CONCLUSION

Geography plays an important role in NTM species distribution in our country. The overall burden of NTM disease in Bulgaria is still low as compared to that of tuberculosis. It is higher in the most urbanized settlements. We are facing an emerging problem. The incidence of current disease cases is strongly underestimated.

COMPLIANCE WITH ETHICAL STANDARDS

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DISCLOSURE OF CONFLICT OF INTEREST

There is no conflict of interest to declare.

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CLINICAL CASE REPORT: PERTUSSIS INFECTION FOLLOWED BY A PARAPERTUSSIS INFECTION IN THE SAME CHILD

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ABSTRACT

Whooping cough is a vaccine-preventable, acute respiratory disease caused by the gram-negative bacterium *Bordetella pertussis*. In recent years there has been a worldwide reccurrence of pertussis infection. The most vulnerable group in society are infants under one year of age, they are at the greatest risk of severe complications or even death. Whooping cough is usually associated with infection caused by *B. pertussis*, but *Bordetella parapertussis* can also cause pertussis-like symptoms. The disease is known as parapertussis. Clinical data alone are not sufficient to differentiate between the two infections. Modern, fast and reliable diagnostic is .needed. *Bordetella holmesii*, viral infections caused by RSV, adenovirus, etc. can present with pertussis-like symptoms and should be diagnosed and treated accordingly.

The aim of this study is to present a clinical case of a child with pertussis infection followed by parapertussis infection. To diagnose and differentiate the two infections, a real-time PCR molecular genetic method was used to detect the genes specific for the causative agents.

Pertussis vaccination does not protect against B. parapertussis infections, and cross-immunity between the two bacteria has not been observed. Therefore, in the presence of pertussis-like symptoms (paroxysms of persistent cough, vomiting after coughing), it is advisable to differentiate between *B. pertussis* and *B.parapertussis* infection, especially in populations with high pertussis vaccination coverage.

Key words: PCR, *Bordetella pertussis, Bordetella parapertussis*, vaccination

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INTRODUCTION

Whooping cough is primarily a vaccine-preventable, acute respiratory disease caused by the gram-negative bacterium *Bordetella pertussis*. Infants under one year of age are the most vulnerable age group in society, with the highest risk of severe illness, hospitalization or even death (1). Since the introduction of pertussis vaccinations (with whole-cell and acellular pertussis vaccines), pathogen prevalence, morbidity and mortality have been significantly reduced worldwide (2). During the last two decades a resurgence of the disease and an increasing incidence of cases have been observed, even in countries with high vaccination coverage (3, 4).

Pertussis infection continues to be widespread, with a proven cyclicity of outbreaks (5). The main reasons for these trends are several: the established genetic differences between vaccine and community-circulating strains, proven antigenic changes leading to stronger immune suppression, improved disease surveillance, improved laboratory diagnostic methods (4).

According to ECDC data for 2018 the reported cases of pertussis in 30 EU/EEA countries were 35636 with 10 deaths. For 2019 there were 38992 cases of the disease and 11 deaths, and for 2020 - 12113 cases and four deaths, respectively (6). The significant reduction in reported cases of whooping cough for

2020 (as for other respiratory diseases) is probably due to the measures limiting the Covid 19 pandemic (lockdown, social distancing measures, imposed isolation, work from home, closure of childcare facilities - nurseries, kindergartens and schools).

Whooping cough affects all age groups in society, but infants under one year of age are the most vulnerable, with the highest risk of severe illness, hospitalization or even death. ECDC reported an incidence rate of 44.4 per 100,000 and three deaths in this age group for 2018 (1). According to CDC data, the pertussis hospitalization rate for patients under 6 months of age is the highest - 30.8% (7).

Whooping cough is usually associated with an infection caused by *B. pertussis*, but *B. parapertussis* can also cause pertussis-like symptoms. Parapertussis is a highly contagious respiratory disease affecting all age groups of the population. The causative agent is the Gram-negative bacterium *B. parapertussis*, which is related to *B. pertussis*, but does not produce pertussis toxin, due to mutations in the promoter region of the genes encoding this toxin (10). Clinical findings in patients infected with parapertussis vary. Some patients are asymptomatic, others show typical pertussis symptoms in terms of severity and duration of the cough, nocturnal attacks and post-cough vomiting, but most patients with *B. parapertussis* have milder symptoms with shorter duration (8, 9).

Infants (under 6 months of age) may experience a more severe course of parapertussis infection. A pertussis and parapertussis co-infection is possible in all age groups.

After an infection with parapertussis, immunity is established, but there is no cross-immunity between pertussis and parapertussis. Therefore, pertussis vaccination does not protect against parapertussis infection (11, 12).

CLINICAL CASE

The clinical case describes a 5-year-old male patient who was diagnosed with pertussis and ten months later - with parapertussis . A parallel molecular genetic assay for the species *B. pertussis* and *B. parapertussis* was conducted.

The clinical signs `during the first infection were a prolonged dry cough for 1 month with severe coughing bouts. The child was afebrile. Treatment with azithromycin was carried out for 6 days before taking the material for genetic testing. The patient had received all doses of pertussis vaccines according to the Immunization Calendar of the Republic of Bulgaria (13).

Clinical symptoms when proving *B. parapertussis* during the second examination were a dry cough for two weeks without severe coughing bouts or fever. Antibiotics had not been applied before taking a sample for molecular genetic testing.

MATERIALS AND METHODS

Two clinical samples (nasopharyngeal secretions) from the patient were obtained in the NRL "Molecular Microbiology" in NCIPD, from which DNA was extracted for the diagnostic of pertussis and parapertussis.

To detect *B. pertussis* and *B. parapertussis*, a Real-Time PCR test was used that detects genes specific for the two causative agents. To prove *B. pertussis*, an analysis was performed to detect the pertussis toxin gene promoter, and to prove *B. parapertussis*, gene IS1001 was analyzed. The assays were performed using an RT- PCR analyzer Gentier96,TIANLONG, China. The simplex PCR assay was performed in 20µl of reaction mixture containing 10µl 1x real-time PCR buffer Takara Premix exTaq, 1,2µl of 10 µM of each primer (F/R), 1,2µl of 5 µM probe, 1,4µl dH_2O and 5µl of extracted sample DNA. The cycling condition included: 95^0C for 30s, 45 cycles: 95^0C for 5s, 62^0C for 30s. Data acquisition of a signal was set at 62^0C during each cycle. Primers and probes used in the RT PCR were:

ptxP-F 5-GCGTGCAGATTCGTCGTAC-3,
ptxP-R 5-TGATGGTGCCTATTTTACGG-3,
ptxP-Probe 5-FAM-
ACACGGCATGAACGCTCCTTCGGC-3 BHQ1;
IS1001-F 5-AGCGGCTTGCCTGGCTGGGGCGATA-3,
IS1001-R 5-CCGGGCCGTCTCGCGTGAGCGTCCT-3,
IS1001-Probe 5- FAM-
AGGCTCGGCTGCGTGCGTCGGTGCGCG-3 BHQ1 (!4).

RESULTS AND DISCUSSION

A parallel analysis for the presence of *B. pertussis* and *B. parapertussis* DNA was performed with both nasopharyngeal secretions from the same child. Analysis of the first material detected the

pertussis toxin gene promoter (a marker for pertussis infection) and negative amplification for IS1001 (for detection of *B. parapertussis*) After the first antibiotic treatment, the follow-up result for *B.pertussis* was negative. The second test ten months later was negative for *B. pertussis* amplification and positive for *B. parapertussis*. The second test, after applied antibiotic treatment, was negative for *B.parapertussis*.

Infections caused by *B. parapertussis* are common but usually overlooked and underdiagnosed. Data indicate that 1% to 35% of *Bordetella* infections are caused by *B. parapertussis* (8).

CONCLUSION

It has been shown that clinical symptoms alone are insufficient to distinguish pertussis from parapertussis infections (15). Some viral infections caused by RSV, adenovirus, etc. may present with similar pertussis-like symptoms. In patients with pertussis-like symptoms, especially in populations with high immunization pertussis coverage, highly specific molecular diagnostic tests are needed. RT-PCR permits to establish a rapid and accurate etiological diagnosis, and in the case of a proven viral infection - to prevent the unnecessary use of antibiotics.

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ANNUAL ANALYSIS OF PARASITIC INFECTIONS IN BULGARIA IN 2022

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ABSTRACT

Introduction: Infections caused by parasites still represent an important global health problem. Although parasitic infections are responsible for significant morbidity and mortality in developing countries, they are also prevalent in developed countries. This study aims to establish the dynamics of the parasitic infections registered in the country (local and imported) and to analyze the situation, based on data from the previous years.

Methods: We used the annual reports of regional health inspectorates, diagnostic laboratories, medical universities, and data from the National centre of infectious and parasitic diseases about all individuals infected with parasitic diseases in the country.

Results: A total of 635,522 persons were examined in 2022, of whom 1.82% were diagnosed with various parasitic infections. In the local helminthic zoonoses such as echinococcosis and trichinellosis, a significant decrease in morbidity was observed,1.3‰ (n = 89) for cystic echinococcosis and 0.16‰ (n = 9) for trichinellosis. For soil-transmitted helminthiases (ascariasis and trichuriasis), the incidence was 6.7‰ and 0.5‰, respectively. Data on enterobiasis does not show any particular dynamics in 2022 either. The registered prevalence for the country was 1.48%, and for children from various childcare facilities - 2.45%. During the year, seven cases of vector-borne parasitic diseases were registered in the country: imported malaria was diagnosed in 5 patients (4 Bulgarian citizens and one foreigner), and autochthonous visceral leishmaniasis - in two persons.

Conclusions: The analysis shows that, in spite of a trend of declining morbidityfor some socially significant protozoan and helminth infections, human parasitic diseases still represent a significant problem with social and medical consequences for the population of our country. Therefore, it is necessary to preserve and strengthen the surveillance and control network by including new personnel in the medical parasitology structures.

Keywords: parasitic diseases, incidence, prevalence, zoonoses

Category of the manuscript: Original article

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INTRODUCTION

Infections caused by parasites still represent an important global health problem. Although parasitic infections are responsible for significant morbidity and mortality in developing countries, they are also prevalent in developed countries. Early diagnosis and treatment of parasitic infections are critical not only to prevent individual morbidity and mortality but also to reduce the risk of spreading in the community (1). The increasing burden of globally distributed parasitic diseases in the era of COVID-19 requires new efforts, targeting not only the clinical manifestations of the diseases but also - rapid diagnostic methods, novel therapeutic approaches, vaccine development and surveillance programs. All these are essential, together with the social, economic and environmental conditions (2).

This study aims to establish the dynamics of the parasitic infections registered in the country (local and imported) and to analyze the situation, based on data from the previous years.

Materials and methods.

Study design

It is a retrospective cross-sectional study of all registered cases of parasitic infections in the country for the period January-December 2022. Data was provided from the annual reports of the Regional Health Inspections (RHI) and the National Reference Laboratory for Diagnosis of Parasitic Infections (NRL)

at the National Centre of Infectious and Parasitic Diseases (NCIPD). Epidemiological indicators such as prevalence (for frequently asymptomatic or oligosymptomatic parasite infections) and morbidity per 100,000 population (for parasitic diseases with pronounced clinical symptoms) were determined. To establish trends in the dynamics of individual nosological units, the data for 2022 were compared with those for previous years.

Methods of parasitological diagnosis

Depending on the level of the laboratories performing parasitological diagnosis in the country (independent medical-diagnostic laboratories, laboratories at RHI, hospitals, Medical Universities, NCIPD), a wide range of diagnostic methods were used: microscopic, immunological, cultural and biomolecular.

Individuals examined for parasitic pathogens

According to prophylactic and epidemiological indications for parasitic diseases were examined children attending nurseries and kindergartens, professionals subjected to annual parasitological examinations, migrants arriving from countries endemic for certain parasitic diseases, and patients referred by other medical institutions.

Ethical considerations

The analysis was based on routine diagnostic procedures in accordance with the rules of good medical practices, containing only aggregated depersonalized data that and are practically retrospective in nature. Therefore, opinion statement and permission from the Institutional Ethics Committee were not required.

RESULTS

In 2022, a significant contingent of 635,522 individuals was examined in parasitological laboratories in the country, of whom 11,543 (1.82%) were diagnosed with various parasitic pathogens. Data on established parasitic diseases with local distribution are presented in Table 1.

Of the registered echinococcosis cases in 2022, 80 (90%) were primary and 9 (10%) were postoperative relapses, with 51 (57.3%) of the affected persons being male and 38 (42.7%) - female . The most

Table 1. Parasitic diseases with local transmission recorded in Bulgaria

Nosological entity	Number of examined	Number of positive	Incidence per 100,000 / Prevalence in %
Zooanthroponoses with epidemic risk			
Echinococcosis	573	89	1.3 per 100,000
Trichinellosis	35	11	0.16 per 100,000
Taeniasis	284,949	2	0.03 per 100,000
Soil-transmitted helminth infections			
Ascariasis	423,577	460	6.7 per 100,000
Trichuriasis	284,949	37	0.5 per 100,000
Community-acquired parasitic diseases			
Enterobiasis	449,741	6,656	1.48%
Giardiasis	430,791	503	7.4 per 100,000
Hymenolepiasis	413,039	99	1.45 per 100,000
Urogenital trichomoniasis	4,166	335	8.0%
Opportunistic parasitic infections			
Visceral leishmaniasis	17	2	0.03 per 100,000
Toxoplasmosis	8,773	1,455	16.58%
Blastocystosis	411,956	1,593	0.39%
Cryptosporidiosis	190	1	0.02 per 100,000
Pneumocystosis	88	10	0.16 per 100,000

affected age group was 30-49 years with 32 (40%) affected persons, followed by 50-69 years with 20 cases (22.5%), and children and adolescents between 5 and 19 years with a total of 19 cases (21.3%). There were no reported cases of echinococcosis in children under 4 years of age. The organ localization of echinococcal cysts was as follows: liver in 65 patients (73%), lung in 13 (14.6%), multiple echinococcosis in 5 (5.7%), and other localization in 6 (6.7%). The regions with the highest incidence of echinococcosis were Sliven - 7.06‰, Razgrad - 5.57‰ and Yambol - 3.4‰. In the regions of Vidin, Vratsa, Gabrovo, Montana, Pernik, Smolyan and Targovishte, there were no recorded cases for 2022.

During 2022, an epidemic outbreak of trichinellosis was reported in the village of Brestovitsa, Plovdiv region in January with nine affected persons (6 males and 3 females), out of 33 who had consumed raw-dried meat from wild boar. *Trichinella* larvae were not detected in the meat and minced meat provided for examination at the NCIPD. Two sporadic cases were also registered in males from the towns of Petrich and Novi Pazar. Both had consumed raw meat purchased from the commercial network

Cases of soil-transmitted helminthiases (ascariasis and trichuriasis) were registered throughout the country, with most cases of ascariasis diagnosed in the regions of Blagoevgrad, Sofia-capital and Burgas, and of trichuriasis - in the regions of Varna and Sliven. The RIH parasitology units reported 153 settlements endemic for soil-transmitted helminthiases in 10 country regions . In 43 (28%) of them prophylactic examinations were carried out, and 130,830 persons were examined, of whom 49 (0.04%) were diagnosed with ascariasis. Etiological treatment was prescribed for all infected, and control tests proved a 100% effectiveness of the treatment.

Imported parasitic diseases

In 2022, 4,920 persons were examined for imported parasitic infections: five Bulgarian citizens and 4,915 foreigners. Parasitic infections were found in 75 (1.5%) persons (71 foreigners and 4 Bulgarians).

A total of 1,993 persons were examined for malaria in 7 regions of the country and in NCIPD, of whom 4 were Bulgarians and 1,989 -foreign citizens (mostly migrants at the refugee centres in Sofia, Haskovo and Sliven regions). In 2022, five cases of imported malaria with causative agent *P. falciparum* were registered in four Bulgarian patients and one foreign citizen. In one of the Bulgarian patients, the disease ended up with a fatal outcome.

In 2022, 2,927 foreign citizens were examined for other imported parasitic infections. In 70 of them the following parasite species were found: *B. hominis* (n = 29), *G. intestinalis* (n =30), *A. lumbricoides* (n = 6), *H. nana* (n = 6), *T. trichiuris* (n = 1), *E. vermicularis* (n = 1). No autochthonous secondary foci were established from the imported infections and all infected individuals were promptly treated.

DISCUSSION

In 2022, with the gradual abatement of the COVID-19 pandemic, a larger volume of medical parasitology activities were carried out in the country as compared to 2021. The number of examined persons was significantly higher (635,522 vs. 596,659 for 2021) and respectively the established prevalence of parasitic pathology for 2022 was 1.82% vs.1.46% for 2021.

Cystic echinococcosis (CE) is a disease of great medical importance for Bulgaria. A study by Rainova et al. (2022), found an average incidence for the period 2011-2021 of 3.7 per 100,000 (3). In the recent years, the disease has shown a permanent downwards trend, both in absolute numbers of registered cases (192 in 2019, 95 in 2020, and 89 for each of 2021 and 2022), and as incidence (2.74;, 1, 37; 1.29 and 1.3 per 100,000 for 2019, 2020, 20121 and 2022 respectively). All age groups were affected, with people between the ages of 30 and 49 years comprising up to 40% of all infected. Unfortunately, the relative share of children and adolescents with CE was high - 21.3%, which in our opinion necessitates an active information campaign among this contingent regarding prevention measures. The relative share of recurrences in 2022 (10%) is comparable to that in previous years (6.7% for 2021 and 9.5 for 2020), (4) which questions the correct application of anti-relapse drug prophylaxis after surgical or PAIR treatment.

Although Bulgaria, together with Croatia occupied leading positions in terms of incidence of trichinellosis among the EU member states in 2021 (5), of the

number of epidemic outbreaks, the number of registered cases and the incidence per 100,000 for 2022 give grounds for slight optimism (Table 1).

Based on many years of hard work, the cases of soil-transmitted helminth infections endemic in the country (ascariasis and trichuriasis) have been reduced to an extent that does not burden the health care system, and in both diseases, there is a decrease in the number of annually registered cases (6).

Sporadic cases of taeniasis caused by *Taenia saginata* have been established in Bulgaria for many years, while those caused by *Taenia solium* and cysticercosis were not been registered in the last five years (4, 6).

Of great medical importance for the country are the community-acquired parasitic diseases (enterobiasis, giardiasis and hymenolepiasis), as they most often affect children and adolescents. After 2018, a significant increase in enterobiasis cases was observed. While in 2017 the prevalence in the age group up to 19 years was 1.56%, it increased to 2.1%, 2.36%, 2.76%, 2.5% (6) and 2.45% for the five following years, respectivelyThese data reveal a poor level of hygiene and health education on the matter among the population The other two infections (giardiasis and hymenolepiasis) have maintained relatively similar morbidity rates in the recent years.

Among the opportunistic parasitic infections, toxoplasmosis is the most widespread. The seroprevalence in the country in the recent years was relatively similar: 19.97% for the period 2015-2017 (7), 18% for 2018 (6) and 16.58% for 2022. Three cases of congenital toxoplasmosis have been reported in children up to 1year old from Plovdiv region. These data show that the laboratory control of toxoplasmosis and the diagnostic algorithms applied in pregnant women are good, and although monitoring is not mandatory, result in few cases of congenital toxoplasmosis, and incidents during pregnancy.

Among opportunistic infections, blastocystosis is in second place with prevalence of 0.39%, and relatively constant distribution among the population in the recent years (0.17%, 2020 and 0.44%, 2021) (4).

Noteworthy, the introduction of PCR methods for detection of *Pneumocystis jirovecii* DNA in NCIPD, the diagnosis of human pneumocystosis has significantly improved, together with the possibility for timely treatment and a favourable outcome of this life-threatening disease. In 2022, there were 10 patients with *Pneumocystis* pneumonia, as compared to 18 and 11 in 2020 and 2021.

Cryptosporidiosis is very rare in the country, and only one case of the disease was confirmed in the past year.

Regarding imported parasitic pathology, malaria is imported into the country every year. In 2022, five cases of imported malaria with the causative agent *P. falciparum* were registered, . Unfortunately, one of the affected Bulgarian citizens, co-infected with SARS-CoV-2, had a fatal outcome due to serious complications that occurred during the treatment of the co-infection. For the period 2000 - 2020, a total of 232 cases of imported malaria were registered in Bulgaria. Of these, 34 (14.7%) developed complications, eight of which were fatal. All patients with complications and fatal outcomes had malaria caused by *P. falciparum*. In all but one of the deaths, the diagnosis was made more than three days after the first clinical manifestations (9).

The rest of the parasitic pathology imported into the country consists of protozoa and helminths affecting the gastrointestinal tract, which also have a local distribution. All were diagnosed in foreign citizens, most of them migrants, and importantly they were diagnosed and treated promptly and the occurrence of autochthonous secondary foci was not allowed.

CONCLUSION

Based on the data for 2022, although considerable number of parasitic diseases are registered in the country every year, none of them exerts serious pressure on the public health care system. With A permanent trend of reduction is observed for the two entities with most serious medical and social impact (cystic echinococcosis and trichinellosis). Never-the-less they require increased attention as our country is still the leader in terms of morbidity among the member states of the EU. Although the import of malaria in the country is low, the disease is still a problem in terms of health knowledge, timely diagnosis and treatment. The presence of illegal migrants, some of whom are parasite carriers, poses a risk of local epidemic outbreaks, not only from malaria but also from other imported pathologies

for which suitable climatic and fauna conditions exist. Therefore, the continuous improvement of the qualification of specialists working in the field of medical parasitology and large-scale health information campaigns among the risk contingents are extremely important.

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Disclosure of conflict of interest

There is no conflict of interest.

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ASSOCIATION OF CLINICAL PRESENTATION WITH SEVERITY AND OUTCOME OF COVID-19

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ABSTRACT

Background. The world continues to struggle with the 2019 coronavirus disease (COVID-19). The pandemic is under control, but the disease exists and it is extremely important to have algorithms for early diagnostic and prognostic guidance. The aim of the study is to find correlations between the spectrum of clinical symptoms with the disease severity and the outcome of COVID-19, aiming to maximally early diagnosis and establishing early predictors for severity and fatal outcome.

Materials and methods. The study included 169 adults hospitalized at the University Hospital St. George, Plovdiv, between September 2021 and December 2022 with a PCR verified diagnosis of COVID-19. The methods of clinical analysis (history and clinical examination) and assessment of oxygen saturation were used. For the purposes of the study, patients were distributed into groups according to age (below and over 60 years); disease severity (moderate or severe/critical clinical course), and outcome (survived or died).

Results. According to analysed data, 92 patients (54.43%) were men, and 69 (40.82%) were under 60 years; 126 patients (74.5%) had a moderate and 43 (25.4%)- severe clinical course. The disease has a gradual onset in 149 (88.1 %). The most frequent initial symptoms were fever and fatigue (60.4 %), followed by fatigue with arthro-myalgias (26.2%). Co-morbidities were documented for 140 patients (82.8%). COVID-19 was severe/critical in 14.3% of patients under 60 years and 34.1% of patients ≥ 60 years ($p < 0.01$), with case fatality rate 7.4% vs. 25% respectively ($p < 0.001$).

Conclusion. Our data highlight the importance of advanced age (over 60 years) and comorbidities (arterial hypertension, diabetes mellitus, cirrhosis hepatis) as high-risk factors for severe course and fatal outcome of COVID-19

Keywords: COVID-19, clinical severity, outcome, symptoms

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INTRODUCTION

The COVID-19 pandemic was declared on March 11th 2020 by the World Health Organization (WHO) as a global threat to humanity. With 770 million confirmed cases of infection and 7 million deaths worldwide as of September 26th 2023, elucidating all aspects of the pathogenesis, thanatogenesis and management of the disease is a global priority.

COVID-19 is a novel disease with extremely varied symptoms, unpredictable course, and a possible sudden collapse of vital functions. The need for hospitalization was observed in 30% of cases. Intensive care was required in 17% as a result of cytokine storm followed by respiratory failure, acute respiratory distress syndrome (ARDS), and possible multiple organ failure (1).

The purpose of this study was to find correlations between the spectrum of clinical characteristics and the severity of the disease as well as the outcome of COVID-19, with the aim of establishing early predictors of a severe course and fatal outcome.

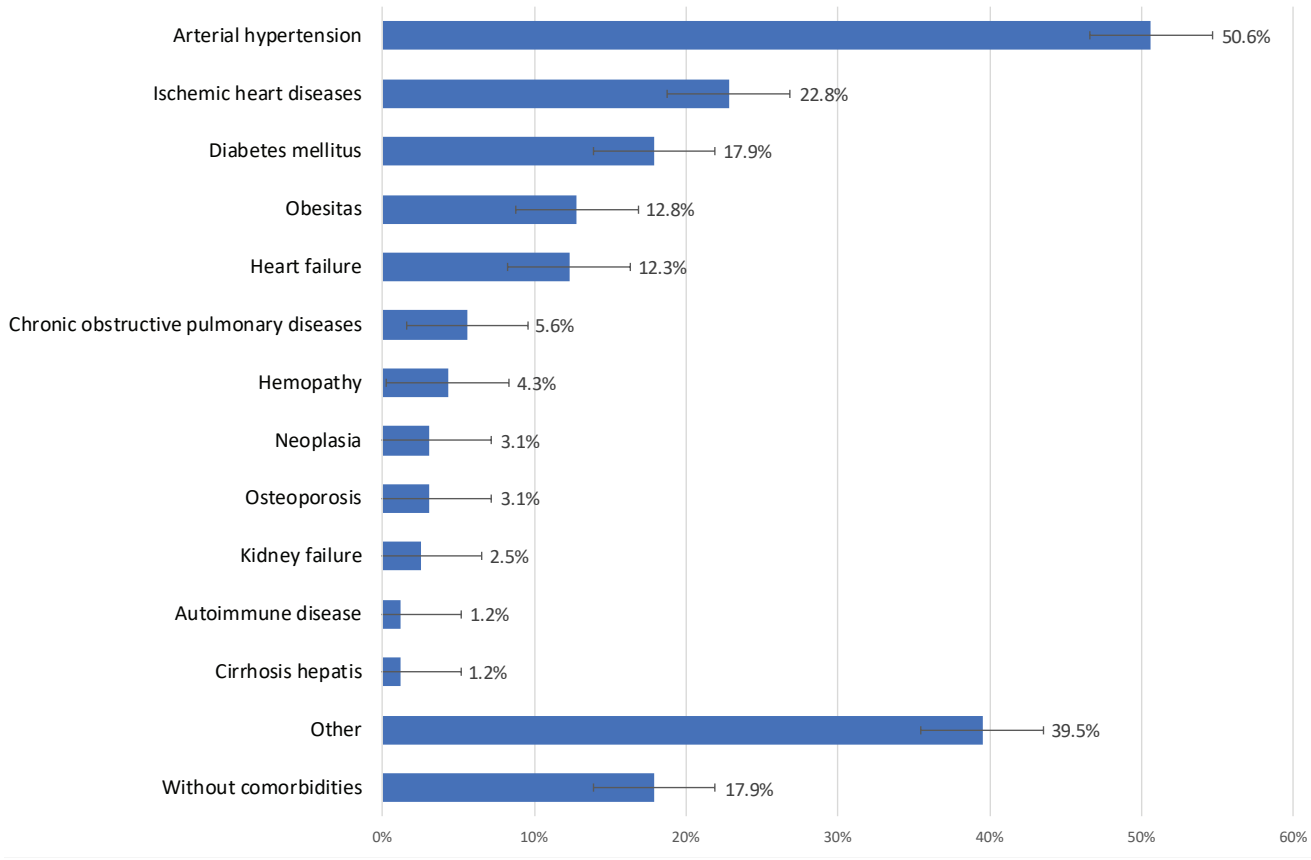

Figure 1. Comorbidities in patients with COVID-19 (n=169).

MATERIALS AND METHODS

This retrospective study included 169 adults hospitalized at the Clinic of Infectious Diseases and Clinic of Rheumatology (as transformed Covid-19 unit) in the University Hospital St. George, Plovdiv between September 2021 and December 2022. All patients had a PCR verified SARS CoV-2 infection. For the purposes of the present study, the patients were distributed into the following groups: up to 60 years (n= 69) and over 60 years old (n=100); with moderate (n=126) or severe/critical (n=43) course, survivors (n=139) or deaths (n=30).

The methods of clinical analysis (history and clinical examination) and assessment of oxygen saturation were used. Disease severity was determined according to WHO recommendations (2). Data were analyzed using IBM SPSS Statistics v.25 software products. Results are presented as proportions and standard error. Values for different groups were compared with a t-test for two independent samples. P<0.05 was considered significant.

RESULTS

Gender-related distribution of patients in the groups with different severity and outcome of COVID-19.

In 76% of the male patients the disease had a moderate clinical presentation, and in 24% (n=92) - severe. Among the women (n=77) the distribution was: moderate course in 72%, and severe/critical course in 28%. Consequently, there was no significant difference in the severity of COVID-19 between men and women (p>0.05).

No significant gender-related differences in the outcome were observed either: 85% of men survived vs. 80% from women (p>0.05). Although the differences were not significant, a more severe course and higher case fatality rate were registered in women.

Patients age in groups with different severity and outcome of COVID-19.

The average age of the patients in the group with moderate form of COVID-19 (n=129) was 61 years. The average age was higher (66.9 years) among patients with severe COVID-19 (n=43). Similarly the

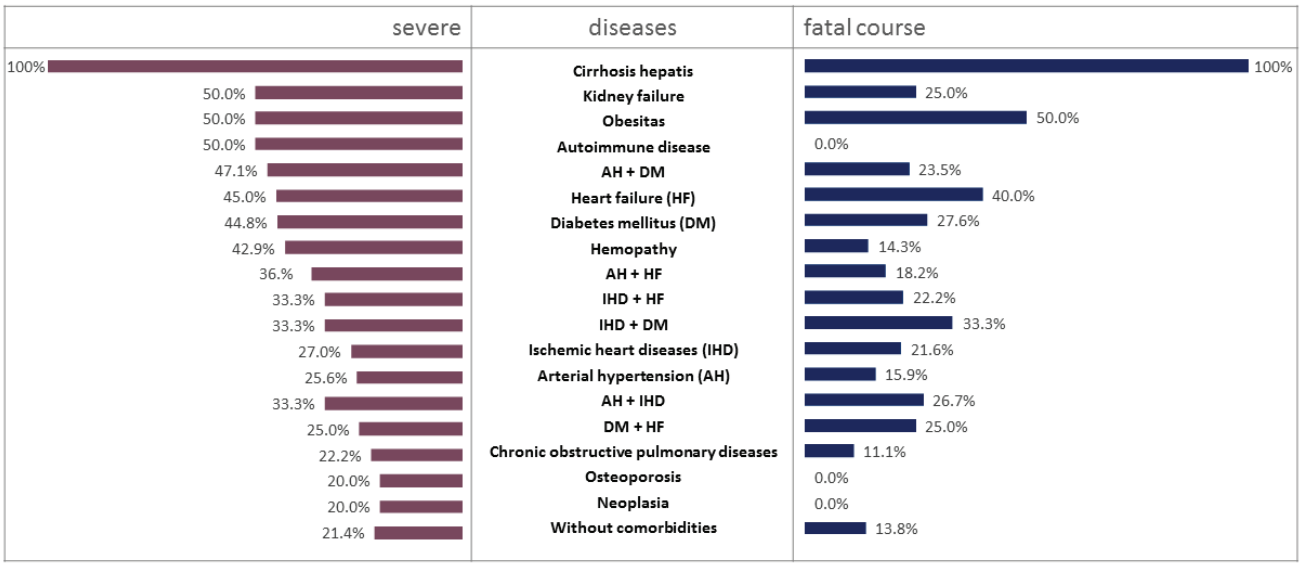

Figure 2. Severe and fatal course in patients (%) with certain accompanying diseases.

average age of survivors (n=139) was 61 years vs. 69 years among the deceased (n=30). Our pilot analysis showed that the age of 60 was critical for the severity and outcome of the disease. The distribution of younger patients according to severity and outcome differed significantly from those in advanced age patients. The severe/critical clinical course of Covid-19 was documented in 14% of patients under 60 (n=69), and in 34% of those over 60 years old ($p < 0.01$). The case fatality rate was 25% in patients over 60 vs. 7.4% for those under 60 ($p< 0.001$).

Co-morbidities

Co-morbidities in our patients are presented in Figure 1. The most common comorbidities were cardiovascular diseases including arterial hypertension (50.6%), ischemic heart disease (22.8%), and heart failure (12.3%). Diabetes mellitus (17.9%) and obesity (12.8%) had also a significant impact.

Among patients with co-morbidities 27.1% had severe COVID-19 vs. 21.4% of patients without comorbidities. Accordingly, the case fatality rate was higher in patients with concomitant diseases (18.8% vs. 13.8%) although the differences were nor significant.

Certain comorbidities (shown in figure 2) were associated with severe and fatal course of COVID-19. All patients with liver cirrhosis had a severe course and a fatal outcome. The prognosis was also unfavorable for patients with renal failure, obesity, diabetes mellitus, heart failure, and hematologic disease.

Diabetes mellitus was significantly more common in the severe patients group (31%) as compared to the moderate form (13.4%) ($p<0.05$). Case fatality rate was higher in patients with diabetes ($p<0.05$). Similarly, severe course was observed in 21.4% of patients with heart failure and moderate course - in 9.2% of those without ($p<0.05$). Case fatality rate was also higher in patients with heart failure (27.6% vs. 9%), ($p< 0.01$).

Epidemiology and clinical data

Epidemiological data for preceding contact with a SARS-CoV-2-positive patient was missing for the majority of patients (77.8%). A probable contact in the family was reported in 11.7%, at the workplace – in 6.2%, and somewhere else in 4.3% of the cases. Only four patients had been vaccinated, but no one had a booster dose.

The disease progressed slowly in 88% of the patients. Initial symptoms were astenia in 78%, fever in 77.4%, myalgias and/or arthralgias 28%, chills 23.2%, nausea or vomiting 17.1%, headache 11.6%, diarrhea 11.4%, shortness of breath 1.2%, sore throat 0.6% and other symptoms 45.7%. Combinations of initial symptoms observed are presented in Table 1.

The fever intensity and duration in patients with COVID-19 have characteristic features. No febrile reaction

Table 1. Combinations of initial symptoms in patients with COVID-19.

	fatique	fever	myalgias and/ or arthralgias	chills	nausea or vom-iting	headache	diarrhea	rapid breathing	sore throat	other symptoms
fatique	78/0% (128)	60.4% (99)	26.2% (43)	22.0% (36)	13.4% (22)	9.1% (15)	6.7% (11)			34.1% (56)
fever	60.4% (99)	77.4% (127)	23.2% (38)	21.3% (35)	12.8% (21)	10.4% (17)	7.9% (13)	1.2% (2)		35.4% (58)
myalgias and/ or arthralgias	26.2% (43)	23.2% (38)	28.0% (46)	14.6% (24)	6.7% (11)	5.5% (9)	4.9% (8)			7,3% (12)
chills	22.0% (36)	21.3% (35)	14.6% (24)	23.2% (38)	3.7% (6)	3.0% (5)	1.2% (2)	0.6% (1)		8.5% (14)
nausea or vomiting	13.4% (22)	12.8% (21)	6.7% (11)	3.7% (6)	17.1% (28)	3.0% (6)	6.1% (10)			5.5% (9)
headache	9.1% (15)	10.4% (17)	5.5% (9)	3.0% (5)	3.0% (6)	11.6% (19)	2.4% (4)			4.9% (8)
diarrhea	6.7% (11)	7.9% (13)	4.9% (8)	1.2% (2)	6.1% (10)	2.4% (4)	10.4% (17)	0.6% (1)		2.4% (4)
rapid breathing		1.2% (2)		0.6% (1)			0.6% (1)	1.2% (2)		0.6% (1)
sore throat									0.6% (1)	0.6% (1)
other symptoms	34.1% (56)	35.4% (58)	7.3% (12)	8.5% (14)	5.5% (9)	4.9% (8)	2.4% (4)	0.6% (1)	0.6% (1)	45.7% (75)

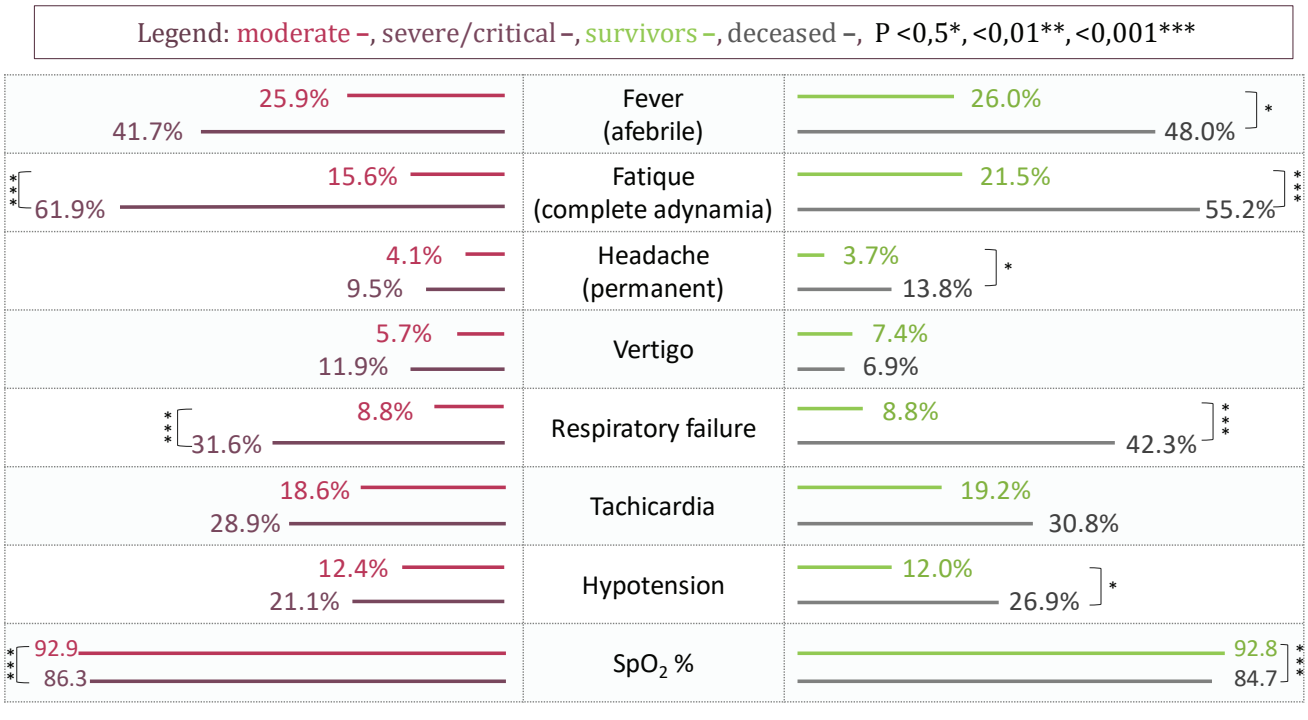

Figure 3. Clinical characteristics in patients (%) with different severity and outcome of COVID-19.

Table 2. Diagnostic and prognostic factors in patients with COVID-19.

Characteristics	Diagnostic factors	Prognostic factors
Epidemiological data	Contact at home or at work	-
Age	-	Over 60 years
Comorbidity	-	Diabetes, cirrhosis, heart failure
Asthenia	Fatigue	Adynamia
Fever	Mild or moderate	Normal
Headache	Headache	Persistent headache
Respiratory system	Respiratory failure	Respiratory failure
Cardiovascular system	Tachycardia	Hypotension

(temperature up to 37 °C) was observed in 27.4% of the patients. Temperature in the range 37 °C - 38 °C was registered in 38.4%, and above 38 °C - in 34.1% of patients. In terms of duration, 40.8% were febrile until the fourth day from the onset of symptoms, 21.7% were febrile until the seventh day, and 7.9% were febrile for more than 8 days.

Some of the clinical characteristics of patients with different disease severity and outcome are presented in Figure 3. Significant differences between patients with moderate and severe/critical course were reported regarding the astenia or complete adynamia (15.6% vs. 61.9%), respiratory failure (8.8% vs. 31.6%) and oxygen saturation (SpO_2 %) with values of 92.9 % in patients with moderate course and 86.34 % in those with severe course ($p<0.001$). Differences in some characteristics were also recorded between survivors and deceased patients. Afebrile reaction, headache, and hypotension were associated with a fatal outcome ($p<0.5$), the same was valid for adynamia (55.2% vs. 21.5% among the survivors, $p<0.001$). Respiratory failure was observed in 42.3% of fatal cases vs. 8.8% of the survivors had ($p<0.001$). Lower SpO_2 values were reported among fatal cases as compared to survivors (84.7 vs. 92.8 %, $p<0.001$).

DISCUSSION

Although COVID-19 has various clinical manifestations, most patients experience very little or no symptoms, especially in the early disease stage (2, 3, 4). In more severe cases, SARS CoV2 infection can cause pneumonia, acute respiratory distress syndrome (ARDS), kidney failure, and even death (4, 5). The need for hospitalization was observed in 20 - 30% of cases, and intensive care is required in about 10 - 17% as a result of cytokine storm and followed by respiratory failure, acute respiratory distress syndrome (ARDS), and possible multiple organ failure (1, 6 - 9). In this prospective study only patients with moderate and severe/critical course of Covid-19 were involved, because only most seriously ill could be hospitalized. In the settings of limited health facilities and a large number of patients referred to hospitals, during a pandemic, the clinical course, and clinical characteristics can be used for risk stratification and prediction of clinical outcomes.

Our data showing that severe/critical clinical course of Covid-19 and fatal outcome were associated with advanced age (over 60 years) are in line with literature data (1,7,10). Only 17.9% of observed patients were without comorbidities, and this proportion was 21.4% from severe/critical forms and 13.8% from fatal cases. Thus, while a distinct effect of comorbidities on the course and outcome of the disease was reported (3, 4), our data only partially confirmed this. Severe/critical forms of Covid-19 were found in 27.1% of the patients with comorbidities vs. 21.4% of those without comorbidities ($p> 0.05$).

The most common symptoms of COVID-19 are fever, dry cough, tiredness, runny nose, and sore throat (4, 5, 8, 9). Fever is one of the most common symptoms. Its incidence varies across studies (56%-82%-94%) (3, 4). According to our data most patients were subfebrile for a short time duration (4 days in 41%)

while in published data the temperature is most often 38.5°C – 39 °C, and in 34% - above 39 °C (5,6).

Our data for afebrile patients with a fatal outcome of the disease are interesting. We found no data on the predictive value of fever but hypothermia is a known poor prognostic factor, and even in adults fever is relatively less common.

The frequency of astenia or adynamia is the same as fever and it is a symptom that very often dominates in the clinical presentation (1). Respiratory failure with low SpO_2 is a syndrome that is not always assessed or may be neglected by the patient (Happy hypoxemia). It is one of the most indicative predictors of a severe course (7). In a summary of our results, the following diagnostic and prognostic factors for COVID-19 patients are proposed (table 2.)

CONCLUSIONS

The analysis of the clinical presentation of COVID-19 allows an orientation towards the diagnosis and early assessment of cases with a high risk of severe course and fatal outcome.

For more definite conclusions, a clinical evaluation needs to be supported by objective laboratory indicators, still it gives the earliest idea about the need for a particular set of laboratory tests.

The stratification allows the selection of an optimal therapeutic regimen, and application of specific antiviral and immunomodulatory therapies. It has a significant effect on the management of resources, patients flow and medical staff of the hospital units involved in the treatment of patients with COVID-19.

Institutional Review Board Statement: This study was approved by the Ethical Committee at the Medical University – Plovdiv, Bulgaria

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Conflicts of Interest: The authors declare no conflict of interest.

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