ABSTRACT
Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne acute viral disease not uncommon in Bulgaria. It belongs to arboviral hemorrhagic fevers. In the country, the disease was first described in 1952 near the region of Stara Zagora. Then, CCHF cases were reported in several provinces: Shumen, Razgrad, Veliko Tarnovo, Plovdiv, Pazardjik, Haskovo, Yamboi and Burgas. In 1968 CCHFV was isolated on suckling mice from blood of two patients. Success was achieved by an inactivated vaccine against this virus prepared according to an original method, which is the only one used now. A unique therapeutic scheme that is commonly practiced in the country includes anti-haemorrhagic hyperimmune immunoglobulin. For the last ten years more than 120 cases have been described. Through the years Bulgarian researchers performed different studies that concerned CCHF and their experience is presented here. This material reviews the history, molecular virology, epidemiology, ecology, pathogenesis, clinical manifestations, laboratory diagnosis, treatment, the control and prevention of CCHF in Bulgaria.

CURRENT STATE OF CRIMEAN-CONGO HEMORRHAGIC FEVER IN BULGARIA

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Studies on Crimean-Congo Haemorrhagic Fever virus (CCHFV) in Bulgaria date back to 1952, when M. Nekludov describes the disease in the region of Stara Zagora (28, 29). Following, cases with CCHF were reported in several regions: Shumen, Razgrad, Veliko Tarnovo, Plovdiv, Pazardjik, Haskovo, Burgas, Gotse Delchev and some other areas of the country (Fig.4). In 1968, S. Vassilenko isolated on suckling mice the CCHF virus from two patients. These two strains were determined to cluster together with Russian strains. Following the isolation, a significant success was achieved by an inactivated vaccine against CCHFV prepared according to an original method. The vaccine was recognized as an invention and is the only one used now (43).

Through the years other Bulgarian researchers (P. Verbev, M. Radev, E. Gacev, B. Monev, S. Vassilenko, V. Serbezov, G. Katsaros, B. Kamarinchew, etc.) performed studies on aetiology, epidemiology, clinical symptoms and pathoanatomy of CCHF and on the ticks – vectors of the virus.

Viral classification and structure
Crimean-Congo Haemorrhagic Fever virus belongs to a genus Nairovirus, which is one of the five genera of the family Bunyaviridae (14). Only three viruses from the family have been determined to cause diseases in humans: Nairobi sheep disease virus, Dugbe virus and CCHF virus, the last being the most significant pathogen for humans.

The viral particle is spherical in shape with a diameter of 80–120 nm, it possesses 3 segments of negative-sense RNA and RNA dependant RNA polymerase packed within a lipid envelope which contains 2 viral glicoproteins [G1 and G2] (22, 23). All nairoviruses replicate inside the cytoplasm of the infected cell, where the virus matures inside the Goldji apparatus (Fig.1 and Fig.2).
Resistance and cell culturing
The resistance of CCHF virus against physical and chemical factors is weak (43), and the virus is inactivated for 72 hours at room temperature. When irradiated with UV light for 15 min or after treatment with 30% ether, chlorophorm, ethyl alcohol, rivanol, potassium permanganate or formalin the virus is inactivated.

The virus of CCHF is successfully cultivated in cell lines MK, Vero, BHK-21, CER, and SW-13. Infected cells demonstrate a weak cytopathic effect 4-5 days after the onset of infection, which can be verified through forming of plaques and the immunofluorescence method (45).

Animals that are susceptible to the virus include rabbits, adult mice, rats, guinea pigs, but best cultivation is achieved after intracerebral inoculation on suckling white mice. The infection causes adynamia, tremor and paralysis of the legs to lethal outcome 6 to 7 days after inoculation (27).

Variability among CCHFV strains
The initial studies based on serology, revealed insignificant differences between CCHF strains. Viral tests such as complement fixation assay (CFA), viral neutralization reaction and interference in cell cultures, demonstrated that specific antigenic differences between the strains from Russia and Africa were not present (3, 4, 6, 41). Recent research based on sequencing of the viral genome and phylogenetic analysis revealed some genetic varieties. The first publication of a sequenced S segment described a virus isolated in China from a sheep as an animal model (24). Then, various research teams sequenced and published differences in the S segment from different strains (30, 32, 34), and the phylogenetic analysis determined that significant changes were present. For example, the initial strain that was isolated in China from a sheep varied 10.0-11.8% in its S segment genomic sequence from the virus that was isolated in 1995 during epidemic in the United Arab Emirates (31). The isolates from Kosovo obtained in 2001 had a difference of 17% in their S segment sequence compared to strains isolated from Nigeria and only 4% compared to a Russian strain isolated from the blood of a patient in Drosdov (30). Interestingly, a strain isolated in Greece from a tick differed significantly from other European strains (24.3% and 25.3% in its nucleotide sequence compared to the isolates from Kosovo and Albania, respectively).

Sequences of CCHFV strains isolated from blood of Bulgarian patients are very closely related to each other. Overall, the Bulgarian sequences based on their nt and aa identities in the S segment were very similar to CCHFV strains from Kosovo (99% and 99%, respectively) and Turkey (97% and 99%, respectively). The phylogenetic analysis confirmed a clear molecular and epidemiological association with Group V, which represents the Europe/Turkey geographic CCHFV lineage (unpublished data).

Epidemiology of CCHF
Crimean-Congo Haemorrhagic Fever is an endemic disease in many African countries (southward from Egypt to the southmost part of the continent, and from Senegal to Madagascar), Europe (Bulgaria, Kosovo, Albania, northern Greece and the European part of Russia), and Asia (the Arab peninsula, Iraq, Pakistan), the central Asian countries that were part of the Soviet Union and the northern provinces of China (Fig. 3).
bushes grow. These conditions are in favour of the endemic process and circulation of the virus has been described in the regions of Shumen, Razgrad, Veliko Tarnovo, Plovdiv, Pazardjik, Haskovo, Yambol and Burgas (Fig. 4). During the last years the dynamics of the endemic areas (an irradiation) and epidemiological activity were also studied outside the previously published areas (3, 26). In April 2008, a cluster of 6 apparent cases occurred in the town of Gotske Delchev in the Blagoevgrad Province of Southwest Bulgaria, an area considered to have low CCHF endemicity. Nevertheless, single cases usually occur among shepherds or agrarians, and small clusters occur among individuals involved in the slaughter of infected animals. Over the years, outbreaks have occurred and a total of 1568 CCHF cases were reported from 1953 to 2008.

**Ecology of CCHF**

The CCHF virus infects a wide range of domestic and wild animals. Many avian species are resistant to infection, but some birds such as ostriches, ravens and partridges are susceptible and may contribute for the wide spread of the infection in the endemic areas. Domestic animals like horses, donkeys, pigs, sheep and goats play a major role in the transmission and maintenance of the disease, serving as reservoirs of the virus (3). Animals are infected with CCHFV through a bite from an infected tick. Ticks are also a reservoir of the virus and more than 22 tick species are determined to be vectors of the pathogen. Most efficient transmission is carried by ticks that belong to genus *Hyalomma* (17). Trans-ovarial (transmission of the virus from infected female ticks to its progeny via eggs) and sexual transmission of the virus have been proven as mechanisms to support the circulation of the virus in nature. In Bulgaria, the most common ways of infection among the patients are by tick bite, removal and crushing the ticks by naked hands, by skinning of wild animals, or by medical procedures dealing with patients. Most of the cases involve stock breeders, agricultural workers, slaughterhouse personnel and veterinarians. Patients are also a source of infection during the acute phase of the disease. For the period of 2006-2010 a CCHFV-tick survey in the regions of Haskovo, Kardzhali and Stara Zagora showed that 4.83%, 2.09%, and 1.46% of ticks, respectively, were infected by CCHFV, and that the most infected tick was *H. marginatum* (15).

Once infected, ticks may transmit the virus to humans, large and small vertebrate animals including some birds. The pathogen circulates in the bloodstream for around one week after the onset of the infection. The disease is marked by spring-summer seasonality associated with the biological activity of these arthropods.

**Pathogenesis**

One of the most detailed studies on clinical features and pathology of CCHF was performed by Swanepoel et al. in South Africa from 1981 to 1987, when 50 cases were diagnosed and described. Fifteen of these patients died (a lethality of 30%), and one patient developed a bacterial meningitis as a complication after a neurosurgery aiming to eliminate brain hemorrhage. Factors that contributed to the lethal outcome included brain hemorrhage; severe anemia; severe dehydration and shock associated with persistent diarrhea; myocardial infarction, pulmonary edema and pleural effusions (38).

Despite the pathogenesis of the disease is not well known, several mechanisms that explain the hemorrhagic diathesis in CCHF are discussed. These include: damage of the central and vegetative neural system (CNS and VNS) with subsequent disturbance in the vassal endothelium; damage of the vassal endothelium and the cardio-vascular system and development of disseminated intravascular coagulation (DIC) caused by the viral replicative cycle.

After initial primary viremia, the virus remains in the organs of the reticuloendothelial system (RES), where it undergoes active viral replication. In the prehemorrhagic period a secondary viremia occurs resulting in intoxication syndrome and damage of the CNS and VNS. This impairs the nervous regulation of blood vessels leading to paresis, dilatation and disturbance in the trophicity with anatomical changes in the vascular wall.

The secondary viremia and the following intoxication impair the adrenal glands, hematopoietic system, internal organs (pituitary, heart, liver) and vascular wall. The impaired adrenals reduce the production of corticosteroids, which leads to decrease in their antihialuronidase action, and consecutively to an increase of vascular permeability. Suppressed bone marrow function leads to leukopenia and thrombocytopenia, which enhances the vascular permeability as a consequence from the deactivated action of the platelet factors on the vessels.

The immune response to the infection is mainly humoral and is based on production of specific viral neutralizing and complement fixing antibodies that appear 6-7 days after the onset of the infection. Cell immunity is not well studied. After the infection, long-lasting immunity is developed.

**Clinical manifestations**

Crimean-Congo Haemorrhagic Fever has only been described in humans (apart from laboratory animal models that employ suckling mice). Other vertebrate animals serve as hosts, where in humans the disease presents as a severe hemorrhagic syndrome. The typical course of the infection develops in four stages: incubation, prehemorrhagic, hemorrhagic and...
convalescence period (19). The duration and symptoms in the different stages may vary significantly.

Duration of the incubation period of the disease depends on the mode of acquisition of the virus. After a tick bite, this period is very short and lasts only 1-3 days, sometimes significantly more. The incubation period when the infection is acquired after a contact with infected blood or tissues is usually 5-6 days (39). It has been hypothesized that the virus and its virulence undergoes changes after transmission from different hosts (16).

The symptoms develop abruptly with fever, myalgia of the limbs and back, dizziness, stiffness of the joints, headache and photophobia. Nausea, vomiting and sore throat, accompanied by diarrhea and abdominal pain may be present. During the following days, the behaviour of the patient may sharply change, with symptoms such as confusion and aggression. After 2-4 days from the initial symptoms, the agitation may be replaced with somnolence and depression.

Other clinical features include tachycardia, lymphadenopathy and petechial rash, caused by bleeding into skin and the mucosal surfaces of the mouth and throat. The petechiae may develop into ecchymoses that cover larger areas of the body or bleeding from the upper gastrointestinal tract may pass to feces and present as melena. Following symptoms involve haematuria, epistaxis and gingival bleeding. In severe and fulminant forms of the disease hepato-renal syndrome and pulmonary insufficiency may develop after the fifth day from the onset of symptoms (39).

Duration of the convalescent period is prolonged, intoxication and hemorrhagic manifestations gradually disappear, but for a long period of time patients have neurasthenic complaints, pains in a single or several nerves, reduced potency and hair loss (19).

Clinical diagnosis
Early and prompt diagnosis is essential when CCHF is suspected and has a major role in order to prevent nosocomial infections. The clinical symptoms, the history of disease, trips to endemic areas, tick bites or contact with animal or human blood are among the first indications for infection with CCHFV. The differential diagnosis should cover different ricketsial diseases, leptospirosis or borreliosis. Also, infections with other pathogens that lead to haemorrhagic diathesis such as bacterial sepsis, hantaviral hemorrhagic fever, malaria, yellow fever, dengue and Omsk fever, should be considered. Cases in Africa ought to be differentiated from Lassa fever and infections caused by filoviruses, Marburg and Ebola fevers.

Laboratory diagnosis
It is of utmost importance that the clinical materials are properly collected, transported and stored at the laboratory. Isolation of the virus needs to be performed within 14 hours from the collection of blood and if possible sent within the first 1-3 days from the onset of the symptoms. Tissue samples obtained after autopsy (peaces of lungs, spleen, liver or kidney) can also be delaminated.

For viral isolation it is obligatory that clinical materials and laboratory tests for detection of CCHFV are handed in specifically equipped laboratories at a BSL3-4. The classical method for viral isolation includes intracerebral inoculation of blood from the patient or pools of collected ticks into suckling mice. Isolation on cell cultures is more simple and fast, but is less sensitive. The virus can be isolated on cultures such as CEP, MK, VERO and BHK-21 from blood or tissues samples during the first five days of the disease. A weak or absent cytopathic effect (CPE) may be observed.

Laboratory methods for diagnosis of CCHF are based on direct detection of the etiological agent or on serological methods, detecting highly specific “antigen-antibody” complex and identifying both virus and antibodies. Efficiency of examination of clinical materials from the patient should comply with certain infectious conditions: the high pathogenicity of CCHFV to humans; lack of viral stability in the external environment; about 10 days of viremia duration; appearance of IgM antibodies 5-7 days after initial symptoms; appearance of IgG antibodies after 7-10 days followed by the increase in titre 15-20 days after the onset of the disease (27).

Immunology based methods testing CCHFV can be performed with complement fixation assay (CFA), reaction precipitation in agar (RPA), immunoflorescence method (IFM) and the enzyme linked immunosorbent method (ELISA) (11). These methods usually require two serum samples: one obtained at the onset of the disease and a second one 14 days later (27, 43). After infection, IgM antibodies are found within four months, while IgG antibodies can be detected within 5 years after the disease. When CCHF develops as a fatal fulminant outcome antibodies are usually not found.

Molecular methods are newly introduced and now routinely used in Bulgaria. Adapted protocols and methods for CCHFV detection with conventional, nested-PCR, SYBR Green I and Taqman Real-time RT-PCRs have been developed (20). Different clinical specimens (blood and serum) from suspected patients are analyzing after viral RNA extraction. The virological tests are used in parallel with serological methods such a CFA and/or ELISA in order to supplement and compare results.

Treatment
It is obligatory that hospitalization is performed under conditions of Biohazard Level III Infections. The treatment regimen should include intensive and supportive care in order to achieve a favourable outcome. A unique therapeutic scheme that is commonly practiced in Bulgaria includes antihaemorrhagic hyperimmune immunoglobulin (KHT-bulin®) (42). It is intended for intramuscular application at a minimal dosage of 18 ml in patients with proven CCHFV infection. A dosage of 6 ml is applied to patients where CCHF is suspected and 3 ml to patients with unclear acute febrile conditions after a tick bite. KHT-bulin is a human immunoglobulin preparation...
with high concentration of specific antibodies against the CCHF virus derived from immunized volunteers or patients. Previously, a similar product termed KHT-venin was used and was intended for intravenous application. In the last years, its application has been suspended. Among available antiviral drugs, Ribavirin is used in other countries, neighboring Bulgaria, but its favourable effect still remains questionable (46). The existing protocols include oral therapy- 500 mg, every 6 hours for 7-10 days; and intravenous therapy- initial dose of 30 mg/kg, followed by 15 mg/kg every 6 hours for 4 days and 7.5 mg/kg every 8 hours for the next 6 days. In vitro studies concerning effect of Ribavirin on the CCHF virus, report antiviral activity when the virus is cultivated on Vero cell culture (45), and when the medication is applied to infected suckling mice, Ribavirin increased the average time for lethal outcome (40). In Bulgaria, Ribavirin is used in the treatment protocols for viral hepatitis C and B, but has not been applied to patients with CCHF.

Applications of Interferons are another therapeutic option that is of particular interest, especially interferons that stimulate production of viral inhibitory proteins from the Mx protein family (18). These peptides are members of the dynamin superfamily of large guanosine triphosphatases. The Mx proteins have displayed antiviral activity against a vast number of RNA viruses, including those in the Bunyavirus and the Orthomyxovirus families.

Essential component from the therapy that is intended to restrain the pathogenic mechanisms includes: transfusion of erythrocytes and platelets; vasoprotective drugs such as rutascorbin, vitamin C and Calcium; corticosteroids; maintenance of the electrolyte homeostasis; infusion of glucose and levulose; and oxygenation.

Overall, it is necessary to thoroughly study the CCHF virus to get to know its evolution and subsequent development of new effective treatment products for this haemorrhagic fever.

**Control and Prevention**

During the last ten years the number of cases with laboratory confirmed CCHF cases in Bulgaria was as follows: 54 in 2002; 19 in 2003; 18 in 2004; 14 in 2005; 7 in 2006; 3 in 2007; 2 in 2008; 1 in 2009; 2 in 2010; and 2 in 2011.

In order to decrease the risk for infection, persons living in endemic areas should use personal protective measures and avoid visiting places such as grasslands, grassy areas with high vegetation and others, where tick vectors are usually found (*Hyalomma* spp. in particular). Professionalists that work with livestock or other animals including birds, in the endemic areas should take particular measures for self-protection. Among these are use of repellents on the skin surface and clothing (for example diethyl-toluamid, DEET), use of leather gloves and other protective clothing in order to prevent the skin from contact with infected tissues and blood.

Hospitalization of CCHF cases is associated with a significant risk for nosocomial spread of the infection. A couple of examples in Bulgaria include nosocomial infections that took place at the Clinics of Infectious Diseases in the City Hospitals of Pazardjik and Gotce Delchev in 2002 and 2008, respectively. It is of utmost importance that when a patient is hospitalized with a haemorrhagic fever, isolation and treatment should be performed under the conditions of Biohazard Level III Infection. Specimens such as blood or tissues, taken for the purpose of laboratory tests and diagnosis, should be collected and handled using universal protective measures and precautions. All biohazard waste including sharps such as needles and other penetrating surgical instruments should be properly decontaminated.

Several nosocomial outbreaks have been described worldwide among nurses, surgeons and laboratory personnel like the Pakistani and South African outbreaks in 1976 and 1985, respectively. According to the American Center for Disease Control and Prevention (CDC) these accidents attributed for classification of CCHF virus as an agent that may lead to a particularly bio-hazardous infection, and necessitates appropriate BSL conditions (31). Healthcare personnel, that is exposed to infectious tissues or blood from patients where CCHF is suspected or has received a laboratory conformation, should be monitored for emerging symptoms of the disease for at least 14 days after the exposure and treated with KHT-bulin.

A unique inactivated vaccine against CCHF derived from brains of mice was developed in 1973 by S. Vasilenko. However, the vaccine has a limited use in Eastern Europe. In Bulgaria it is mandatory for healthcare workers, livestock breeders (for professionals that grow sheep, goats and cattle), mowers, military personnel, forest workers, geologists and people that go on camping trips. All these are selected based on the risk for tick bites or exposure to infected blood from patients (5).

The initial immunization dose includes two applications of 1 ml each (1 ampoule) in an interval of 30-45 days. First re-immunization (booster dose) is performed with one application of 1 ml one year after the initial dose, and a second re-immunization should be carried out after 5 years. The immunization should commence during the pre-endemic period (March-April). The vaccine is applied subcutaneously in the region of the scapula.

It is important to note that according to the governmental policy the Ministry of Health of Republic of Bulgaria provides free vaccination to contingents at risk in the endemic foci through the regional Health Offices. CCHF mortality is approximately 30-50% in different geographic areas, in Bulgaria it was reduced to 11,4% (5, 27), probably due to vaccination.

**Conclusions**

The clinical features of CCHF, especially at the onset of the disease, are non-specific. Thus, early clinical diagnosis of this and other haemorrhagic fevers is difficult and sometimes even impossible. The symptoms are hard to be distinguished from
the symptoms of many other acute viral and bacterial infections. Namely critical here is rapid and accurate etiological diagnosis. On the other hand, high pathogenicity of CCHFV brings in concerns its application as a potential weapon of bioterrorism. The National Institute for Allergy and Infectious Diseases in the United States has placed CCHFV as a Category C priority pathogen (new agents that are potentially dangerous in the future) because of its transmission from human to human; high contagious and lethality index; ability to cause large epidemics; and may disorganize the administrative and health system (1). CCHFV infection is a serious threat to public health in Bulgaria. Development of effective control measures and continued accumulation of information regarding the molecular basis of these epidemics are crucial for prevention of its spread to other European countries.

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