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Tick-borne virus diseases of human interest in Europe

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ABSTRACT

Several human diseases in Europe are caused by viruses transmitted by tick bite. These viruses belong to the genus *Flavivirus*, and include tick-borne encephalitis virus, Omsk haemorrhagic fever virus, louping ill virus, Powassan virus, *Nairovirus* (Crimean-Congo haemorrhagic fever virus) and *Coltivirus* (Eyach virus). All of these viruses cause more or less severe neurological diseases, and some are also responsible for haemorrhagic fever. The epidemiology, clinical picture and methods for diagnosis are detailed in this review. Most of these viral pathogens are classified as Biosafety Level 3 or 4 agents, and therefore some of them have been classified in Categories A–C of potential bioterrorism agents by the Centers for Disease Control and Prevention. Their ability to cause severe disease in man means that these viruses, as well as any clinical samples suspected of containing them, must be handled with specific and stringent precautions.

Keywords *Flavivirus*, louping ill virus, *Nairovirus*, Omsk haemorrhagic fever, Powassan virus, review, tick-borne encephalitis virus

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TICK-BORNE ENCEPHALITIS VIRUS DISEASES

After the recognition of tick-borne encephalitis (TBE) as a distinct disease entity by Schneider in 1931 [1], the causative tick-borne encephalitis virus (TBEV) was discovered by Zilber in 1937 in far-eastern Russia [2]. TBEV and antigenically closely related viruses have since been isolated in regions stretching from northern Asia to central and western Europe. The data show that TBEV is present in at least 25 European and seven Asian countries.

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Virus properties and taxonomy

TBE virions have an average diameter of 50 nm and possess two membrane-anchored surface

proteins, i.e., the envelope glycoprotein E and the small membrane protein M. The genome consists of a positive single-stranded RNA molecule of *c.* 11 000 nucleotides that encodes a large polypeptide (*c.* 3400 amino-acids), which is co- and post-translationally cleaved and processed by host-cell and virus enzymes to yield three structural and seven non-structural proteins [3]. The open reading frame of all flaviviruses is flanked by 5′ and 3′ untranslated RNA regions that form secondary stem–loop structures; these probably serve as *cis*-acting elements for genome amplification, translation and packaging [4–6]. According to the classification scheme of the International Committee for Taxonomy of Viruses, TBEV (family Flaviviridae, genus *Flavivirus*) is a single virus species with three subtypes, designated European, Siberian and Far-Eastern, respectively [7].

Ecology

TBE is an infectious zoonotic disease that occurs in so-called natural foci, risk or endemic areas, all of which refer to the occurrence of a specific agent during a constant period of time within the borders of a particular location. Virus activity within such risk areas and the geographical distribution of agents within the foci may vary [8,9]. TBEV is maintained in nature in a cycle involving ticks and wild vertebrate hosts.

The vectors are haematophagous ticks that remain infected throughout their life cycle. The ticks transmit the disease agent to vertebrate hosts when feeding, having picked up the agent from reservoirs which may or may not be in a viraemic state. Although many different species of tick are biologically competent to transmit TBEV in the laboratory, in nature only *Ixodes ricinus* in Europe, and *Ixodes persulcatus* and *Haemaphysalis concinna* in Russia, appear to play a significant role in virus maintenance, largely because of specific ecological limiting factors [10]. Virus transmission from infected to uninfected ticks occurs through the migratory skin cells of vertebrates [11]. This process forms an important focus of virus replication in the absence of systemic viraemia [12] and occurs when naïve ticks feed on vertebrate hosts close to infected ticks, a process known as co-feeding. In the laboratory, this has been shown to be an efficient means of transmission of TBEV and other tick-borne viruses among ticks [12–16], and the typical pattern of aggregated distributions of larval and nymphal ticks within rodent populations ensures a sufficient level of transmission in nature [17,18]. Obviously, the feeding of naïve ticks on viraemic vertebrates may also play a role in the natural cycle of TBEV. The relative importance of each mechanism has been modelled [17], but has not yet been determined clearly in the field. In order for transmission to occur, the virus must be capable of multiplication within the vector. The vector then carries the pathogen to a range of hosts that play different roles within natural foci. A host may be a carrier of the pathogen without automatically contributing to the cycle of pathogen transmission. Therefore, it is helpful to apply specific terms to divide the hosts into reservoir, indicator and accidental hosts.

The reservoirs are wild-living vertebrates capable of transmitting infection. Within natural foci, these reservoirs are present in high numbers, and have a high reproduction rate and a rapid generation turnover. In the case of TBEV, they must be receptive to the virus and enable the virus to multiply and be delivered to feeding ticks. If viraemia develops with a high virus titre, it should not cause host death before the ticks have completed their blood meal [16]. These are not characteristics seen in small rodent species infected with TBEV, where, if viraemia develops, it is short-lived (several days) [19] and can be fatal before ticks complete their blood meal [20]; in hosts shown to infect a high proportion of feeding ticks, virus titres reach only low levels. Experiments on non-viraemic transmission have shown that viraemia is not a condition necessary for successful transmission to ticks. For ticks, in contrast to insect vectors, a reservoir host plays an active (albeit accidental) role in the vector–reservoir relationship by sweeping questing ticks from vegetation; indeed, with relatively immobile ticks, it is the host's movement that is instrumental in picking up ticks [21]. In the case of TBEV, small rodent species are short-lived reservoirs of the virus, whereas ticks maintain the virus within natural foci for many months or even years.

Indicator hosts cannot transmit the virus to other vectors, either because they can endure only a brief period of viraemia with low virus titres, or because they lack the necessary cell-based mechanisms to support non-viraemic transmission [12]. Experimental work during the 1990s showed that this statement is not true—*Apodemus* mice are

recognised as the most significant transmission hosts in the field, yet develop only low virus titres. Thus, there must be other reasons why indicator species are not competent to transmit, e.g., they lack the necessary cell-based mechanisms. Man, and large animals such as goats, cows, sheep, deer, dogs and swine, can become infected accidentally, but there is experimental evidence that ungulates are not competent to transmit the virus back to feeding ticks [22]. Indirectly, these species support virus circulation by enabling the ticks themselves to survive and reproduce [23]. Seroprevalence in these large vertebrates may represent an indirect means of measuring the intensity of TBEV transmission within a geographical region [24]. Therefore, they constitute valuable sentinel species for antibody detection in epidemiological studies.

Accidental hosts are species that can be infected by the pathogen and can develop viraemia, but generally neither participate in virus circulation nor form a significant nutrition source for ticks.

Epidemiology

Since ticks remain infected throughout their life cycle, the epidemiology of TBE is clearly related closely to the ecology and biology of ticks, with regard to not only their distribution but also the seasonality of tick feeding activity [25]. The seasonal dynamics of ticks determine the potential for co-feeding transmission among ticks, and also the risk to humans from infected questing nymphal ticks. *I. ricinus* is the dominant hard tick species across Europe and is the most important vector for European TBEV. *I. persulcatus* inhabits forest regions of the Urals, Siberia and far-eastern Russia, and is the main transmission vector for TBEV of the Siberian and Far-Eastern subtypes. *I. persulcatus* exists also in parts of the Baltic States (Estonia, Latvia). In Russia, TBEV has also been isolated sporadically from 18 other tick species, such as *Dermacentor* spp. and *Hyalomma* spp. [26]; however, these species probably do not contribute significantly to the epidemiology of human disease. The prevalence of ticks infected with TBEV in endemic areas in Europe usually varies from 0.5% to 5% [27,28], although prevalence rates of 40% have been recorded in certain regions of Russia [24]. It is important to note that methods for measuring virus prevalence in ticks have not yet been standardised.

Human cases of TBE usually occur between April and November, when infected ticks are questing for hosts. TBEV infections in humans can show considerable year-to-year variation; for instance, certain regions of Russia, that typically reported 700–1200 cases annually, recorded up to 10 000 cases in the post-Perestroika era, probably because of a large increase in outdoor activities [29] and a simultaneous drastic decrease in the use of pesticides. The highest incidence has been registered in Latvia, the Urals and western Siberian regions of Russia, where attack rates range from 115 to 199 reported cases/100 000 inhabitants/year [30]. However, attack rates in the range of 50–60/100 000 inhabitants are more typical (V. I. Zlobin, unpublished results). It is estimated that *c*. 3000 cases occur in western European countries annually, giving a typical incidence of <4 cases/100 000 inhabitants (http://www4.tbe-info.com/epidemiology/). Nevertheless, the incidence of TBE has increased during the past 20 years, and the virus is now found in previously unaffected areas. A link between observed changes in climate and changes in vector distribution and TBE incidence has been suggested [31,32], but the increase in TBE in Sweden from 1984 onwards seemed to occur independently of the increase in recorded temperatures [33], which began in 1989.

The European subtype comprises almost all known isolates from Europe and has been found in *I. ricinus* as well as in *I. persulcatus* (Latvia) [28]. Strains of the Siberian subtype are typically isolated in the Urals, Siberia and far-eastern Russia, while the Far-Eastern subtype is isolated in far-eastern Russia, China and Japan [34]. However, Siberian and Far-Eastern subtypes of TBEV have been isolated recently in Europe [28,35,36].

Routes of TBEV infection

The most likely way for a human to become infected with TBEV is to be bitten by an infected tick during outdoor activities in forest areas where dense vegetation can sustain large numbers of ticks. However, 70–95% of human infections in endemic regions are either sub-clinical or totally asymptomatic [24,37]. The major factor contributing to the incidence of the disease in man is an abundance of ticks containing a sufficient dose of infectious TBEV [29]. Although the highest incidence of human infections

coincides with seasonal peaks in the feeding activity of *I. ricinus* (May–June and September–October) and *I. persulcatus* (May–June), a substantial proportion of patients do not report a history of tick bite [38]. TBEV infections have been diagnosed serologically or by virus isolation in Austria, Belarus, Bosnia, Croatia, China, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Japan, Kazakhstan, Latvia, Lithuania, Norway, Poland, Romania, Russia, Serbia, the Slovak Republic, Slovenia, Sweden, Switzerland and the Ukraine [2,24]. Epidemiological and surveillance data regarding TBE from 27 European countries and the Far East, including China and Japan, have been reviewed elsewhere [39].

A second natural route for the acquisition of TBEV infection is associated with the consumption of raw goats' milk [24,40]. TBEV has been isolated repeatedly from the milk of infected goats for up to 25 days after collection; infectivity is maintained in various milk products such as yoghurt, cheese and butter. Persistent infectivity in gastric juice is observed after ingestion of such products for up to 2 h [41], while pasteurisation prevents milk-borne TBEV infection [2]. However, confirmation based on recent experimental data is currently lacking. Data regarding the alimentary route and other transmission routes for TBEV not involving tick bites have been reviewed elsewhere [39].

Laboratory-acquired cases of infection have been reported in the context of accidental needlestick injuries or aerosol generation and contamination through the olfactory pathway [42,43].

Clinical and standard laboratory features

Acute phase

After an incubation period of 7–14 days (with extremes of 4–28 days), the first phase consists of the sudden onset of an uncharacteristic influenza-like illness with fever, headache, joint and back pain, and accompanying symptoms such as nausea and vomiting; this phase usually lasts for 4 days (range 1–8 days) [44,45]. Biologically, this phase is commonly characterised by thrombopenia, leukopenia and hyperalbuminorachia. A biphasic course is common and occurs in 74–87% of cases [46]. After an 8-day symptom-free interval (range 1–33 days), a second phase occurs in which meningoencephalitis presents in 20–30% of infected patients [47]. In the second phase, neurological signs occur, and this is usually the time when patients with fever and severe headache consult a physician. In the remaining individuals (13–26%), the disease resolves without a second phase. It is important to note for the anamnestic history that a substantial proportion of TBE patients recognised the tick bite. Encephalitic symptoms during the second phase include meningeal signs, ataxia and cognitive disorders such as impaired concentration and memory, dysphasia, altered consciousness, confusion, irritability, tremor, and paralysis of cranial nerve and respiratory muscles. The fatality rate in Europe is $< 1\%$.

Long-term morbidity

Sequelae characterised by neurological and neuropsychiatric symptoms are often reported. Time to recovery is extended over several months, generally with a good outcome [48]. Common symptoms are various cognitive and focal neurological signs. Hearing defects have been reported with high frequency in certain studies [49,50]. Post-encephalitis syndrome includes diverse manifestations such as spinal nerve paralysis, neuropsychiatric complaints, dysphasia, ataxia and paresis; occurrence of such a syndrome has been correlated with increased age, impaired consciousness during the acute phase, with or without ataxia and paralysis, a history of assisted ventilation, abnormal findings on magnetic resonance imaging, pleiocytosis of > 300 cells/μL in cerebrospinal fluid (CSF), and impairment of the blood–CSF barrier [45,51,52].

Our understanding of the pathogenic mechanisms of TBE is incomplete. The difficulties associated with detecting virus RNA in CSF during the encephalitic phase strongly suggest that virus replication may be inhibited or reduced when neutralising antibodies appear in serum and CSF, although the virus may be located in neurones [53]. Low levels of neutralising serum antibodies correlate with a severe course of the disease. The theory of antibody-dependent enhancement, which originated from clinical studies of dengue fever [54,55], has been considered, but there are no laboratory data indicative of a similar phenomenon in human TBE.

A remarkable characteristic of TBEV infections is the existence of chronic forms [56,57]. This seems to be an Asian–Russian phenomenon, and such chronic forms have not so far been observed in western Europe. Further investigations are

needed into the clinical, virological and pathophysiological features, but these forms are characterised by neurological symptoms that appear progressively without being preceded by a recognised acute phase. Symptoms are progressive neuritis, lateral sclerosis, dispersed sclerosis, Parkinson-like disease or progressive muscular atrophy [24]. In such cases, TBEV has been identified as the aetiological agent only by virus isolation [58–60]. Molecular diagnostic methods that will allow the possibility of excluding cross-contamination during virus isolation procedures are now available.

Association of pathogenesis with TBEV subtype and geographical location

Despite their antigenic similarities, it is possible to distinguish three variants of TBEV, i.e. Central European, Siberian and Far-Eastern, on the basis of serological tests. Nucleotide and amino-acid sequence analysis has validated the divergent nature of these three variants despite their close biological similarities [3,61,62]. Human infections in far-eastern regions are usually severe with frequent encephalitic signs, a fatality rate of 5–35% [2] and an absence of chronic forms [24]. In contrast, TBEV infections in Siberian–Ural regions present as a less severe disease (fatality rate of 1–3%), but chronic forms seem to be more frequent. Experimental evidence for the association of Siberian strains with chronic forms has been derived from monkey and Syrian golden hamster model systems [58–60,63–65]. Infections caused by European strains are typically biphasic and are characterised by a viraemic phase with fever, malaise, headache, myalgia, leukocytopenia, thrombocytopenia and elevated liver enzymes; after a 1-week latency period, 25% of patients develop clinical signs of neurological involvement [44,47,66]. Residual sequelae are observed in *c*. 25–50% of patients, but $<2\%$ of cases are fatal [45].

A cluster of eight fatal cases was reported recently in the Novosibirsk region of Russia (Siberia); these cases were caused by strains with the Far-Eastern genetic pattern. Surprisingly, these patients suffered a pronounced haemorrhagic syndrome with massive gastrointestinal bleeding and multiple haemorrhages in mucosa and internal organs [67]. Numerous experimental data demonstrate pathogenic differences between European and Far-Eastern strains on the one hand, and between Far-Eastern and Siberian strains on the other. More specifically, Far-Eastern strains have a clear tropism for neurones, thereby accounting for the degenerative manifestations associated frequently with infection. However, the molecular mechanisms responsible for the different pathogenic features remain obscure [24]. Increasing numbers of clinical and subclinical cases of TBE have also been documented in dogs [68].

Collection and preservation of samples

TBEV is classified as a Biosafety Level 3 (BSL-3) agent. Therefore, all procedures involving biological samples must be performed according to stringent safety rules.

Serum or heparinised plasma should be collected during the acute febrile stages of the disease. The samples must be frozen on dry ice or in liquid nitrogen because storage at temperatures above $-40°C$ results in progressive loss of infectivity. Direct diagnosis was previously achieved by virus isolation (see below), but may now be performed with an RT-PCR assay. If inoculation into cell culture (or suckling mice) or RT-PCR has to be delayed for >24 h, the plasma or buffy coat layer should be frozen in liquid nitrogen (or at $-70°C$ if intended for isolation procedures). Storage at $-20°C$ is suitable for molecular methods.

For serological diagnosis, blood samples should be collected early in the course of the disease, with a second sample obtained after a further 1–2 weeks. If a four-fold rise in antibody titre has not occurred, a third serum sample, collected after 4–6 weeks, may be useful. Samples collected for serological diagnosis can be kept at $-20°C$. TBEV-specific antibodies are usually detected by enzyme-linked immunosorbent assay (ELISA). When results are being interpreted, it should be remembered that TBEV is a flavivirus and therefore shares several antigenic determinants with mosquito-borne viruses such as Dengue virus, Japanese encephalitis virus or West Nile virus. Specific identification can usually be achieved with neutralisation tests.

Field studies often result in the collection of ticks that are suitable for analysis for the presence of TBEV. Ticks should be tested for the TBEV genome with RT-PCR techniques, since large numbers of samples can be tested in a short time

period with high sensitivity. Classically, ticks are crushed in 500–1000 μL of phosphate-buffered saline containing fetal bovine serum 20% v/v; an aliquot is used for total RNA extraction and subsequent RT-PCR, while the remainder is stored at −80°C for virus isolation in those cases where the TBEV genome is detected. A detailed protocol is available upon request from the corresponding author.

Tools available for diagnosis

TBEV isolation can be achieved through virus propagation after inoculation of serum and/or CSF into mammalian cell cultures (Vero, BHK-21, PK, RH, 293 or A549 cells) or the brains of suckling mice. Alternatively, the TBEV genome can be detected with RT-PCR from either blood or CSF during the first phase of the disease. However, these techniques are of minor diagnostic importance in practice, since most patients consult a physician only during the second phase of the disease, when the virus has already been cleared from blood and CSF. Moreover, in contrast to many other virus infections, including those with other flaviviruses, RT-PCR is not very useful for the laboratory diagnosis of TBE, since even the most sensitive molecular methods frequently fail to detect virus RNA during the first phase [53,69]. Newer real-time RT-PCR techniques merit further evaluation of their usefulness in TBE diagnostic procedures [2,70]. RT-PCR for the detection of TBEV RNA has been used successfully in epidemiological surveys of virus prevalence in ticks and for the investigation of viraemia in vertebrate hosts [71–74]. However, it is important to underline that virus isolation and molecular techniques do not play a significant role in the routine diagnosis of TBE, which is based mainly on the presence of specific antibodies, usually detected at the beginning of the second phase.

Before the 1980s, paired sera were tested with complement fixation [75] or haemagglutination inhibition tests [76]. For rapid diagnosis, specific IgM antibodies were detected by 2-mercaptoethanol reduction in haemagglutination inhibition tests. ELISA is now the method of choice for serological diagnosis on paired sera. ELISAs may be performed on serum or CSF. Rapid diagnosis is performed by detecting IgM with capture ELISA, which avoids false-positive results caused by the interference of rheumatoid factor or heterophilic antibodies [69]. Detection of IgM antibodies to TBEV in serum or CSF by ELISA has been shown to be the most reliable serological test [77]. However, the suitability of IgM for assessment during early diagnosis is questionable, since IgM antibodies can persist for up to 10 months in vaccinees or individuals who acquired the infection naturally. Therefore, confirmation by detection of specific IgG with ELISA or a seroneutralisation test is recommended.

It should be noted that the results of antibody tests may be negative in the early phase of infection, and the tests should therefore be repeated 1–2 weeks later, and that commercial ELISAs show great variation with regard to specificity and sensitivity [78]. For a more satisfactory analysis of the immune response against TBEV, knowledge of previous infections with other flaviviruses (Dengue, etc.) and/or vaccinations (yellow fever, Japanese encephalitis, TBE, etc.) is helpful because of the cross-reactivity of the antibodies [79]. Verification of the diagnosis with specific IgG detection is necessary. However, for cases with other flavivirus contacts (e.g. vaccination against yellow fever or Japanese encephalitis; Dengue virus infections), a neutralisation assay is necessary because of the interference of flavivirus cross-reactive antibodies in ELISA and haemagglutination inhibition tests [80]. Moreover, both IgG and IgM antibodies perform well in neutralisation tests.

Therapy and prevention

There is no drug with demonstrated efficacy against flaviviruses. Other than the avoidance of exposure to the bite of an infected tick, vaccination is the most effective means of disease prevention. Two vaccines are available currently in western Europe (FSME-IMMUN, Baxter Vaccine AG, Vienna, Austria; and Encepur, Chiron-Behring, Marburg, Germany), prepared with Austrian and German strains, respectively, that are closely related genetically. FSME-IMMUN is the vaccine used most widely in Europe; this vaccine has been improved progressively over time, and consists of whole purified virus of the European TBEV subtype, propagated in chick embryo cells and inactivated with formaldehyde [81,82]. The basic immunisation protocol consists of two vaccinations given approximately 1 month

apart, followed by a third vaccination after 1 year. Booster immunisations are recommended every 3–5 years, giving a protection rate of 96–98% [83]. A so-called 'accelerated schedule' has been proposed, consisting of three doses at days 0, 7 and 21 [84], with a further dose after 1 year to complete the basic immunisation protocol. Following mass vaccination in Austria, active surveillance revealed a dramatic decline in the incidence of TBEV infection [83,85]. Currently, there are also two vaccines available for children that contain half of the adult dose, FSME-IMMUN Junior (Baxter) and Encepur Kinder (Chiron-Behring) [81,82]. Normally, there is a very low rate of non-serious and serious adverse reactions other than post-vaccination fever [81]. Both vaccines have been used widely to immunise infants and children in the high-risk areas of Austria and Germany [82,84,86]. Although the full recommended vaccination schedule requires 1 year, an accelerated schedule (at days 0, 7 and 21) achieves a similar efficacy, despite a slight increase in side effects [84]. Although the vaccine is manufactured exclusively from the European subtype, immune protection could be provided against all three subtypes [87]. Accordingly, it is reasonable to recommend immunisation for unexposed individuals travelling to endemic areas, and for laboratory workers performing TBEV propagation.

In Russia, tissue culture inactivated vaccines were developed in 1960 [88], and the results of extended field trials of these vaccines have been reported [89]. Currently, two vaccines are available, prepared from the Far-Eastern subtype (205 and Sofjin strains) of TBEV. ENCEVIR (Virion, Tomsk, Russia) has been available since 2001, while the other has been produced at the Institute of Polyomyelitis and Viral Encephalitis (Moscow, Russia) since 1984; both are certified for children. These vaccines consist of formaldehyde-inactivated, purified and concentrated TBEV, and have been used for mass vaccination (up to 7 million doses) in Russia. For both vaccines, the primary course of vaccination consists of three doses.

In Russia, prevention of TBE also includes the use of specific immunoglobulins, which are active when administered to tick-bitten persons within 3 days post-bite. This therapy is reported to be effective in 98% of cases in a curative context and 100% in a prophylactic context (i.e., administered to individuals before visiting natural foci of TBEV (V. A. Lashkevich, personal data)). In Europe, TBE immunoglobulin first lost its licence for children aged up to 14 years because of a suspected association between post-exposure application of immunoglobulin and very severe forms of the disease. It was then withdrawn completely from the market and is no longer available. It is likely that such preventive and/or curative methods will have little future because of safety regulations regarding the use of human-derived products for treating humans.

OMSK HAEMORRHAGIC FEVER

Agents and vectors

Omsk haemorrhagic fever virus (OHFV) was first recognised following several outbreaks during 1943–1945 in the rural region of the Omsk district in Siberia. The virus was first isolated from a patient's blood in 1947, and later from ticks belonging to the species *Dermacentor reticulatus* (*Dermacentor pictus*), muskrats and other vertebrates and arthropods. According to the International Committee for Taxonomy of Viruses, OHFV is a unique species of the genus *Flavivirus* within the family Flaviviridae [7]. This delineation is based on a clear-cut antigenic difference between OHFV and TBEV, demonstrated with monoclonal antibodies [90]. These findings were corroborated by genetic distances calculated from complete coding sequences between OHFV and TBEV of 10.2–11.6% at the amino-acid level [91] (R. N. Charrel, personal data). However, there is no morphological difference between OHFV and TBEV; both have virions with an average diameter of 50 nm that possess two envelope glycoproteins (E and M). The genomic characteristics are also similar to those of TBEV (see previous sections).

The natural foci of OHFV are in the Omsk and Novosibirsk regions, and also in Kurgan and Tyumen (western Siberia), which comprise forested areas and open wetlands. The typical landscape associated with OHFV is forest–steppe. The highest incidence of OHFV disease was observed in the years following World War II; subsequently, a substantial decline in case numbers has been recorded, probably linked to the immunisation of the local population against TBEV and OHFV, and a decrease in the vector population.

The classic route of transmission is a tick bite during outdoor activity in forest areas and nearby wetlands situated in the endemic region. Typically, cases occur from April to December. Spring cases correlate with the activity of the vector *D. reticulatus*. A second peak may be observed during August–September, correlating with the feeding activity of a second vector, *Dermacentor marginatus* [92]. Collaborative studies led by the Institute of Poliomyelitis and Viral Encephalitis and the Omsk Institute for Natural Focal Infections [93] established that *D. reticulatus* was the natural reservoir, in which OHFV was transstadially and trans-ovarially maintained throughout the tick life cycle.

However, in recent years, most human cases have been related to direct contact with muskrats (*Ondatra zibethica*), and usually occur during the hunting season from September to October [94]. Of 165 cases of OHFV recorded in 1988–1997, only ten involved the classic route of transmission (tick bite) [95]. Muskrats can become infected through both the alimentary and respiratory routes (the virus has been isolated from urine and faeces), and OHFV transmission in muskrat populations results in a prevalence rate as high as 14%. Therefore, muskrats possess all the characteristics of a potent amplifier host for OHFV. In contrast to other arthropod-borne viruses, OHFV is highly pathogenic for its main host (muskrat). It is interesting that muskrats were first introduced into Siberia from Canada in 1928. This suggests that OHFV has been in contact with its new host for only 75 years, during which time it may have shown some degree of co-evolution. For unknown reasons, the OHFV endemic area is much smaller than the areas in which *Dermacentor* ticks and muskrats are distributed.

Clinical and standard laboratory features

The incubation period is usually 3–7 days, with extremes of 1–10 days. In contrast to TBEV, OHFV infection does not involve the central nervous system (hence the absence of major neurological signs), and the principal disorders are vascular and circulatory, with capillary damage being responsible for the haemorrhagic manifestations. The onset is sudden, with fever lasting for 5–12 days. There may be remission of the fever, after which 30–50% of patients experience a second febrile phase, commonly more severe than the first. Common features include fever, headache, myalgia, cough and gastrointestinal symptoms. Haemorrhage (epistaxis, bleeding gums, metrorrhagia, haematemesis) is not severe and does not impair the prognosis, but in some cases, signs of vascular fragility, such as petechial rash and bruises at the puncture sites, can be observed. Blood analysis often reveals leukopenia and thrombocytopenia. During the second phase, patients can develop meningeal signs, but neurological involvement has not been reported. Recovery is usually slow, but sequelae are unusual. Mortality rates range from 0.5% to 3% [96]. More extensive information can be obtained from reviews published previously [97,98].

Collection and preservation of samples

The procedures for collection and preservation of samples are essentially as described above for TBEV. The only difference is that OHFV is classified as a BSL-4 agent in most European countries except France, where it is considered as a BSL-3 agent.

Tools available for diagnosis

The tools available for diagnosis are as described above for TBEV.

Therapy and prevention

Therapy and prevention are as described above for TBEV. There is evidence of cross-protection between OHFV and TBEV, so TBEV vaccines are likely to be reasonably effective in the prevention of OHFV infections, although this has not yet been formally demonstrated.

CRIMEAN-CONGO HAEMORRHAGIC FEVER

Agents and vectors

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* within the family Bunyaviridae. Morphologically, CCHFV resembles other bunyaviruses, with a spherical, enveloped virion 90–120 nm in diameter. The CCHFV genome consists of three molecules of negative-sense single-stran-

ded RNA, each encapsulated separately. The virion particle contains three major structural proteins, i.e., two envelope glycoproteins G1 and G2, and a nucleocapsid protein. The virus glycoproteins play a role in: (1) the recognition of receptor sites on susceptible cells; (2) the haemagglutination process; and (3) the induction of an immune response by a vertebrate host. The nucleocapsid protein is involved in the synthesis of complement-fixing antibody.

CCHFV is distributed widely in Africa, the Middle East and central and southwestern Asia. It has also been found in parts of Europe, specifically Rostov, Stavropol, Astrakhan and some other southern provinces of Russia [99–102], Bulgaria [103], Greece [104], the Kosovo province of the former Yugoslavia [105,106] and Albania [107]. There is also very limited serological evidence for the presence of the virus in parts of Hungary, France and Portugal. In general, the prevalence of antibodies to CCHFV in livestock and human populations coincides with the distribution of *Hyalomma* ticks.

CCHFV causes disease in man, but generally asymptomatic infections in other mammals, except newborn laboratory mice, rats and Syrian hamsters (A. M. Butenko, personal data). The virus has been isolated from at least 30 species of tick, including 28 Ixodidae and two Argasidae spp. However, it is unlikely that argasids are capable of serving as vectors, since CCHFV does not replicate in these ticks following intra-coelomic inoculation. Many ixodid tick species, e.g., *Hyalomma marginatum*, *Rhipicephalus rossicus* and *D. marginatus*, display numerous properties that make them principal (or efficient) vectors: (1) they are capable of acquiring CCHFV infection through feeding on viraemic hosts; (2) infection can persist trans-stadially from one stage of the tick life cycle to the next (larva, nymph, adult), and thereafter be transmitted successfully to a second host; (3) trans-ovarial transmission to their progeny has been observed; (4) co-feeding has also been demonstrated to be an important mechanism for tick-to-tick infection, as for tick-borne flaviviruses; and (5) infected male ticks are able to transmit CCHFV to the female via the venereal route, and subsequent trans-ovarial dam-to-progeny transmission has been observed. While many ixodids are capable of transmitting CCHFV, members of the genus *Hyalomma* are the most efficient vectors. Epidemiological data, based on reports of human CCHFV infections and the results of serological studies, confirm that *Hyalomma* ticks are the principal transmitters of the infection in nature [99].

Seroepidemiological studies conducted in different endemic regions of Europe, Africa and Asia have shown that large herbivores (the principal hosts of adult *Hyalomma* spp.) exhibit the highest antibody prevalence, and that birds generally lack antibodies (birds appear to be refractory to CCHFV infection, but it is not clear whether this simply reflects lack of exposure), with the major exception being ostriches, which are known to be parasitised by *Hyalomma* ticks. This is particularly important given the increasing importation of ostriches to breeding farms in western Europe. Evidence of infection of other birds (hornbills, starlings and guinea fowl) has been obtained in Senegal [108].

Many animal species, when viraemic for CCHFV, have been shown to infect feeding ticks. From an epidemiological point of view, small vertebrates such as hares may be the most important host for the perpetuation of the virus in nature, since, unlike large vertebrates, they are infested by immature ticks that are more likely to transmit CCHFV trans-stadially, and thus act as amplifying hosts. CCHFV infection in man can occur: (1) through tick bite or crushing infected ticks in ungloved hands; (2) via the nosocomial pathway in homes and hospitals [109–111]; (3) from contact with blood or other infected tissues of livestock; (4) by drinking raw milk from infected animals [112]; or (5) via the transcutaneous or respiratory pathway in laboratory workers. Most cases are reported in individuals, e.g., shepherds, dairy workers, veterinarians, farmers and, occasionally, slaughterhouse staff, who have occupational contacts with livestock.

Clinical and standard laboratory features

After an incubation period, estimated at 2–7 days, onset is sudden, with fever, chills, headache, dizziness, neck pain, nuchal rigidity, photophobia, retro-orbital pain, myalgia and arthralgia. Non-focal digestive manifestations, such as nausea, vomiting, diarrhoea and abdominal pain, are encountered frequently. Haemorrhagic manifestations occur after several days of illness, and include petechial rash, ecchymoses, haematemesis and melena, and are often associated with

thrombocytopenia and leukopenia. Hepatitis is associated frequently with jaundice, hepatomegaly and elevated levels of transaminase enzymes. Generally, petechiae precede the haemorrhagic signs, which consist of bleeding from venepuncture sites, and large bruises and ecchymoses in the axilla and inguinal regions. The mortality rate is *c*. 30% (varying from 10% to 60%, depending on region and transmission route). Death occurs mainly after 6–10 days of illness as a result of profuse haemorrhages, haemorrhagic pneumoniae, and disturbance of vital cardiovascular functions. Cardiovascular disturbances include tachycardia, bradycardia and low blood pressure. Individuals who recover do not usually experience sequelae other than a persistent asthenia. Although there is a lack of documentation regarding non-hospitalised cases, it is believed that there are few subclinical cases; further investigations are required to confirm this hypothesis.

Several biological parameters can be affected during the course of the disease, of which the most frequently reported are: (1) leukopenia; (2) thrombocytopenia; (3) elevated levels of aspartate transaminase, alanine transaminase, γ-glutamyl transferase, lactic dehydrogenase, alkaline phosphatase, creatine kinase, bilirubin, creatinine and urea; and (4) declining levels of serum proteins. Disorders in the coagulation pathways can lead to elevation of thrombin time, fibrin degradation products and a decrease in the amount of fibrinogen. The appearance of all these disorders early in the course of the disease suggests that disseminated intravascular coagulation is an early and central event in the pathogenesis of CCHFV infection.

Collection and preservation of samples

As CCHFV is a BSL-4 pathogen, all procedures involving patient management and handling of infectious materials must be performed with extreme caution in accordance with appropriate national guidelines and legislation (see [113] for those applicable in the USA).

Direct diagnosis

Serum or heparinised plasma should be collected during the acute febrile stages of the disease, and the samples must be frozen on dry ice or in liquid nitrogen. Storage at a temperature above −40°C will result in progressive loss of infectivity. Classically achieved by virus isolation, direct diagnosis may now be performed with RT-PCR. When inoculation (for isolation in cell culture or suckling mice) or RT-PCR has to be delayed for >24 h, plasma or buffy coat layer should be frozen in liquid nitrogen or at −70°C for isolation procedures, or at −20°C for molecular methods.

Indirect diagnosis

Samples collected for serological diagnosis can be kept for long periods at −20°C or below without degradation of antibodies. A blood sample should be collected early in the course of the disease, with a second sample after 1 or 2 weeks. If a four-fold rise in antibody titre has not occurred, a third serum sample taken after 4–6 weeks can be useful. Blood obtained in the early convalescence period may be infectious, despite the presence of antibodies, and therefore should be handled with appropriate precautions.

Tools available for diagnosis

Serology

IgG and IgM antibodies can be detected with ELISA and indirect immunofluorescence tests from about day 7 of illness. Specific IgM declines to undetectable levels by 4 months post-infection, but IgG remains detectable for at least 5 years. Serological diagnosis is made: (1) by demonstration of IgM antibodies with IgM antibody capture (MAC)-ELISA, even in the first (single) serum sample; (2) by demonstration of IgM and IgG antibodies in the second and third samples; or (3) by demonstration of a four-fold or more significant increase (or decrease) in the titres of specific antibodies in paired sera. Interestingly, sera collected from patients who succumb to the disease rarely show a demonstrable antibody response. ELISA methods (MAC-ELISA and ELISA IgG) are quite specific and much more sensitive than immunofluorescence and neutralisation tests [114,115]. Serological protocols based on the nucleocapsid protein expressed from the cloned gene, using ELISA [116] or immunofluorescence [117], have also been developed.

Cultivation

Blood taken during the febrile period and inoculated immediately into newborn mice usually results in infection. Viraemia generally continues for 7–8 days, but sometimes until day 12, after the

onset of illness. Infected blood kept at 4°C remains infective to newborn mice for 10 days, but usually yields negative results subsequently. Blood and plasma from patients, as well as autopsy material (especially lung, liver, spleen, liver, bone marrow, kidney and brain) may be used for CCHFV isolation. Post-mortem material should be taken within 11 h of death [118]. For long-term storage, blood and post-mortem material should be frozen on dry ice, in liquid nitrogen or at −70°C for subsequent direct isolation procedures, or at −20°C for RT-PCR, provided that the latter is performed shortly after storage. CCHFV replicates poorly or not at all in most cell lines, with no visible cytopathic effect, the only exception being SW-13 cells derived from human adrenal adenocarcinoma [119,120]. However, virus isolation can be achieved in cultured Vero cells. Virus isolation can be achieved in 2–5 days, but cell cultures lack sensitivity, and usually only allow detection of the relatively high viraemia encountered during the first 5 days of illness. Virus identification can be achieved subsequently through immunofluorescence tests with polyclonal antisera and/or monoclonal antibodies. Intracerebral inoculation of suckling mice necessitates specific facilities, and presents greater risks for the laboratory worker, but is much more sensitive, and virus can be recovered up to 13 days after the onset of illness.

Molecular methods

Classic RT-PCR methods have been described and are becoming the method of choice for rapid laboratory diagnosis of CCHFV infection [105,121–123]. The quantitative assay described by Drosten *et al.* [121] demonstrated that these assays are easily capable of detecting the levels of virus RNA typically present in acute serum samples. Attempts to design and develop real-time RT-PCR methods are hindered by the extreme genetic diversity of CCHFV strains and the lack of sequence data for the virus polymerase gene, which would be the gene of choice, since it probably exhibits greater homogeneity than other virus genes.

Treatment, prevention and control

Standard treatment consists of intensive monitoring and supportive care. There is no evidence that immune plasma from recovered patients has any beneficial effect. Ribavirin inhibits the growth of CCHFV *in vitro* and in mice infected experimentally [124,125]. There is anecdotal evidence that it is effective in patients following oral and intravenous administration, but no formal trials of its efficacy have been conducted [105,126]. An inactivated mouse brain vaccine has been produced and used on a small scale in the former Soviet Union and Bulgaria, but continuation of such production and development of a safe modern vaccine have both been inhibited by the limited potential demand.

OTHER EUROPEAN TICK-BORNE VIRUS DISEASES

The following viruses have also caused disease in man, but few cases have been reported in the literature.

Eyach virus (EYAV)

EYAV was isolated from *I. ricinus* in southwestern Germany in 1976 [127]. Neutralisation and complement fixation test results indicated that EYAV and Colorado tick fever virus (CTFV; the type species in the genus *Coltivirus* within the family Reoviridae) are related antigenically to each other, but are distinct [127]. In 1981, other strains of EYAV were isolated in France from *Ixodes ventalloi* and *I. ricinus* ticks [128]. EYAV was also incriminated indirectly in cases of encephalitis and polyradiduloneuritis in the former Czechoslovakia, since antibodies to the virus were identified in the sera of patients with neurological syndrome, but without a formal identification of the virus [129]. EYAV particles are non-enveloped, are 70–80 nm in diameter, and possess two concentric capsid shells with a core that is *c.* 50 nm in diameter. The EYAV genome is composed of 12 segments of double-stranded RNA.

The reservoir of EYAV is thought to be the European rabbit (*Oryctolagus cunniculus*), but the natural cycle of the virus is still unclear and it is not known whether it circulates continuously in France and Europe [130]. The virus was re-isolated in Baden Württemberg (Germany) in 2003, and serological surveys detected antibodies to EYAV in rodents, including the European rabbit (0.9%). Serological surveys in higher mammals, including ovines, deer and caprines, in the southern half of France, identified anti-EYAV

antibodies in 1.35% of the tested animals [131]. Following the original isolation of EYAV in France, serum from a farmer (0.2% of the tested population) was found to be positive [128]. The presence of anti-EYAV antibodies in sera and CSF from 12% of patients in the former Czechoslovakia is an indication of the widespread distribution of the virus in Europe [129]. Most of these patients were diagnosed originally as suffering from a TBEV infection.

To date, it has not been possible to propagate EYAV in mammalian cell lines, and virus isolation can be achieved only by intracranial injection of suckling mice. Complement fixation and neutralisation assays for EYAV have been described, but are time-consuming and difficult to perform. Determination of the full-length genome sequence of EYAV has allowed PCR primers to be designed that detect as few as ten virus particles [132], as well as the construction of a recombinant protein used in an ELISA for detection of anti-EYAV antibodies.

Powassan virus (POWV)

POWV, a member of the genus *Flavivirus*, was isolated originally in Ontario (Canada) in 1958 from a fatally infected boy aged 5 years. Cases have also occurred in Russia, where the virus is transmitted by *I. persulcatus* and various *Haemaphysalis* ticks. POWV is known to have life cycles in both ticks and mosquitoes, and this might account for the wider geographical distribution of this virus compared with other tick-borne flaviviruses [133]. *Apodemus* mice and *Microtus* voles are the principal vertebrate hosts. After an incubation period that may last for several weeks, clinical manifestations include fever, headache, retro-orbital pain and photophobia, which are usually accompanied by neurological signs such as lethargy, generalised or focal seizures, paresis, paralysis and focal neurological signs.

POWV has been recovered from the brains of patients following fatal cases of the disease. Serological diagnosis can be achieved by detection of specific IgM antibody in acute serum or spinal fluid, or by observing seroconversion. However, because of possible cross-reactions with other flaviviruses, neutralisation assays are required for aetiological confirmation.

Louping ill virus (LIV)

LIV is another member of the genus *Flavivirus* that mostly causes encephalitis in sheep. The term 'louping ill' is ancient, and was used in the 18th century to describe a disease of sheep, occurring in the Border counties of England and Scotland, where sheep were farmed intensively on the hillsides [134]. LIV variants circulate in European countries other than the UK, such as Spain (Spanish sheep encephalomyelitis virus), Turkey (Turkish sheep encephalomyelitis virus) and Greece (Greek goat encephalomyelitis virus). These are probably derived from TBEV, and have evolved in different ecological niches [133]. They are antigenically and genetically related closely to TBEV and LIV, and are all capable of infecting man. LIV is transmitted by *I. ricinus* ticks. Naturally-acquired human infections have occurred mainly in individuals with occupational exposure, such as sheep farmers, veterinarians, slaughterhouse workers or butchers who had direct contact with animals. Thus, a seroprevalence rate of 8% has been reported in abattoir workers, indicating that exposed individuals often develop asymptomatic infection. Laboratory-acquired infections are common, suggesting that the virus might be transmitted by direct mucous or respiratory pathways. Tick-transmitted cases are scarce, but have also been reported. Seventeen human cases of natural infection and 26 of laboratory-acquired infection had been described by 1991 [135].

The clinical picture is very similar to that of the biphasic meningitis typical of western European TBEV. After an incubation period, generally 4–7 days, patients present with a self-limited influenza-like illness with fever, headache, dizziness, retro-orbital pain, articular pain and myalgias [135]. This first phase is followed by clinical improvement and, in 50% of cases, by an encephalitic phase. Some patients develop a petechial rash, with leukopenia in the first stage and leukocytosis in the second. Infection should be suspected in a patient presenting with neurological manifestations and an occupational context, and can be confirmed by demonstration of specific IgM antibody or a four-fold rise in antibody level in serum or CSF.

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