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Molecular diagnostics of viral hemorrhagic fevers

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Abstract

This review addresses the diagnostics of viral hemorrhagic fevers (VHFs). In the first part, an overview is given on classical methods of VHF diagnostics as well as novel molecular diagnostic tools. Currently available polymerase chain reaction (PCR) assays for diagnosis of VHF are summarized and discussed. In the second part, VHF diagnostics are described in particular for Lassa fever, yellow fever, and Crimean-Congo hemorrhagic fever, based on cases that were imported into or occurred within Europe. The third part is focussed on important differential diagnoses of VHF.

Keywords: Viral hemorrhagic fevers; VHF diagnostic tool; Molecular diagnostic tool

1. Part I

1.1. Introduction

The increase in travel activities to tropical countries and the international commitment in conflict and disaster situations have made the import of viral hemorrhagic fevers (VHFs) into Europe a more likely event than in the past. The possible threat of attacks with biological warfare agents has additionally risen the interest in VHF pathogens. Some of the viruses causing VHF can cause epidemics due to human-to-human transmission and have to be identified or excluded fast and reliably. Several European countries have established facilities of biosafety level (BSL) 4 for handling and diagnosing contagious VHF agents, like Ebola and Marburg viruses, Lassa virus, or Crimean-Congo hemorrhagic fever (CCHF) virus in the recent years. Others are taking the initiative to establish such laboratories.

Molecular diagnostic tools like the polymerase chain reaction (PCR) and other nucleic acid amplification techniques (NATs) meet the necessity to rapidly identify VHF or biological warfare agents. These techniques are suitable for identification of viruses, bacteria, parasites, and fungi. In contrast to classical diagnostic methods which are based on the detection and identification of the intact organism, NAT detect the genetic material of a pathogen and thus reduce the contact with infectious material to a minimum. The Bernhard-Nocht-Institute of Tropical Medicine has established a facility for VHF diagnostics in the recent years. While PCR techniques serve as the first-line tool, the full diagnostic spectrum involves classical methods such as virus culture and serological testing. In particular the latter techniques require work in BSL-3 and BSL-4 laboratories. The facility is consulted by hospitals from Germany and also from other European countries when VHF is suspected.

In the first part, this review addresses the clinical and virological aspects of VHF and provides an overview on tools for VHF diagnosis. In the second part, VHF diagnostics is described particularly for Lassa fever, yellow fever, and CCHF cases that were imported into or occurred within Europe. The third part is focussed on important differential diagnoses of VHF.

1.2. Viral hemorrhagic fevers

The term “viral hemorrhagic fever” describes a variety of viral diseases which are characterized by fever and bleeding in humans. This syndrome is caused by RNA viruses belonging to the families Filoviridae (Ebola virus and Marburg virus), Arenaviridae (Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus), Bunyaviridae (CCHF virus, Rift Valley fever (RVF) virus, hantaviruses), and Flaviviridae (yellow fever virus and dengue virus) (Table 1). After transmission from their reservoir host or vector to humans, these viruses cause an acute infection and there is no evidence of chronic courses.

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Table 1
Relevant VHF viruses

Virus (host or vector)	Disease	Endemic region
Lassa virus (rodents)	Lassa fever	Africa: Guinea, Sierra Leone, Liberia, Nigeria (also Ghana, Ivory Coast, or Burkina Faso)
Junin virus, Guanarito virus, Machupo virus, Sabia virus (rodents)	Argentine, Venezuelan, and Bolivian hemorrhagic fever	South America: Argentina, Venezuela, Bolivia, Brazil
Ebola virus (unknown host)	Ebola hemorrhagic fever	Africa: Gabon, Democratic Republic of Congo, Uganda, Sudan, Ivory Coast Asia: Philippines
Marburg virus (unknown host)	Marburg hemorrhagic fever	Africa: Uganda, Zimbabwe, Kenya, Democratic Republic of Congo
Crimean-Congo hemorrhagic fever virus (*Hyalomma* ticks)	Crimean-Congo hemorrhagic fever	Africa and Near-, Middle-, Far East (Pakistan, Afghanistan, China) Europe: Balkan countries
Rift Valley fever virus (mosquitoes)	Rift Valley fever	Sub-Saharan Africa, Egypt, first outbreak outside Africa in 2000 in Yemen and Saudi Arabia
Yellow fever virus (*Aedes* mosquitoes)	Yellow fever	Tropical Africa and Central-/South America (Asia is still free of yellow fever)
Dengue virus (*Aedes* mosquitoes)	Dengue fever, dengue hemorrhagic fever, dengue shock syndrome	Tropical and subtropical areas world-wide (increasing incidence in Latin America and Asia)
Hantavirus (rodents)	Hemorrhagic fever with renal syndrome (HFRS)	Europe and Asia (China, Korea)

The clinical symptoms in the early phase of a VHF are very similar irrespective of the causative virus and resemble a flu-like illness or a common enteritis. Headache, myalgia, gastrointestinal symptoms, and symptoms of the upper respiratory tract dominate the clinical picture. Hepatitis is also common. Therefore, especially in the early phase virological testing is of utmost importance in diagnosis. The late phase of a VHF is more specific and characterized by organ manifestations and organ failure. Hemorrhage, the hallmark of a VHF, is present only in a fraction of patients depending on the virus species or even the virus strain. Mild and subclinical courses seem to occur in all hemorrhagic fevers. However, if the disease is symptomatic, the case fatality ranges between 5 and 30%, but may be as high as 80% in Ebola fever.

With a few exceptions, currently there exists no specific and effective therapy for VHF. The drug ribavirin is effective against Lassa virus, Hantaan virus, and possibly CCHF virus as well. Cases of Lassa fever and yellow fever recently imported into Europe demonstrate that even state-of-the-art intensive care cannot prevent a fatal outcome (see Part II). Vaccines have been developed against yellow fever virus (Theiler and Smith, 1937), Junin virus (Maiztegui et al., 1998), and RVF virus (Pittman et al., 1999).

1.3. Epidemiology of VHF

Each VHF is endemic in specific geographic regions (Table 1). Most but not all VHF are restricted to tropical and subtropical areas. In Europe, only hantavirus and CCHF virus are endemic. The specific geographical distribution of a VHF is explained at least in part by the distribution of the reservoir host and the vector species which are required to maintain the transmission cycle of the virus. Reservoirs of VHF viruses are rodents (arenaviruses) and other vertebrates (yellow fever virus, RVF virus, and CCHF virus). Vectors are arthropods (mosquitoes (flaviviruses and RVF virus) and ticks (CCHF virus)). A reservoir and possible vector of Ebola and Marburg virus has not yet been identified. It is of particular epidemiological relevance to know whether a VHF virus can be transmitted from human to human. In the endemic regions, human-to-human transmission has been observed with Ebola virus, Lassa virus, and CCHF virus. While Ebola virus seems to remain genetically stable after repeated transmission among humans (Rodriguez et al., 1999) and has caused large epidemics after single transmissions from the reservoir to humans (Khan et al., 1999), CCHF virus and possibly also Lassa virus may become attenuated after a few passages in humans. There are specific conditions which have facilitated outbreaks of Ebola and Lassa fever in Africa in the past, especially poor sanitary conditions in the hospitals and the absence of proper barrier-nursing techniques (Fisher-Hoch et al., 1995b; Khan et al., 1999).

The incubation period of a VHF ranges from 2 days for Ebola virus (Ndambi et al., 1999) up to 3 weeks, for example for Lassa virus (McCormick et al., 1987a). During this period of time and whilst first non-specific symptoms are present, the infection may be unperceivedly imported into non-endemic regions. If a VHF is suspected in a traveler, the travel route can give the decisive clue concerning the causative virus. However, travelers have also acquired VHF agents outside of hitherto established endemic regions. Examples are cases of Marburg fever in Zimbabwe (Gear et al., 1975), of Ebola fever in Côte D'Ivoire (Le Guenno et al., 1999) and of Lassa fever in an area covering Burkina Faso, Côte D'Ivoire, and Ghana (Günther et al., 2000).

1.4. Laboratory diagnosis of VHF

Isolation of the virus in cell culture or laboratory animals, PCR, virus antigen detection, electron microscopy, and detection of specific antibodies in the patient's serum are common methods for laboratory diagnosis of a VHF.

1.4.1. Virus culture and antigen testing

The inoculation of cultured cells with serum, cerebrospinal fluid (CSF), or other body fluids or tissue extracts is the classical method to isolate and detect VHF viruses. Filo-, bunya-, arena-, and flaviviruses grow in culture cells (often Vero cells are used). An alternative is the initial passaging of the isolate in a laboratory animal, which may be more sensitive than cell culture. A cytopathic effect is sometimes an unspecific sign of virus growth. Specific detection of the isolate may be accomplished by PCR on the culture supernatant or cells, detection of virus antigen in cells by immunofluorescence using virus-specific antibodies, or electron microscopy. Detection of a virus is independent of virus strain or species if broadly cross-reactive antibodies or electron microscopy (if the titer is high and the virus has a characteristic morphology) are available. Furthermore, substances inhibiting enzymatic reactions such as PCR usually do not affect viral growth in tissue culture. Finally, virus isolation opens the possibility to characterize the isolate in detail. A disadvantage is the time (days to weeks) required to isolate the virus and the need of BSL-3 of BSL-4 facilities. Direct demonstration by electron microscopy of viruses which show a specific morphology, such as filoviruses, is possible in organ sections and in serum, but high virus concentrations are needed for this purpose ($>10^7$ particles/ml serum; 10^5 ml^{-1} after ultracentrifugation) (Biel and Gelderblom, 1999).

Antigen tests are based on detection of virus proteins using specific antibodies, either after antibody-mediated capture of the antigen or directly in tissue by immunohistochemistry. Antigen tests are hardly influenced by virus variability and robust, which is advantageous under suboptimal laboratory conditions. The high virus concentration in Ebola and Lassa fever patients often facilitates antigen detection, although the tests are clinically less sensitive than PCR (Bausch et al., 2000; Leroy et al., 2000a). A simple diagnostic test for Ebola virus is the detection of antigen by immunohistochemistry in skin biopsies (Zaki et al., 1999).

1.4.2. Serological tests

Indirect immunofluorescence using virus-infected cells is a common antibody test for VHF viruses. ELISA tests using recombinant protein or infected cells as antigen have also been developed (ter Meulen et al., 1998; Ksiazek et al., 1999; Bausch et al., 2000; Saijo et al., 2002). Although the interpretation of immunofluorescence requires experience, the assay has advantages over other methods. Firstly, there is a long standing experience with this technique, which is important in rare diseases when only a limited number of sera is available to evaluate new assays. Secondly, all proteins of a virus serve as antigen, and thirdly, each virus generates a characteristic fluorescence pattern which adds specificity to the assay compared to an ELISA readout. A difficulty (or an advantage if a virus-specific test is not available) with immunofluorescence tests is that they often detect cross-reacting antibodies resulting from infections with related viruses. The relevance of antibody testing in acute VHF depends on the virus and the duration of illness. Early during illness, specific IgM is often not present yet, and patients who die of VHF can fail to seroconvert at all. However, in dengue virus and hantavirus infections, specific IgM often is the only detectable marker because virus is rapidly cleared. If only specific IgG is detectable, at least a four-fold increase in the titer should be demonstrated to prove an acute infection. The prevalence of VHF virus-specific IgG antibodies in the population of endemic areas may be remarkably high, especially for Ebola virus (Johnson et al., 1993), CCHF virus (Tikriti et al., 1981), RVF virus (Johnson et al., 1983) and Lassa virus (McCormick et al., 1987b). Demonstration of specific IgG at the beginning of the disease is of prognostic relevance in dengue virus infections, because pre-existing IgG, indicating previous infection with another dengue serotype, increases the risk of dengue hemorrhagic fever (DHF).

1.5. Molecular diagnostics—PCR

This review will focus on PCR-based methods. Other molecular diagnostic techniques such as nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA), which are summarized by the term NAT, have not yet been described for VHF agents.

1.5.1. Specimens and RNA preparation

The common clinical material used for VHF PCR is serum or plasma. Collection of blood in EDTA tubes ensures the highest PCR efficiency compared with serum, heparin, or citrate tubes (Holodniy et al., 1991; Dickover et al., 1998). Testing of CSF is useful when neurological symptoms are present. For example, Lassa virus was detected in CSF, but not in serum, in a patient with an atypical course of Lassa fever (Günther et al., 2001) (see Part II), and there are reports on the detection of dengue virus in CSF (Hommel et al., 1998). Since many viruses replicate in the upper respiratory tract, throat washings can be used to detect VHF viruses. Lassa virus was detected in such specimens (Monath et al., 1974; Johnson et al., 1987; Schmitz et al., 2002). Testing of urine is appropriate even after recovery from Lassa virus infections (Monath et al., 1974; Lunkenheimer et al., 1990). Similarly, Ebola virus RNA has been found in seminal fluid for long periods of time after recovery (Rodriguez et al., 1999). In asymptomatic Ebola virus infections, RNA was detected in PBMC (Leroy et al., 2000b).

Viral RNA from cell-free fluids can reliably be isolated with silica column affinity chromatography. Lysis is

accomplished by guanidinium salts. Chromatography methods are fast—which is a relevant aspect in VHF diagnostics—reproducible, and largely remove inhibitory substances. These purification techniques are also used for detection of RNA viruses in critical settings like blood donor screening (Roth et al., 1999). Volumes between 100 and 200 μl of body fluid are usually extracted, and the resulting nucleic acids are dissolved in volumes of about 50–100 μl. The maximum volume of nucleic acid solution in the PCR should be not more than 20% of the reaction volume to limit the input of residual substances which inhibit PCR (alcohol, salt, hemoglobin, heparin, etc.).

1.5.2. Primer design

The design of primers for PCR of VHF viruses can pose considerable problems. VHF agents are RNA viruses characterized by a high degree of genetic variability. The presence of up to four internal mismatches between primer and template seems to have little effect on product yield. However, five or more internal mismatches or a single mismatch at the 3′ position can drastically reduce PCR efficiency (Kwok et al., 1990; Christopherson et al., 1997). Therefore, it is important that as many sequences as possible from various strains and phylogenetic lineages are included in the primer design. Unfortunately, sequence information on VHF viruses is often limited, and in the past PCR assays had to be established on the basis of very few sequences. In this review, published primers were evaluated in light of novel sequence information and some assays were found to require revision (see Tables 2–5). Frequent mismatches at the same position may be compensated for by add-in primers. Reliable primers are those which are able to bind to several species of a virus genus or even family. For example, the 5′ and 3′ ends of the arenaviruses genomes are extremely conserved and therefore represent an ideal target sequence.

1.5.3. Reverse transcription PCR

Since all VHF viruses are RNA viruses, their detection requires reverse transcription of the RNA into cDNA prior to PCR (so-called RT-PCR). In almost all diagnostic PCRs for VHF viruses, cDNA synthesis is performed in a separate reaction vial before PCR. This procedure is referred to as two-step RT-PCR.

The recent development of one-step RT-PCR systems can make RT-PCR faster and more sensitive. Reverse transcription and PCR has first been performed in one reaction tube using Tth polymerase, a thermostable DNA-dependent DNA polymerase from bacterium *Thermus thermophilis* which also exhibits reverse transcriptase (RT) activity. This system has already been applied to filoviruses (Sanchez et al., 1999; Gibb et al., 2001a,b). An alternative one-step system is based on a mixture of a professional retroviral RT and inactivated Taq polymerase. The reversible inactivation of the Taq polymerase prevents the generation of by-products during the RT step, which would otherwise hamper specific amplification during the PCR step. Upon heating to PCR temperature, the Taq polymerase is activated (so-called hot-start enzyme). Such systems are reliably used in high-throughput screening for HIV in the blood bank setting (Drosten et al., 2001) and have been applied for a variety of VHF viruses (Drosten et al., 2002a; Preiser et al., 2002).

1.5.4. Modified conventional PCR

Nested or semi-nested PCR can increase the sensitivity (more cycles are possible than in a single-round PCR) and specificity of a reaction (additional oligonucleotides have to recognize the target sequence), and is therefore often used in VHF diagnostics (Schwarz et al., 1996; Deubel et al., 1997; ter Meulen et al., 1998; Leroy et al., 2000b; Sall et al., 2001; Scaramozzino et al., 2001). Alternatively, PCR products can be subjected to Southern blotting which reaches comparable sensitivity and specificity (Lunkenheimer et al., 1990; Trappier et al., 1993; Demby et al., 1994; Burt et al., 1998; Leroy et al., 2000a). The disadvantages of these methods are the increase in processing time and the increased risk of DNA contamination due to additional manipulations. Novel PCR techniques can obviate the need of nested PCR or Southern blotting. First, one-step RT/Taq polymerase formulations can reach the sensitivity of nested PCR in a single round (C. Drosten, unpublished data) and second, real-time PCR probes (see subsection 1.5.5) can yield a similar specificity as “nested” primers.

Because a wide spectrum of agents has to be tested in suspected VHF cases, the application of multiplex PCR seems to be a reasonable means to save time. However, multiplexing generally reduces the sensitivity of each individual test due to interference between the oligonucleotides and impedes optimization if each pair of primers requires other reaction conditions. This is probably the reason why no multiplex tests have been developed to diagnose VHF. As an alternative, PCRs for several VHF agents have been optimized on the basis of uniform cycling conditions. The reactions are performed simultaneously but in separate tubes, which speeds up testing (Drosten et al., 2002a).

1.5.5. Real-time PCR

So far, only few real-time PCR assays for VHF viruses have been published (Garcia et al., 2001; Gibb et al., 2001a,b; Drosten et al., 2002a). However, as this exciting new technique has several advantages over conventional diagnostic PCR, it is described here in more detail. In real-time PCR, the synthesis of the amplification product is monitored during the reaction allowing quantification of the virus RNA in the sample. The specificity of the reaction can be increased by using specific detection probes. Furthermore, the risk of contamination is greatly reduced as the PCR product is detected within the closed tube.

Real-time detection is accomplished by various fluorescence detection methods which are either sequence-dependent or sequence-independent. The latter work with dyes intercalating into double-stranded DNA, like SybrGreen or

Table 2
Filovirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	Filovirus polymerase	Two-step	Filo-B Filo-A	atgtggtgggttataataatcactgacatg (g) atcggaatttttctttctcatt (ag)	11/0/0[b] 9/0/0[b] family	Clinical 100% GS: virus isolation, antigen test	Sanchez et al. (1999)
2	Filovirus polymerase	Two-step SB	-"-	-"-	-"-	Clinical 100% GS: antigen test, serology	Leroy et al. (2000a)
3	Filovirus polymerase	One-step Real-time	-"-	-"-	-"-	95% detection limit: 2647 copies/ml 15 copies/PCR	Drosten et al. (2002a)
4	Ebola GP	Two-step	EBO-GP2 EBO-GP1	ttttttagtttcccagaaggcccact (g) aatgggctgaaaattgctacaatc (ag)	16/0/0 18/0/0	clinical 100% GS: virus isolation, antigen test	Sanchez et al. (1999)
5	Ebola–Reston NP	One-step	RES-NP2 RES-NP1	caagaaattagtcctcatcaatc (g) gtatttggaaggtcatggattc (ag)	2/0/0[c] 2/0/0[c]	Clinical 100% GS: antigen test	Sanchez et al. (1999)
6	Ebola–Zaire NP	One-step	ZAI-NP2 ZAI-NP1	gcatattgttggagttgcttctcagc (g) ggaccgccaaggtaaaaaatga (ag)	8/0/0[d] 8/0/0[d]	Analytically more sensitive than PCR no. 1	Sanchez et al. (1999)
7	Ebola–Zaire/Sudan GP	One-step Real-time	EBOGP-1Dfwd EBOGP-1Drev EBOGP-1ZPrb EBOGP-1SPrb	tgggctgaaaaytgctacaatc (ag) ctttgtgmacatascggcac (g) FAM-ctaccagcagcgccagacgg-TX VIC-ttacccccaccgccggatg-TX	12/0/0[e] 12/0/0[e]	10–100 fg viral RNA/PCR 3–8 pfu/PCR	Gibb et al. (2001a)
8	Marburg GP	One-step Real-time	MBGGP3fwd MBGGP3rev MBGGP3prb	ttccccttggaggcatc (ag) ggaggatccaacagcaagg (g) FAM-cgatgggctttcaggacaggtgt-TX	6/0/0 6/0/0	2–5 pfu/PCR	Gibb et al. (2001b)

Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; GS, gold standard for determining clinical sensitivity; pfu, plaque forming units; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.

[a] Homology of primers to published virus sequences (GenBank); X/Y/Z score indicates: X, number of published sequences overlapping the primer binding site (the more the better); Y, sequences containing ≥5 mismatches (the lesser the better); Z, sequences containing ≥2 mismatches within five bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better); "family" indicates that the primer binding site is even conserved within the virus family.

[b] Ebola and Marburg sequences.

[c] Only Ebola–Reston sequences.

[d] Only Ebola–Zaire sequences.

[e] Only Ebola–Zaire and –Sudan sequences.

Table 3
Bunyavirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	CCHF	Two-step	F2	tggacaccttcacaaactc (ag)	19/0/0	Clinical 25%	Schwarz et al. (1996)
	NP	Nested	R3	gacaaattccctgcacca (g)	19/0/0	GS: suspect in outbreak	
			F3	gaatgtgcatgggttagctc (nested ag)	18/0/0		
			R2	gacatcacaatttcaccagg (nested g)	31/0/0		
2	CCHF	Two-step	F2	tggacaccttcacaaactc (ag)	19/0/0	Clinical ~60%	Burt et al. (1998)
	NP	SB	R3	gacaaattccctgcacca (g)	19/0/0	GS: serology	
			Probe	Nick-translated probe			
3	CCHF	One-step	CCS	atgcaggaaccattaartcttggga (ag)	70/0/0	95% detection limit: 2779 copies/ml 15 copies/PCR	Drosten et al. (2002a)
	NP	Real-time	CCAS	ctaatcatatctgacaacatttc and ctaatcatgtctgacagcatctc (1:1 g)	37/0/0		
4	RVF	Two-step	RVF1	gactaccagtcagctcattacc (ag)	3/0/0	0.5 pfu/PCR	Ibrahim et al. (1997)
	G2	Nested	RVF2	tgtgaacaataggcattgg (g)	21/0/0		
			RVF3	cagatgacaggtgctagc (nested ag)	21/0/0		
			RVF4	ctaccatgtcctccaatcttgg (nested g)	21/0/0		
5	RVF	Two-step	NSca	ccttaacctctaatcaac (g)	2/0/0	Clinical 70%	Sall et al. (2001, 2002)
	NSs	Nested	NSng	tatcatggattactttcc (ag)	2/0/0	GS: virus isolation; 0.5 pfu/PCR	
			NS3a	atgctgggaagtgatgagcg (nested g)	2/0/0		
			NS2g	gatttgcagagtggtcgtc (nested ag)	2/0/0		
6	RVF	Two-step	S432	atgatgacattagaaggga (ag)	20/0/0	100 copies/PCR	Garcia et al. (2001)
	NSs	Real-time	NS3m	atgctgggaagtgatgag (g)	2/0/0		
			CRSSar	FAM-attgacctgtgcctgttgcca-TX			
7	RVF	One-step	RVS	aaaggaacaatggactctggtca (ag)	43/0/0	95% detection limit: 2835 copies/ml 16 copies/PCR	Drosten et al. (2002a)
	G2	Real-time	RVAs	cacttcttactaccatgtcctccat (g)	21/0/0		
			RVP	FAM-attgacctgtgcctgttgcca-TX			

Abbreviations: g/ag, genomic/antigenomic primer; SB, Southern blot; GS, gold standard for determining clinical sensitivity; pfu, plaque forming units; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.

[a] Homology of primers to published virus sequences (GenBank); X/Y/Z score indicates: X, number of published sequences overlapping the primer binding site (the more the better); Y, sequences containing ≥5 mismatches (the lesser the better); Z, sequences containing ≥2 mismatches within five bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better).

Table 4
Arenavirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	Lassa GPC	Two-step SB	36E2 80F2	accggggatcctaggcattt (g) atataatgatgactgttgttctttgtca (ag)	Family 11/2/0	Clinical 100% GS: virus isolation; 1–10 copies/PCR	Demby et al. (1994)
2	Lassa GPC	One-step Real-time	-”-	-”-	-”-	95% detection limit: 2445 copies/ml 14 copies/PCR	Drosten et al. (2002a)
3	Lassa GPC	Two-step SB	G2 G1 probe	cagaatctgacagtgtcca (ag) gtgtgcagtacaacatgagt (g) gctcccaccccaagccatcc (p)	7/0/0 7/1/4	Clinical ~85% GS: virus isolation	Trappier et al. (1993)
4	Lassa NP	Two-step SB	N2 N1 N3	ctgcccctgttttgtcagacatgcc (g) ggggctcgggctgggagagatggag (ag) aatgcagagttgctcaataatcagttcgggacc (p)	7/3/5 7/4/2	10^2 $TCID_{50}$ per PCR	Lunkenheimer et al. (1990)
5	Lassa GPC	Two-step SB	GPC2 GPC1 GPC3	ggatggcttggggtgggagctactacat (g) ataaccgatgggagatggtctcgag (ag) ggcagtgatcttcccaggttgtattttggattatc (p)	7/0/0 7/4/3 7/1/0	-”- 50 $TCID_{50}$ per PCR	-”-
6	Lassa NP	Two-step Nested	N2+ N1+ N2 N4	tgtactgcatcattcagtcaac (g) aagtgcaggtgtctatatggg (ag) ctgcccctgttttgtcagacatgcc (nested g) caacctaagctcacagcaacttgac (nested ag)	7/3/0 7/3/5 7/5/4		ter Meulen et al. (1998)
7	Junin S RNA	Two-step	J2 1	ggcatccttcagaacat (g) cgcacagtggatcctaggc (ag)	5/0/0 Family	PCR 7 and 8 combined	Lozano et al. (1995)
8	Junin S RNA	Two-step	1 J3	cgcacagtggatcctaggc (ag) caaccacttttgtacaggtt (g)	Family 43/0/1	Clinical 98% GS: serology	-”-
9	Arenavirus	Two-step	RT PCR2 PCR3 PCR4	cgcaccgdggatcctaggc (g/ag) cgcaccgaggatcctaggcatt (g/ag) cgcaccggggatcctaggcaatt (g/ag) cgcaccggggatcctaggctt (g/ag)	Family Family Family Family	Positive with hightitered serum and CSF	Günther et al. (2000)

Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; GS, gold standard for determining clinical sensitivity; $TCID_{50}$: 50% tissue culture infectious dose; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.

[a] Homology of primers to published virus sequences (GenBank); X/Y/Z score indicates: X, number of published sequences overlapping the primer binding site (the more the better); Y, sequences containing ≥5 mismatches (the lesser the better); Z, sequences containing ≥2 mismatches within five bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better); “family” indicates that the primer binding site is even conserved within the virus family.

Table 5
Flavivirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Reference
1	Flavivirus NS5/3′-NCR	Two-step	1 2	ggtctcctctaacctctag (g) agtggatgaccacggaagacatg (ag)	Genus Genus	Tanaka (1993)
2	Flavivirus NS3	Two-step	DV1 DV3	ggracktcaggwtctcc (g) aartgigcytcrtccat (ag)	Genus Genus	Chow et al. (1993)
3	Flavivirus NS5	Two-step	FG1 FG2	tcaaggaactccacacatgagatgtact(g) gtgtccatcctgctgtgtcatcagcataca(ag)	Genus Genus	Fulop et al. (1993)
4	Flavivirus NS5/3′-NCR	Two-step	EMF1 VD8	tggatgacsackgargayatg (g) gggtctcctctaacctctag (ag)	Genus Genus	Pierre et al. (1994)
5	Flavivirus NS5	Two-step	CFDJ9977 FUDJ9166	gcatgtcttccgtcgtcatcc (g) gatgacacagcaggatgggac (ag)	Genus Genus	Chang et al. (1994)
6	Flavivirus NS1	Two-step	DJA DJS	tccatcccatacctgca (g) gacatggggtattggat (ag)	Genus Genus	Meiyu et al. (1997)
7	Flavivirus NS5	Two-step	MA cFD2	catgatgggraaragrgarrag (ag) gtgtcccagccggcggtgtcatcagc (g)	Genus Genus	Kuno (1998)
8	Flavivirus NS5	Two-step Nested	MAMD cFD2 FS778	aacatgatggraaragrgaraa(g) gtgtcccagccggcggtgtcatcagc (ag) aargghagymcdgchathtggt (nested g)	Genus Genus Genus	Scaramozzino et al. (2001)
9	Yellow fever NS5/3′-NCR	Two-step Nested	EMF1 VD8 NS5YF	tggatgacsackgargayatg (g) gggtctcctctaacctctag (ag) atgcaggacaagacaatggt (nested g)	Genus Genus 17/0/0	Deubel et al. (1997)
10	Yellow fever, West African	One-step	269R	tgaaaggcgcggaacg (g)	39/0/0	Preiser et al. (2002) and personal communication
	Strains Envelop	Real-time	127F 150T	ccagttcaagccgccaaatag (ag) FAM-cggtgtttggctctgctttcagg-TX	25/0/4[b]	
11	Yellow fever 5′-NCR	One-step Real-time	YFS YFAS YFP	aatcgagttgctaggcaataaacac (g) tccctgagctttacgaccaga (ag) FAM-atcgttcgttgagcgattagcag-TX	31/0/0 32/0/0	Drosten et al. (2002a)

Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.

[a] Homology of primers to published virus sequences (GenBank); X/Y/Z score indicates: X, number of published sequences overlapping the primer binding site (the more the better); Y, sequences containing ≥5 mismatches (the lesser the better); Z, sequences containing ≥2 mismatches within five bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better); “genus” indicates that the primer binding site is even conserved within the virus genus.

[b] West African strain sequences only.

SybrGold. These dyes detect any PCR product, e.g. both specific and unspecific products. The specific product may be discriminated from by-products at the end of the reaction by melting point analysis (Wittwer et al., 1997). However, a positive signal must be confirmed by other methods such as sequencing, especially in VHF diagnostics.

Sequence-dependent detection is accomplished by binding of dye-labeled hybridization probes to the PCR product. This increases the specificity of PCR compared to SybrGreen or conventional agarose gel detection. So-called 5′ nuclease or TaqMan probes have a fluorescence dye on each end (Livak et al., 1995; Lie and Petropoulos, 1998), one of which is excited by light and transfers the energy to the second dye (fluorescence quenching). The energy is emitted from the second dye as long-wave light. If a specific PCR product has been synthesized, the probe is cleaved upon hybridization by the 5′–3′-exonuclease activity of Taq polymerase. Now, as the dyes are spatially separated, quenching is no longer possible and the energy is emitted directly from the first dye as short-wave light. This emission is recorded during PCR (real-time detection).

Fluorescence resonance energy transfer (FRET) or kissing probes (Wittwer et al., 1997; Didenko, 2001) are a pair of probes that hybridize in close proximity to each other to the PCR product. Two dyes, one attached to each probe, facilitate transfer of the energy from the first to the second dye if the probes hybridize to the specific product. The second probe emits the energy as light of specific wavelength which is measured.

Molecular beacons are stem-loop configured oligonucleotides that contain dyes at each end, providing a fluorescence quenching system as long as the stem is closed (Piatek et al., 1998). When a PCR product is generated, the loop hybridizes to the product, opening the stem. Quenching is reversed and short-wave light is emitted. Molecular beacons require highly conserved binding sites to be bound. Therefore, they are less suitable for detection of highly variable viruses like VHF viruses.

Only SybrGreen intercalation and 5′-nuclease probes have been implemented in PCR assays for VHF viruses so far (Garcia et al., 2001; Gibb et al., 2001a,b; Drosten et al., 2002a). While 5′-nuclease probes provide much more specificity than SybrGreen, the former require a conserved binding site (though more mismatches are allowed than with beacons). Some VHF viruses like Lassa virus are too variable for probe detection. In these cases, SybrGreen can facilitate real-time PCR (Drosten et al., 2002a).

Real-time PCR thermocyclers are equipped with a light source for excitation and a fluorimeter. High-throughput machines are the 7000, 7700, and 9700 sequence detection systems (Applied Biosystems), the i-cycler (BioRad), and the Opticon System (MJ Research). They utilize the 96-well format, except of the 9700 machine, which processes 378 samples. Low-throughput machines include the LightCycler (Roche), the SmartCycer (Cepheid), and the Rotorgene instrument (Corbett Research) in which 32, 16, and 36 (optional 72) samples, respectively, can be processed. These machines perform PCR much faster than conventional thermocyclers (an important aspect in VHF diagnostics) due to small reaction volumes and very rapid temperature changes. The SmartCycler can simultaneously perform up to 16 different cycling profiles. Only the 7700 machine can detect the full spectrum of visible light and is therefore the most flexible if new dyes become available. All other machines detect fixed wavelengths, limiting the range of possible dyes.

The number of PCR cycles necessary to yield a detectable PCR product (threshold cycle) depends on the template RNA concentration (Bustin, 2000). This concentration can be calculated from the threshold cycle using a standard curve. The standard curve is generated by amplification of serial dilutions of quantified virus stock or in vitro transcribed viral RNA (Garcia et al., 2001; Drosten et al., 2002a). Quantification of viral RNA in serum may be of interest as a prognostic parameter, in therapy monitoring, and in the risk assessment of virus transmission to contact persons. Studying the clinical significance of quantitative PCR will be an interesting future topic in the VHF field.

1.5.6. Sensitivity of VHF PCR

In VHF it is often impossible to determine the clinical sensitivity of a diagnostic method (for instance PCR-positive per actual number of infected patients) because large panels of samples from patients with established diagnosis are not (generally) available. However, at least the analytical sensitivity of the test can be determined, for example, in terms of RNA copies, 50%-cell culture infectious doses, or plaque forming units. A statistically precise determination of the analytical sensitivity (usually expressed as the ≥95% detection limit) is possible by testing a large number of replicate samples with different template RNA concentrations, and by subjecting the results (number of positive per number of tested) to probit regression analysis (Finney, 1971; Saldanha, 1993, 1999, 2001; Damen et al., 1996; Drosten et al., 2000, 2001, 2002b; Smieja et al., 2001). If a few clinical samples from VHF patients are available, it can be tested whether the concentration of viral RNA in the clinical samples is sufficiently high above the ≥95% detection limit of the assay (e.g. ≥100-fold). This allows to estimate if a new PCR assay is suitable for diagnostics even if the clinical sensitivity cannot be determined.

1.5.7. PCR inhibition

PCR inhibition has been reported to occur in 0.34 and 2.1% of serum samples tested by RT-PCR for HIV-1 and HCV, respectively (Drosten et al., 2001; Nolte et al., 2001). Inhibition can be caused by bad sample preparation, leaving residual substances like phenol, salts, and alcohol in the RNA solution. Furthermore, inhibitory substances may originate from the specimen, for example heme (Akane et al., 1994), leukocyte DNA (Morata et al., 1998), heparin (Satsangi et al., 1994), bile, lactoferrin, and IgG (Al-Soud et al., 2000). To detect inhibitory substances, an aliquot

of the clinical specimen can be spiked with a low amount of viral RNA (in vitro transcribed RNA or virus) prior to RNA preparation and processed in parallel with the original sample (Roth et al., 1999; Drosten et al., 2002a). If this external inhibition control is negative, the specimen should be diluted prior to RNA preparation to lower the concentration of inhibitors and be re-tested. Complete inhibition has indeed been observed with samples from patients with yellow fever and Ebola hemorrhagic fever (Drosten et al., 2002c) (see Part II for details). Inhibition control can be made more reliable using an artificial control RNA which is processed and amplified together with the sample RNA in one tube (internal control) (Cone et al., 1992; Kolk et al., 1994; Kox et al., 1994). This method is more sophisticated as the control RNA must differ from the target RNA between the primer binding sites to facilitate distinction of both amplicons, for example by probe hybridization after PCR (Kox et al., 1996; Bassiri et al., 1997; Pham et al., 1998; Rosenstraus et al., 1998; Johanson et al., 2001) or during real-time PCR (Drosten et al., 2000, 2001; Lachnik et al., 2002). Although technically feasible, internal inhibition control has not yet been implemented in VHF PCR.

1.6. Published PCR methods for detection of VHF viruses

1.6.1. Filoviruses

Ebola and Marburg viruses can be detected by Filoviridae-specific primers binding to the polymerase gene. These primers target sites that are highly conserved among the virus family (Sanchez et al., 1999) and were applied in PCRs of conventional and real-time format (Table 2, PCR 1–3). The glycoprotein gene of Ebola virus is used as a target to detect all four subtypes of Ebola virus (Zaire, Sudan, Ivory Coast, Reston), but not Marburg virus (Table 2, PCR 4) (Sanchez et al., 1999). Furthermore, real-time PCRs in the glycoprotein gene for differentiating Zaire and Sudan strains, as well as for detecting Marburg virus are available (Table 2, PCR 7, 8) (Gibb et al., 2001a,b). PCR tests targeting the nucleoprotein gene detect and differentiate Ebola subtypes Zaire and Reston (Table 2, PCR 5, 6). However, differentiation between filovirus species or subtypes is not required in the clinical situation.

Sensitivity studies have been mainly carried out using the polymerase gene-specific PCR. The clinical sensitivity of this PCR was 100% in two studies and thus higher than virus isolation or antigen capture assay (Sanchez et al., 1999; Leroy et al., 2000a). Furthermore, even in patients with silent seroconversion, Ebola virus RNA can be detected in PBMC by a nested format of the polymerase gene PCR (Leroy et al., 2000b). The viral RNA concentration in a serum of a convalescent and an acutely infected patient was 3×10^3- and 3×10^5-fold, respectively, above the detection limit of the real-time format of the polymerase gene PCR (Drosten et al., 2002a). Taking together the data on clinical and analytical sensitivity, the polymerase gene PCR can be considered a reliable tool to diagnose filovirus infections.

1.6.2. Bunyaviruses

CCHF virus is generally detected by RT-PCR targeting the S RNA segment which is best characterized among the three genomic segments (Table 3, PCR 1–3). However, the virus could be detected by PCR in only 2/3 of retrospectively (serologically) confirmed cases (Burt et al., 1998). RNA concentration in serum was tested in one case of CCHF virus infection and found to be 7.7×10^5 copies/ml of plasma by real-time PCR (Drosten et al., 2002a), which is about 3×10^2 times above the detection limit of that assay. According to the current data, PCR is a useful tool in the diagnosis of CCHF, but must be complemented by virus isolation and serology.

For detection of Rift Valley fever virus, assays targeting the M- and S-segment are available (Table 3, PCR 4–7). Quantitative real-time PCR has also been developed (Garcia et al., 2001; Drosten et al., 2002a) but has not yet been applied to clinical samples. Only one clinical evaluation study has been conducted (Sall et al., 2002), showing a sensitivity of 70.6% (Table 3, PCR 5). All samples negative by PCR but positive by virus culture had detectable IgM, suggesting that a combination of IgM and PCR testing seems to be at least equivalent to virus isolation (Sall et al., 2002).

Similar to dengue fever, hantavirus infections causing renal syndrome are usually diagnosed serologically because the virus is rapidly cleared. Therefore, hantavirus PCR assays are not further discussed here.

1.6.3. Arenaviruses

All current diagnostic PCRs target the S RNA segment (Table 4). For some Lassa virus PCRs, clinical evaluation data is available showing 85 and 100% sensitivity (Table 4, PCR 1, 3). These studies also demonstrate that virus is detectable beginning at least from day 3 of onset of fever. Quantitative real-time PCR was established (Table 4, PCR 2) and used to monitor virus RNA concentrations during the course of disease in two cases of Lassa fever (Fig. 1, and Part II, Lassa fever cases no. 1 and 2) (Drosten et al., 2002a; Schmitz et al., 2002). The RNA concentrations in serum of these patients were 5×10^2–2×10^6-fold above the detection limit of the assay. Extensive sequence information for the S RNA segment of Lassa virus has recently become available and reveals considerable genetic variability of the virus (Bowen et al., 2000; Günther et al., 2000, 2001). In light of these new sequences, some PCR primers, which had been designed mainly on the basis of isolates from Guinea, Sierra Leone, or Liberia, require revision (Table 4, PCR 3–6).

PCR assays are published for the New World arenavirus Junin (Table 4, PCR 7, 8). Clinical sensitivity of the combination of the two different Junin PCRs was 98%.

A PCR predictably amplifying any member of the Arenaviridae has been developed by targeting the highly conserved termini of the S RNA segment (Günther et al., 2000).

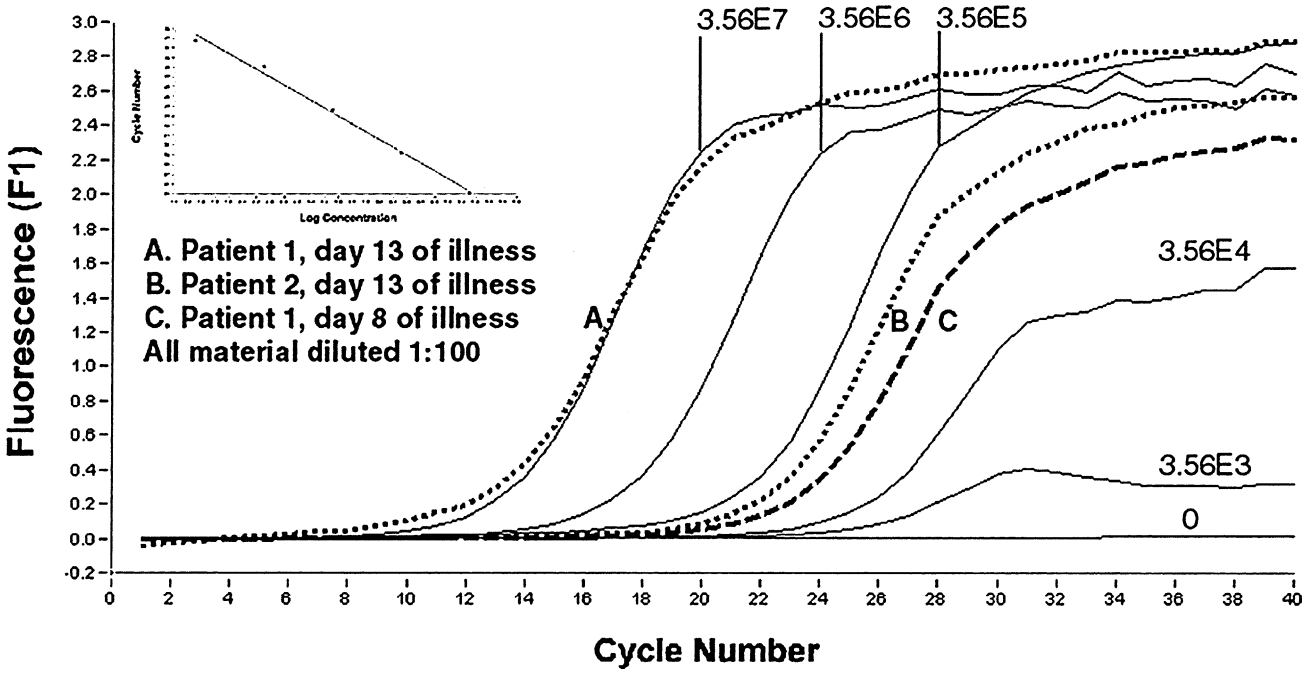

Fig. 1. Detection and quantification of Lassa virus in clinical specimens (A, B, and C) by real-time PCR using SybrGreen detection. Sera were diluted 1:100 to save material. The graphs show the real-time detection of the specific PCR products by fluorescence. A log 10 dilution series of in vitro-transcribed Lassa virus RNA was amplified as a standard; the copy number/ml plasma is shown at the curves. The insert in the upper left corner depicts the standard curve (*x*-axis: RNA concentration; *y*-axis: cycle number at first detection of PCR product) (modified from Drosten et al., 2002a).

Although this PCR amplifies the whole 3.4-kb S RNA, it was able to detect Lassa virus RNA in clinical samples (see Part II). The L RNA segment of arenaviruses contains highly conserved regions which are now being used to develop a PCR assay that is able to detect various Old World arenaviruses such as Lassa virus and lymphocytic choriomeningitis virus (S. Vieth and S. Günther, unpublished data). In conclusion, suspected Lassa virus infections will be identified by PCR with high probability after day 3 of illness, but primers may fail, due to the variability of the virus.

1.6.4. Flaviviruses

Yellow fever virus is usually detected using a universal flavivirus PCR (Table 5, PCR 1–8). Most of these universal assays target the NS5 which is highly conserved among the flavivirus genus. Data on clinical sensitivity of these assays for yellow fever are largely lacking. Only few yellow fever virus-specific PCR tests have been published (Table 5, PCR 9–11). A quantitative real-time PCR specific for yellow fever virus exists, and in two cases (see Part II) the viral RNA concentration in serum has been determined by this assay (Drosten et al., 2002a,c). It was 2×10^2- and 1×10^3-fold above the PCR detection limit. Therefore, yellow fever may be reliably diagnosed by PCR, but virus isolation and serology must be performed in parallel.

Dengue hemorrhagic fever (DHF) is rare compared to dengue fever (DF) and is almost exclusively seen in endemic areas. The molecular diagnosis of dengue infection has been extensively reviewed elsewhere (Guzman and Kouri, 1996; Gubler, 1998). Noteworthy are recently developed real-time PCR assays, which either detect all dengue virus subtypes (Drosten et al., 2002a) or differentiate between the four subtypes (Laue et al., 1999; Callahan et al., 2001; Houng et al., 2001). Because dengue virus is rapidly cleared during infection, DF and DHF are usually diagnosed by detection of specific IgM (μ-capture EIA), which may be complemented by PCR and virus isolation. In a few imported cases of dengue fever the diagnosis was established by PCR before the development of specific IgM (C. Drosten, unpublished data).

Apart from yellow fever and dengue virus, rare flaviviruses such Kyasanur forest virus or Alkhurma virus can cause a hemorrhagic fever (Pavri, 1989; Charrel et al., 2001). Use of universal flavivirus PCRs is appropriate when these infections are suspected and (other) typical VHF agents have been ruled out.

2. Part II

2.1. Introduction

In the second part of this review we will illustrate clinical, diagnostic, and epidemiological aspects of viral hemorrhagic fevers by means of cases which were imported to Europe from endemic countries or which occurred within Europe during the recent years. The lessons learnt from these cases will be helpful in the management of viral hemorrhagic fevers in the future. A list of hemorrhagic viral

Table 6
Viral diseases (VHF) imported into Europe in the recent years

Date	Country of origin	Imported to	Pathogen	Number of cases/fatalities	Business/tourist
November 1994	Ivory Coast	Switzerland	Ebola virus	1/1	Business
April 1996	Brazil	Switzerland	Yellow fever virus	1/1	Unknown
February 1998	Zimbabwe	UK	CCHF virus	1/1	Unknown
August 1999	Ivory Coast	Germany	Yellow fever virus	1/1	Business
January 2000	Ghana, Ivory Coast, or Burkina Faso	Germany	Lassa virus	1/1	Tourist
February 2000	Sierra Leone	UK	Lassa virus	1/1	Business
March 2000	Nigeria	Germany	Lassa virus	1/1	Nigerian citizen
June 2000	Sierra Leone	The Netherlands	Lassa virus	1/1	Business
March 2001	Chile/Argentina	France	Hantavirus (Andes virus)	1/0	Tourist
May 2001	Kenya	Germany	Dengue virus (hemorrhagic symptoms)	1/0	Tourist
November 2001	The Gambia	Belgium	Yellow fever virus	1/1	Tourist

The data were kindly provided by M. Niedrig, European Network for Imported Viral Diseases (ENIVD).

diseases imported into Europe during the last years is shown in Table 6. The Bernhard-Nocht-Institute in Hamburg has been involved in the diagnosis of three cases of Lassa fever, two cases of yellow fever as well as one case of Crimean-Congo hemorrhagic fever. Following an introduction into the life cycle of the three viruses and a general description of the disease and its therapy and prevention, the patient cases will be described. For more detailed information on other hemorrhagic fever viruses such as Ebola and Marburg virus, the reader is referred to accompanying articles in this issue.

2.2. Lassa fever

2.2.1. Classification and morphology of the virus

Lassa virus belongs to the family Arenaviridae which comprises only the genus arenaviruses. For excellent reviews on all aspects of arenaviruses, see Oldstone (2002a,b). Arenaviruses are classified as segmented negative strand RNA viruses, although the genes are oriented in both negative and positive sense on the RNA genome (a coding strategy which is called ambisense). The Arenaviridae are phylogenetically closely related to other segmented negative strand RNA viruses such as the Bunyaviridae and Orthomyxoviridae with which they share several common features of the replication cycle.

Arenaviruses are divided phylogenetically, serologically, and geographically into two major complexes, the Old World complex (e.g. Lassa virus, lymphocytic choriomeningitis virus (LCMV)) and the New World complex (e.g. Tacaribe virus, Junin virus, or Machupo virus). With the exception of the prototype arenavirus LCMV, all other species show a specific geographical occurrence. The restricted local occurrence of arenavirus species is at least partially explained by the geographical distribution of the respective natural host or reservoir species which are nearly exclusively rodents. In addition to Lassa virus, Junin, Machupo, Guanarito and Sabia virus can cause hemorrhagic fever in humans, while LCMV mostly causes a mild illness, usually meningitis. Propagation of the former viruses requires laboratories of biosafety level 4, which are available in only few countries world-wide.

Arenaviruses share a characteristic morphology. In electron microscopic pictures, the interior of virion shows a typical granular pattern (name bearing: arenosus = sandy), which is surrounded by an envelope.

2.2.2. Genome structure

The single-stranded arenavirus genome consists of two segments, a small (S) and a large (L) RNA fragment, with a size of 3.4 and 7 kb, respectively (Fig. 2). The S RNA encodes the viral glycoprotein precursor protein (GPC) and the nucleoprotein (NP). GPC is posttranslationally cleaved into GP1 and GP2. The L RNA encodes the viral polymerase (L protein) and a small, zinc-binding (Z) protein. The genes are located on the RNA in opposite directions separated by an intergenic region. The intergenic region of both segments predictably folds into an stable secondary structure. The terminal 19 nucleotides at the 3′ and 5′ ends of the RNA segments are complementary to each other and are highly conserved among all arenaviruses. They are believed to function as a binding site of the viral polymerase. The viral RNA which is predominantly found within virions is defined as the genomic RNA. The genomic and antigenomic RNAs themselves are not infectious after introduction into cells.

2.2.3. Viral life cycle

Lassa virus enters the cell via the receptor molecule alpha-dystroglycan (Kunz et al., 2002). Replication and transcription of the genome takes place in the cytoplasm of an infected cell. During genome replication, a full-length copy of the genomic S and L RNAs is synthesized yielding the corresponding antigenomic S and L RNAs. Expression of proteins requires transcription of mRNA from both the genomic and antigenomic RNA within a ribonucleoprotein complex (Fig. 2).

NP is the most abundant protein of the ribonucleocapsid, followed by Z protein and L protein. NP and L protein are sufficient for genome replication and transcription

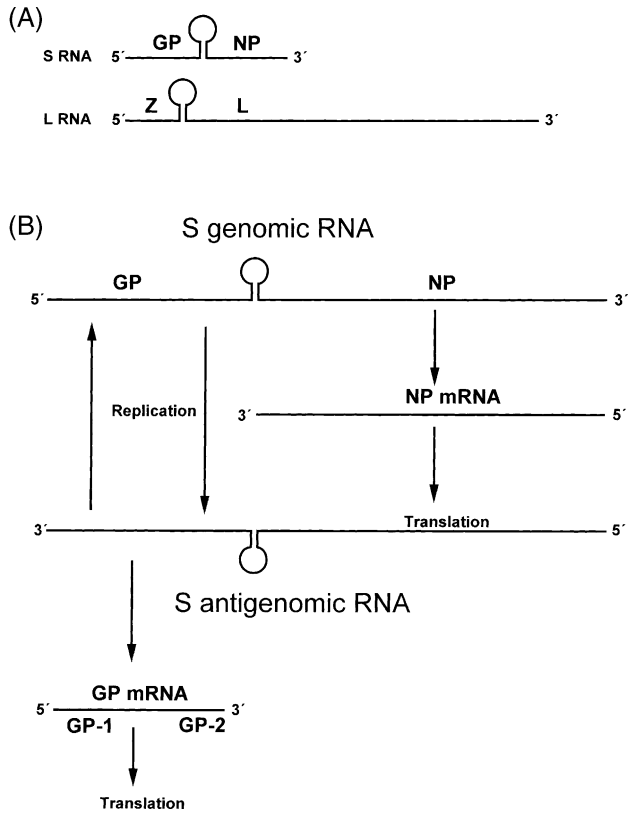

Fig. 2. (A) S and L RNA segments of the Lassa virus genome. (B) Model of replication and gene expression of Lassa virus. The stem-loop structure in the intergenic region is schematically shown. For details see the text (modified from Meyer and Southern, 1993).

(Lee and de la Torre, 2002). Besides of a structural function (Salvato et al., 1992)—Z protein may function analogous to matrix proteins—it seems to have a regulatory function during infection due to its interaction with a variety of cellular proteins (Borden et al., 1998a,b). The large L protein most likely represents the viral RNA-dependent RNA polymerase. Within a central domain, it shares conserved motifs with the catalytic domain of other viral RNA polymerases (Lukashevich et al., 1997).

GPC is directed posttranslationally into the endoplasmic reticulum. Cleavage of GPC into GP1 and GP2 occurs at a later stage of the secretory pathway. The cellular protease SKI-1/S1P was shown to be responsible for cleavage (Lenz et al., 2001). Cleavage of GPC is required for incorporation of glycoproteins into the virion envelope and thus for release of infectious Lassa virus. The glycoproteins are transported to the cell membrane where budding and release of the virus takes place.

2.2.4. Epidemiology

Lassa fever is endemic in West Africa. The disease is highly endemic in Sierra Leone, Guinea, Liberia, and Nigeria. One of the imported cases suggest that the virus is endemic in larger areas of West Africa (see below). The natural host of Lassa virus is the African rodent *Mastomys natalensis*, although there is some dubiety (Salazar-Bravo et al., 2002). The rodents shed the virus in urine and contamination by unprotected food is a likely mode of transmission. A risk factor for transmission is also hunting of *Mastomys* as a food source, which is associated with a close contact with the rodent (ter Meulen et al., 1996). In Africa, secondary infections from human to human occur frequently in the hospital setting causing epidemics with high fatality (Carey et al., 1972). Poor medical practice is one reason for nosocomial spread of the virus (Fisher-Hoch et al., 1995b). Barrier nursing techniques and adherence to minimal standards of hygiene can effectively prevent transmission of Lassa virus in the hospital setting (Fisher-Hoch et al., 1985; Helmick et al., 1986).

The incubation period of Lassa fever may last up to 3 weeks. Therefore, the virus may be imported into other regions of the world, while the infected person is asymptomatic or shows early unspecific signs of Lassa fever. So far, about 20 cases of imported Lassa fever have been reported world-wide (Johnson and Monath, 1990). The risk of secondary infections was investigated in cases of Lassa fever imported into the U.S. and Great Britain (Zweighaft et al., 1977; Cooper et al., 1982; Holmes et al., 1990). In contrast to the situation in Africa, no clinically apparent secondary cases were observed. Serological testing of contact persons also disclosed no asymptomatic infections. Therefore, the risk of human-to-human transmission due to imported Lassa fever is considered low. Ribavirin, which is therapeutically effective (McCormick et al., 1986a), has been used for post exposure prophylaxis in some cases (Holmes et al., 1990).

2.2.5. Clinical manifestation and pathogenesis

Lassa fever is associated with a wide spectrum of clinical manifestations. Initially, flu-like and gastrointestinal symptoms are present in most cases and the disease can, clinically, hardly be distinguished from other viral, bacterial, or parasitic infections. Hemorrhage and organ failure occurs in a subset of patients and is associated with high mortality. Neurological complications such as confusion, tremor, convulsion, and coma are frequent in critically ill patients who often die after the onset of these symptoms (Solbrig and McCormick, 1991; Cummins et al., 1992; Solbrig, 1993). Sensorineural deafness is a neurological complication of the reconvalescence phase (Cummins et al., 1990). Typical clinical courses as well as a new manifestation of Lassa fever—an encephalopathy with the virus being detectable only in CSF during the late phase of infection—are illustrated by the case reports.

The pathogenesis of Lassa fever is poorly understood. The virus can be isolated from essentially all organs, but histopathological examination often reveals only lesions which are not sufficient to explain organ failure and death (Walker et al., 1982). Measurements of cytokines in two cases of imported Lassa fever may point to an important role of proinflammatory cytokines in pathogenesis (Schmitz et al., 2002).

2.2.6. Antivirals, therapy, and vaccination

A variety of compounds has shown antiviral activity against arenaviruses in cell culture (Huggins et al., 1984; Rodriguez et al., 1986; Burns et al., 1988; Andrei and De Clercq, 1990, 1993; Nair and Ussery, 1992; Smee et al., 1992; Candurra et al., 1996; Garcia et al., 2000; Wachsman et al., 2000; Bartolotta et al., 2001). However, only the broad-spectrum antiviral agent ribavirin, which significantly reduces replication of Lassa virus and other arenaviruses in cell culture (Jahrling et al., 1980; Huggins et al., 1984; Rodriguez et al., 1986; Huggins, 1989), was tested in animals. Early treatment of monkeys was fully protective, while initiation of the therapy at day 7 conferred only partial protection (Jahrling et al., 1980, 1984). In treated animals, viremia developed more slowly and peaked at lower titers than in untreated controls.

A clinical trial showed a therapeutic effect of the drug in humans with Lassa fever (McCormick et al., 1986a). In patients who had risk factors for a fatal outcome of the disease at admission, like high liver enzyme levels, and were treated within the first 6 days after the onset of fever, the case fatality rate decreased from 55 to 5%. Similarly, in patients showing high viremia as a risk factor, the therapy reduced the case fatality from 76 to 9%. Even in patients treated at day 7 or later, the case fatality could be reduced in these risk groups from 55 to 26% and from 76 to 47%, respectively. A problem associated with ribavirin are the side effects, i.e. reduction of haematocrit due to reversible anaemia. In Lassa fever patients, brief episodes of rigor toward the end of the treatment course were reported (Fisher-Hoch et al., 1992).

Another treatment option may be the application of neutralizing Lassa virus antibodie. However, neutralizing antibodies develop only very late (months) after convalescence, if at all, usually show only a low titer, and are poorly cross reactive between different Lassa strains (Jahrling et al., 1985). Guinea pigs and monkeys were protected from Lassa fever by high dose treatment with plasma containing neutralizing activity (Jahrling, 1983; Jahrling et al., 1984). There are also case reports which may suggest effective treatment of Lassa fever patients with reconvalescent sera (Frame et al., 1984). However, a large study which included controls did not reveal a beneficial effect of treatment with reconvalescent sera (McCormick et al., 1986a). Since the neutralizing titer of the sera used for treatment was not known, it cannot be excluded that neutralizing antibodies are effective. The problem is to get sera with sufficiently high neutralizing activity. Actually, even horses have been employed as producers of neutralizing antibodies to Lassa virus (Krasniaskii et al., 1997).

Currently, there is no vaccine available for humans, although several experimental vaccines have been successfully tested in monkeys (Fisher-Hoch and McCormick, 2001).

2.3. Imported cases of Lassa fever

2.3.1. Clinical description

Case 1 (Fleischer et al., 2000; Schmitz et al., 2002): A 22-year old female art student from Germany traveled though Ivory Coast, Ghana and Burkina Faso (i.e. countries not known as Lassa endemic regions). The travel route during the incubation period is shown in Fig. 3. In Abidjan (Ivory Coast) she had sudden onset of high fever (39 °C) and flu-like symptoms on January 2, 2000. She had been vaccinated against yellow fever but had not taken malaria prophylaxis. The diagnosis of malaria was made at a local hospital

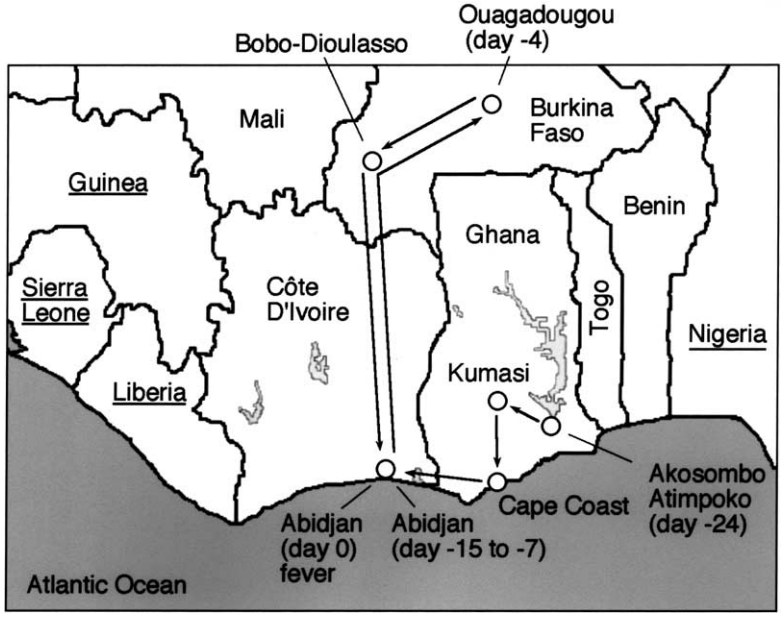

Fig. 3. Map of West Africa and travel history of the patient 1 before the onset of febrile illness (day 0). Countries where Lassa fever is endemic are underlined (modified from Günther et al., 2000).

and she was given artesunate. She returned to Germany on day 6 of illness by public flight and was admitted at the hospital at Schwäbisch Hall with high fever (40 °C) and tonsillitis. Several blood smears were negative for malaria. On day 9 she was transferred to a hospital specialized in tropical diseases in Würzburg. On admission, a severe pharyngitis, ulcerative tonsillitis, shortness of breath with cough, high fever, and diarrhea were noted. She received ciprofloxacin. Lassa fever was considered and a serum sample was sent to the BSL-4 laboratory. RT-PCR for Lassa virus was positive, while Lassa virus-specific IgG or IgM antibodies were not detected (also not during the further course). Intravenous ribavirin treatment was started. Liver enzymes were elevated and peaked at day 11 (aspartate aminotransferase (AST) up to 1500 U/l and alanine aminotransferase (ALT) up to 250 U/l). She developed a large pleural effusion. Despite therapy, encephalopathy developed, and an increased serum lipase level pointed to pancreatitis. Due to progressive renal failure and hypovolemia, the patient was haemodialysed and received volume expansion. A massive hemorrhage developed which could not be corrected by 19 blood donations. The patient experienced seizures and died from hemorrhagic shock and organ failure on day 14. Histological post-mortem examination of the liver showed only rare necrotic cells in the intermediate zone of some lobuli.

Case 2 (Schmitz et al., 2002): A 48-year-old male surgeon worked for 5 months at a hospital in rural Sierra Leone. He was healthy until July 10th, 2000, when he developed fever and malaise. He had been vaccinated against yellow fever and had not taken malaria prophylaxis. He received artesunate for presumed malaria from a local hospital without subsequent improvement of his symptoms. On day 4, he returned to The Netherlands and was hospitalized at the Leiden University Medical Center with high fever. On the following day he was admitted to the hospital. He complained of nausea, crampy watery diarrhea, myalgia, arthralgia, and headache. Chest, abdomen, and extremities were normal, except for a faint rash on the trunk. Thick blood smears were negative for malaria. On suspicion of typhoid fever, cefamandol and netilmicin were given. He improved with resolution of headache, nausea, and diarrhea, while his temperature remained elevated at 38.5 °C. Since cultures were negative for bacteria, cefamandol and netilmicin were stopped and doxycycline was started. On day 11, he developed a mild encephalopathy and renal dysfunction. The clinical diagnosis of Lassa fever was made and intravenous ribavirin was started immediately. Liver enzymes were elevated and peaked at day 12 (AST up to 3000 U/l and ALT up to 500 U/l). Serum samples were sent to the laboratory, where Lassa virus RNA was detected by RT-PCR. Subsequently, he developed progressive renal failure and hypoxia with diffuse pulmonary infiltrates. He was transferred to the intensive care unit on day 15, where he required intubation. The next day he died of respiratory failure.

Case 3 (Günther et al., 2001): A 56-year-old Nigerian male was seen on March 21, 2000, at the Emdee Medical Center at Jos, Nigeria, because of a 2-week history of fever (38.2 °C) and diarrhea. Treatment with antibiotics was initiated. On March 23, he was admitted at the Life Camp Clinic Abuja, Nigeria. His temperature was 39.6 °C; he was drowsy and intermittently disoriented. Flu-like symptoms and diarrhea were absent. The liver enzymes were slightly elevated (AST 58 U/l and ALT 80 U/l). On March 25, he experienced a 30-minute episode of generalized seizures without loosing consciousness. The temperature declined and, on March 27, he was transferred to the neurology department of the Dr.-Horst-Schmidt-Kliniken in Wiesbaden, Germany. On admission, he was afebrile, disoriented in time and place, and with a slight depression of his conscious level. Meningeal signs were absent. The electroencephalogram showed no epileptic signs, and nuclear magnetic resonance tomography of the brain was normal. CSF findings demonstrated a dysfunction of the blood–brain barrier. The patient received methylprednisolone and heparin as a thrombosis prophylaxis. Further seizures were not observed and the orientation improved. Five days after admission, the patient suddenly died with signs of pulmonary embolism. There was clinically no sign of Lassa fever. Diagnosis of Lassa virus infection was established shortly after the death of the patient by laboratory screening. Lassa virus was detected by RT-PCR in CSF but not in serum. Lassa virus-specific IgM and IgG were detected in serum and CSF.

These three cases demonstrate the great variety in the clinical manifestations of Lassa virus infection and the challenge to early diagnosis of this deadly disease. Even in classical courses of Lassa fever, as in cases 1 and 2, the unspecific signs of the disease make a clinical diagnosis difficult. This is the main reason why the diagnosis was established only late during illness. In case 1, the fact that the patients did not travel through known endemic regions (Fig. 3) further complicated the diagnosis.

Patient 3 experienced an uncommon course of Lassa fever. For this reason, neither in Nigeria nor in Germany Lassa fever was clinically suspected. Encephalopathy has been the chief manifestation during the late phase of infection and Lassa virus was detected in CSF but not in serum, a clinical syndrome which has not been reported in Lassa fever patients before. This makes it clear that Lassa fever should be considered in West African patients and in those returning from this region with fever and neurological signs.

On the other hand, all three patients had early signs which, retrospectively, turn out to be typical symptoms of Lassa fever: diarrhea, pharyngitis, liver enzyme elevations, and AST levels which were much higher than ALT levels (McCormick et al., 1986b, 1987a). Especially an AST/ALT ratio much greater than 1 is common in Lassa fever and should be an alarming sign.

2.3.2. Diagnostic procedure

The laboratory diagnosis was based on three methods: RT-PCR, virus isolation in cell culture, and detection of specific IgM and IgG. For RT-PCR, a protocol (Demby et al.,

1994) was chosen in which the primers were based on a large number of Lassa virus sequences from various geographical regions. Therefore, this PCR was expected to be robust with respect to virus variability. However, subsequent sequence analysis of the Lassa virus strain isolated from the CSF revealed seven nucleotide exchanges in a primer binding site which definitely reduced amplification efficiency. Nevertheless, the PCR showed a signal because of the high RNA concentration. This underscores that existing PCR protocols for VHF agents must be continuously revised in view of novel sequence information. An RT-PCR amplifying the whole S RNA fragment was used as a diagnostic tool in parallel to the short-range PCR. The primers bind to the highly conserved RNA termini. Therefore, different arenaviruses can be amplified (Fig. 4A and B). Although this PCR is clearly less sensitive than a short-range PCR, it amplified the S RNA directly from serum in case 1 (Fig. 4C) and, in a retrospective analysis, also from CSF in case 3 (Günther et al., 2001).

In addition, Lassa virus was isolated in cell culture. In case 2, the virus concentration was so high that virus was detected in the cells by immunofluorescence at 2 days after inoculation. Virus culture is still a timely method that is hardly influenced by virus variability and allows a detailed characterization of the causative agent. However, it may be time-consuming. Isolation of the virus from CSF in case 3 required about 2 weeks.

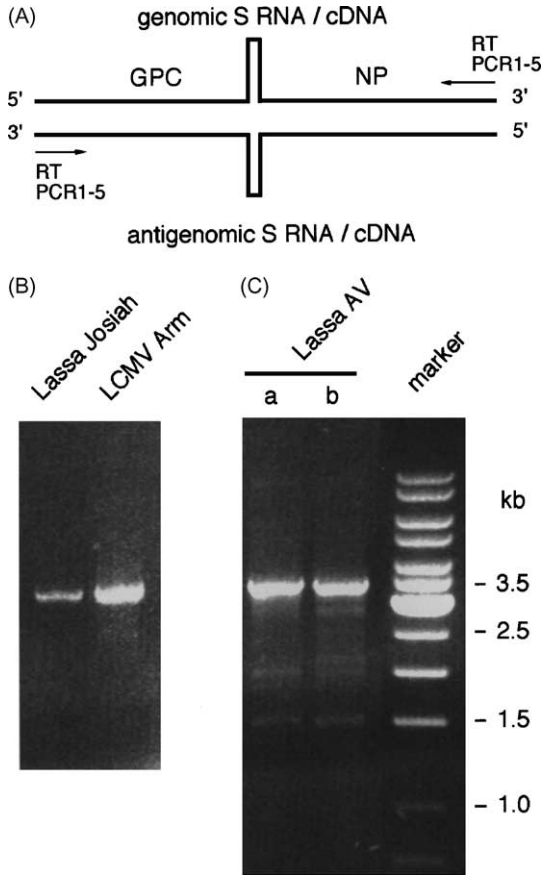

Fig. 4. Reverse transcription and PCR amplification of full-length S RNA. (A) Position of the RT and PCR primers at the termini of S RNA. (B) Different arenaviruses such as Lassa virus (strain Josiah) and LCMV (strain Armstrong) can be amplified by the S gene PCR. Virus RNA was isolated from the supernatant of infected cells. (C) Amplification of full-length Lassa virus S RNA directly from serum of patient 1 (Lassa virus strain AV) (modified from Günther et al., 2000).

Lassa virus-specific antibodies were detected by immunofluorescence using Vero cells infected with a laboratory strain of Lassa virus. In case 1, specific antibodies were never detected throughout the course showing that a Lassa infection may be missed if only antibodies are tested for. Furthermore, IgG or IgM antibodies are detectable only in about half of Lassa fever patients on admission to the hospital (Johnson et al., 1987).

Virus RNA concentrations were monitored retrospectively using real-time PCR (Fig. 5). In patient 1, an initial concentration of 10^6 RNA molecules/ml serum was found. The virus concentration increased by several orders of magnitudes until day 10 when the viremia reached a plateau phase. This phase coincided with the development of serious organ manifestations and bleeding. This finding is consistent with the observation that the level of viremia in Lassa fever patients is highly correlated with the risk of fatal outcome (Johnson et al., 1987). In contrast, in patient 2 who had less severe symptoms and no hemorrhagic signs, the virus concentration steadily decreased during the late stage of illness. However, despite a decreasing virus load, the disease progressed and the outcome was fatal.

2.3.3. Management of contacts

To determine the risk of secondary transmission after import of Lassa fever into Europe, contact persons of patient 1 were identified and kept under surveillance (W. Haas and S. Günther, unpublished data). The level of exposure of these persons was evaluated and they were categorized as high risk contact (unprotected exposure of skin or mucous membranes to blood or secretions), close contact (direct physical contact with the index patient), and casual contact (in the same room with the index patient or travelling on the same flight). About 30 high risk and close contacts were identified and several of these had been administered ribavirin, which is recommended for prophylaxis. No symptomatic secondary infections were observed. Furthermore, no spread of the virus during the initial phase of the symptomatic illness (<9 days) of the index patient was disclosed by serological testing, although numerous high risk exposures occurred during this period. However, Lassa virus-specific IgG antibodies were detected in the serum of a physician who had made a physical examination of the patient on day 9 (when viremia in the index patient had increased, Fig. 5) and who had been exposed to cough of the patient. The physician took ribavirin prophylactically and did not develop symptoms of Lassa fever. Even if the transmission could not definitively be proven by IgM detection or an increase in IgG titer, the possibility that a transmission took place should be considered in the management of imported

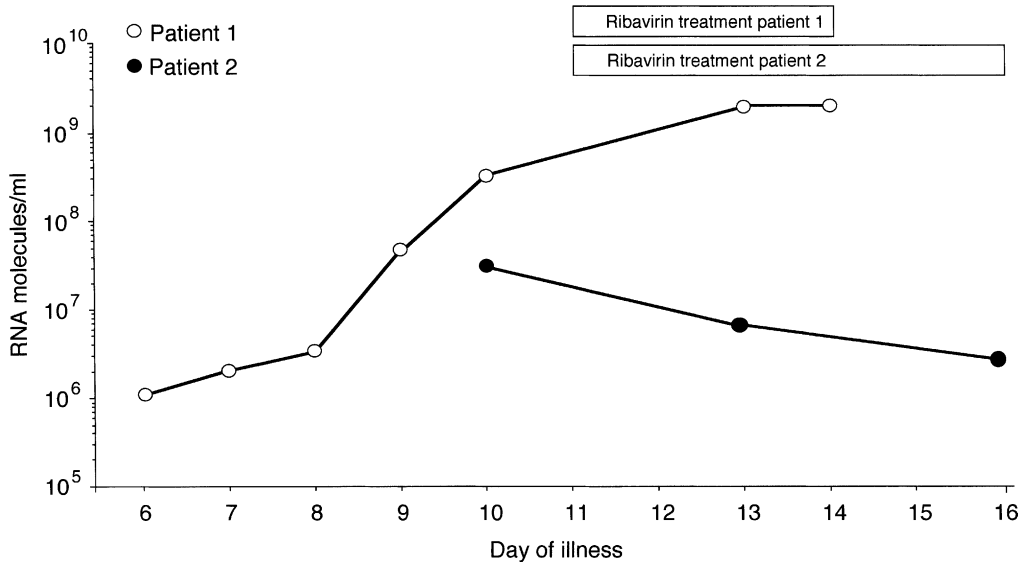

Fig. 5. Measurement of the Lassa virus RNA concentration in consecutive serum samples of patients 1 and 2. Quantification was performed by real-time PCR using SybrGreen detection (see Fig. 1) (modified from Schmitz et al., 2002).

Lassa fever. In particular, the stage of illness and/or the level of viremia at the time point of exposure should be included in the risk assessment of transmission. Overall, the data from this case are in agreement with previous studies suggesting that the risk for human-to-human transmission of Lassa virus outside endemic areas is extremely low (Zweighaft et al., 1977; Cooper et al., 1982; Holmes et al., 1990). It can be concluded from all this that Lassa virus is unlikely to have the potential to cause epidemics in Europe.

2.4. Yellow fever

2.4.1. Introduction

Yellow fever virus is the prototype of the genus flavivirus within the family Flaviviridae. Most flaviviruses are arthropod-borne and replicate in both vertebrates and arthropods. Yellow fever virus is transmitted via chronically infected mosquitoes (reviewed in Burke and Monath, 2001). Since the discovery of yellow fever virus as a filterable agent transmissible by mosquitoes about a century ago, much progress has been made in our understanding of the virus life cycle, the virion and genome structure as well as the functions of the individual viral proteins. Although vaccination has been available for almost 65 years, yellow fever virus still remains a public health problem due to its endemic persistence in sub-Saharan Africa and South America.

2.4.2. Virion morphology and genome structure

Virions are spherical and enveloped with a diameter of 40–60 nm. The envelope surrounds a spherical nucleocapsid, which consists of a single-stranded RNA genome complexed with capsid (C) protein. Embedded in the envelope are two proteins, the envelope protein (E) and the membrane (M) protein. PrM is the unprocessed precursor of the M protein found in intracellular viruses, whereas M is predominant in released virus particles. Released virions have a greater infectivity than those remaining intracellularly.

The genome is a capped RNA of positive polarity with a length of approximately 11 kb. Flanked by short 5′ and 3′ non-translated regions, the RNA comprises a single open reading frame. The translated polyprotein is processed co- and posttranslationally by host cell and viral proteases into individual viral proteins. The protein order in the polyprotein is NH2-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, where C, prM(M), and E represent the structural proteins or their precursors and NS1 through NS5 represent the nonstructural (NS) proteins (Fig. 6) (reviewed by Lindenbach and Rice, 2001). The genomic flavivirus RNA is infectious after introduction into cells.

2.4.3. Viral life cycle

Binding of the virions to the cell surface is probably mediated by the E protein, but a specific cellular receptor has not yet been identified. After uptake of the virions by endocytosis, uncoating occurs via membrane fusion in a pH-dependent manner. The nucleocapsids are released into the cytoplasm where translation and RNA replication occurs (Fig. 6). Translation of the genomic RNA can start immediately and results in the formation of the polyprotein. Cleavage in the structural protein region is mainly mediated by host cell proteases. The majority of processing events within the NS protein region are mediated by the viral NS2B-3 protease. The catalytic activity of the viral protease is localized in the N-terminal part of NS3 and requires NS2B as a cofactor (Chambers et al., 1991; Falgout et al., 1991). NS3 also possesses an RNA helicase and RNA triphosphatase activity (Warrener et al., 1993; Wengler and

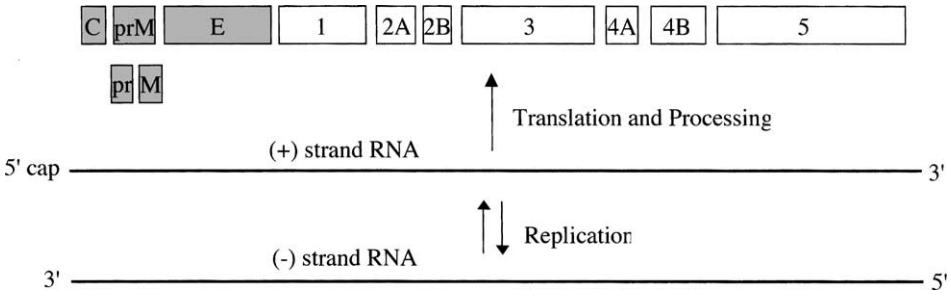

Fig. 6. Model of replication and gene expression of yellow fever virus. For details see the text.

Wengler, 1993). NS1 plays a role in early RNA replication and interacts with NS4A (Lindenbach and Rice, 1997, 1999). NS2A seems to play a role in the production of infectious particles (Kümmerer and Rice, 2002), whereas no specific function has been attributed to NS4B yet. NS5 represents the RNA-dependent RNA polymerase required for replication of the genome (Koonin, 1993; Tan et al., 1996). After assembly of the replication complex, complementary RNA negative strand is synthesized, which then serves as template for synthesis of additional genomic plus-strand molecules. Progeny virions assemble by budding through intracellular membranes and are released via exocytosis.

2.4.4. Reverse genetics

Since the RNA genome of yellow fever virus is per se infectious, it is possible to create manipulated viruses with modified phenotypes by introducing mutations in an infectious full-length cDNA clone. The first infectious yellow fever virus RNA synthesized in vitro was described in 1989 (Rice et al., 1989). Meanwhile, several infectious full-length cDNA clones have been described for different flaviviruses (Ruggli and Rice, 1999). The cDNA clones usually contain the complete genomic sequence under control of an SP6 or T7 promoter. After linearization of the cDNA at the 3′ end of the genome, RNA is transcribed in the presence of a cap structure analogue by SP6 or T7 RNA polymerase. Transfection of the in vitro synthesized RNA into cells results in production of recombinant virus. Recently, it was exemplified with poliovirus whose RNA is per se infectious as that of flaviviruses, that an infectious clone can be assembled from chemically synthesized oligonucleotides (Cello et al., 2002). Thus, for the first time an infectious virus was generated solely on the basis of sequence information. It is noteworthy that Ebola virus can also be rescued from cloned cDNA and can be genetically modified (Volchkov et al., 2001).

2.4.5. Epidemiology

Yellow fever occurs in tropical South America and sub-Saharan Africa, but not in Asia. The virus is maintained in two transmission cycles, known as jungle yellow fever and urban yellow fever. In the first transmission cycle, the virus circulates in the forest mainly between non-human primates and various mosquito species; humans are only occasionally infected. In the urban cycle, the virus spreads into the human population and solely circulates between humans and the mosquito *Aedes aegypti*. In this case, large epidemics may occur if the vaccination coverage of the human population is below 80%. Yellow fever is not transmitted from human to human.

Based on sequence differences within the E gene, three genotypes can be distinguished which cluster geographically: genotype I in Central and East Africa, genotype IIA in West Africa, and genotype IIB in America (Chang et al., 1995).

2.4.6. Pathogenesis and clinical features

The virus first replicates in regional lymph nodes and spreads to liver, spleen, bone marrow as well as cardiac and skeletal muscles. The major target organ is the liver. The incubation period is only 3–6 days. The clinical spectrum varies from very mild illness to severe hemorrhagic disease, which is lethal in 20–50% of the patients. Often a biphasic course is observed. In addition to fever, the first period is characterized by less specific symptoms such as chills, headache, myalgia, nausea, and vomiting. This phase lasts approximately 3 days. After a decrease of temperature, symptoms of organ manifestation and organ failure appear in severe and fatal cases: jaundice (“yellow” fever), liver and renal failure, and bleeding.

2.4.7. Vaccination and therapy

For yellow fever, vaccination with the live-attenuated 17D strain is available. Attenuation of the yellow fever virus was obtained during serial passage of the parental Asibi strain in chicken embryo cells (Theiler and Smith, 1937). The vaccine strain has been used effectively for almost 65 years. However, several fatalities associated with vaccination have been reported recently (Chan et al., 2001; Martin et al., 2001; Vasconcelos et al., 2001). In view of the fact that several hundred million doses have been given without serious side effects, the vaccine is still considered safe and effective and WHO has not changed its recommendation regarding a universal childhood vaccination in endemic countries (Anonymous, 2002). Vaccination is also required when travelling to endemic areas. It is unknown which of the amino acid exchanges present in the vaccine strain are responsible

for attenuation. Comparison of several 17D lineages with the parental Asibi strain revealed 22 amino acid substitutions, 8 of which are localized in the E protein and are potentially related to attenuation (Galler et al., 1997). The availability of an infectious full-length clone of yellow fever virus 17D represents a tool to determine the importance of single amino acid exchanges in attenuation and to genetically engineer further attenuated vaccines in the future.

There is no specific therapy for yellow fever virus.

2.5. Imported cases of yellow fever

2.5.1. Clinical description

Case 1 (Teichmann et al., 1999): A German cameraman spent 2 weeks in the Comoé National Park of Côte D'Ivoire. Malaria prophylaxis was taken. He returned to Germany on August 1, 1999, where he had fever over 39 °C with chills, malaise, and weakness, and went to a local hospital. Myalgia, headache, and nausea developed. Coagulation parameters were impaired and liver enzymes were strongly elevated (AST 22,000 U/l and ALT 8700 U/l). The patient stated repeatedly that he had been immunized against yellow fever in 1993. Because a VHF was suspected, he was transferred to a specialized clinic in Berlin on day 3 of his illness and samples were sent for virological testing. Physical examination of the fully oriented febrile patient revealed an enanthema, a conjunctival injection with discrete jaundice, petechiae on both arms, multiple residues of mosquito bites, and an enlarged liver. Antibiotic treatment as well as therapy with ribavirin were initiated. Fluid and clotting factor replacement were given. On day 4, the coagulation status deteriorated (prothrombin level below 10% and PTT 94 s), he had thrombocytopenia and an impaired renal function which progressed to complete anuria. The patient gradually fell into coma and died on day 5 after onset of his illness. Yellow fever virus was detected in Vero cell culture and by RT-PCR, while specific antibodies were not detected. Necropsy showed evidence of acute liver and kidney failure (tubular necrosis) and cerebral oedema.

Case 2 (Colebunders et al., 2002): A Belgian traveler became ill on November 7, 2001, in The Gambia with high fever, chills, headache, myalgia, and asthenia. She had not been vaccinated against yellow fever. Next day she returned to The Netherlands. On day 3, she developed diarrhea and complained of nausea, and was admitted on day 4 at the intensive care unit of a local hospital. Laboratory tests on admission showed extremely high liver enzyme levels (AST 49,000 and ALT 23,000). The fever disappeared, there were no bleeding signs and a neurological examination was normal. On day 5, she remained anuric and hemodialysis was started. The coagulation status was seriously impaired (prothrombin level 15%) and bleeding occurred from injection sites. The clinical suspect of yellow fever was confirmed by RT-PCR. Liver transplantation was considered and the patient was transferred to a specialized hospital. On day 7 she vomited dark blood ("vomito negro", a synonym of yellow fever). A hypovolaemic shock developed. Despite massive transfusion of plasma, red blood cells, platelets, and clotting factors, the hemodynamic situation did not improve. She became comatose and died on day 8. Necropsy confirmed that death resulted from massive gastrointestinal bleeding.

2.5.2. Diagnostic procedure

These two cases are classical courses of yellow fever. Diagnostically indicative during the early phase are the extremely high elevations of the liver enzymes. Similar to what happens in Lassa fever, the AST is higher than the ALT. In both cases the laboratory diagnosis was challenging.

In case 1, the patient stated that he had been vaccinated against yellow fever. Therefore, a transmissible hemorrhagic fever such as Lassa fever, CCHF or Ebola fever was suspected and the patient was treated under high security conditions. Consequently, a yellow fever PCR was not included in the first set of PCRs that were run. Specific antibodies were not detected but this is not uncommon in vaccinees. The virus culture proved to be very helpful in that case because 48 h after inoculation virus was grown which reacted with flavivirus-specific polyclonal antibodies (which are broadly cross-reactive), and in a subsequent screening also with a yellow fever-specific monoclonal antibody. A yellow fever PCR was run in parallel and reacted positive.

In the second case, despite of the very high viral RNA concentration in the plasma sample (as turned out subsequently), the initial PCR tests were negative (Drosten et al., 2002c). However, a duplicate sample that was spiked with in vitro transcribed yellow fever virus RNA and processed in parallel was also negative. This indicated the presence of substances that inhibit RT-PCR. Therefore, the plasma was diluted in log 10 intervals. The RNA was prepared from this material and re-tested. Quantitative real-time RT-PCR clearly detected yellow fever virus RNA in the plasma diluted 1:100, 1:1000 and 1:10,000, but not in the undiluted sample (Fig. 7A). The undiluted material was estimated to contain $>10^6$ RNA copies/ml (Fig. 7A). A similar inhibition was seen with plasma from a moribund patient with Ebola hemorrhagic fever from Gulu, Uganda (Fig. 7B). Viral RNA concentration in this case was 6.9×10^8 copies/ml. No hemolysis, which often causes inhibition of PCR (Akane et al., 1994), was observed in the plasma of both patients. These two observations suggest that in patients with severe hemorrhagic fever substances may circulate which strongly inhibit RT-PCR. Therefore, especially in these cases appropriate inhibition controls are essential.

2.6. Crimean-Congo hemorrhagic fever

2.6.1. The virus

Crimean-Congo hemorrhagic fever virus is a negative-strand RNA virus which belongs to the genus Nairovirus of the family Bunyaviridae. The RNA genome of CCHF virus, like that of other bunyaviruses, consists of three segments: S, M, and L. The S segment encodes the virus nucleocapsid

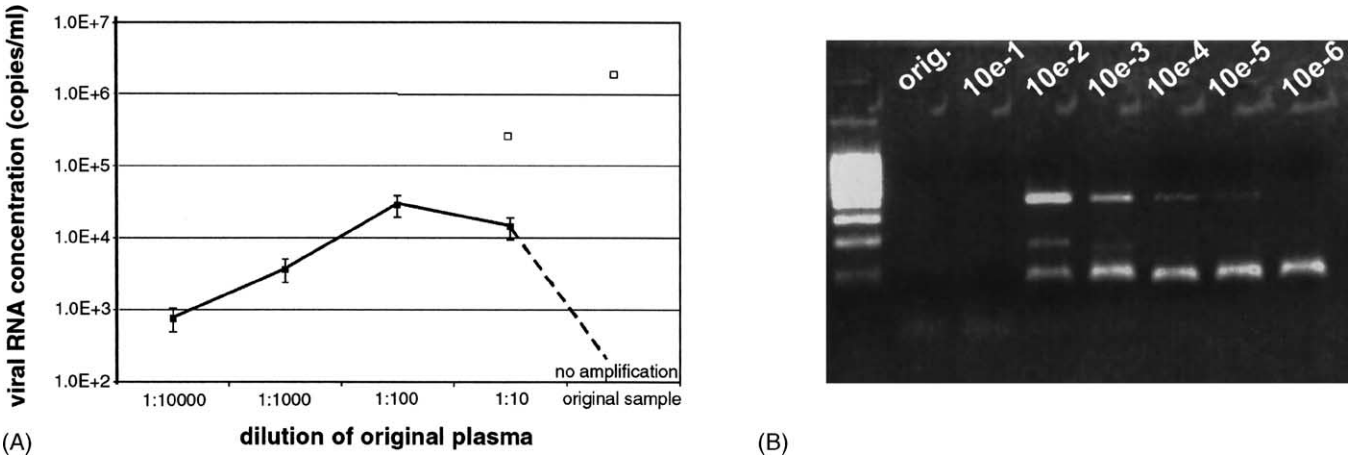

Fig. 7. Inhibition of VHF RT-PCR. (A) Original and diluted plasma from yellow fever case 2 was tested by quantitative real-time RT-PCR for yellow fever virus (Drosten et al., 2002a). Closed circles depict the experimentally determined values. PCR was completely inhibited with the undiluted plasma. In the 1:10,000–1:100 dilution steps, the measured viral RNA concentration correlates with the actual concentration. Open circles represent the extrapolated RNA concentration in the undiluted and the 1:10 diluted sample. (B) Ebola virus RT-PCR on original and diluted plasma from a patient with Ebola fever. The dilution factor is shown above each lane. Amplification was only possible when the sample was diluted 1:100 (modified from Drosten et al., 2002c).

protein, the M segment the glycoprotein precursor, and the L segment the polymerase protein. It was recently shown that the glycoprotein precursor of CCHF virus is posttranslationally cleaved into the mature glycoproteins G2 and G1, and that cleavage is most likely mediated by the cellular subtilase SKI-1/S1P (or a related protease; Sanchez et al., 2002), which also cleaves the Lassa virus glycoprotein (Lenz et al., 2001). The use of similar biochemical host cell pathways by CCHF virus and Lassa virus would be consistent with a close evolutionary relationship between nairoviruses and arenaviruses (S. Günther, unpublished data) and may open the possibility to design specific inhibitors that may act against both viruses.

2.6.2. Epidemiology

The common vector for CCHF virus are ticks of the genus *Hyalomma*. The virus is transmitted to humans either directly by *Hyalomma* ticks or by contact with infected domestic animals. CCHF virus is primarily a zoonosis, which means that the transmission cycle mainly involves ticks and wild or domestic animals. Cattle, sheep and goats do not become ill after infection but are viremic for about 1 week. During this period of time the virus may be transmitted to humans which have close contact to these animals such as agricultural workers, slaughterhouse workers, and veterinarians. Furthermore, the virus may be spread into other geographical regions via infected livestock. The virus may also be transmitted from human to human which occurs primarily in the hospital setting. Health care workers are mainly at risk.

CCHF has been reported from the Near, Middle and Far East (Central Asian republics of the former Soviet Union, Iraq, Pakistan, United Arab Emirates, Kuwait, Oman, China), from several African countries but also from South-East Europe (Bulgaria, the former Yugoslavia, northern Greece).

2.6.3. Clinical picture and therapy

If the virus is transmitted via tick bite, the incubation period appears to be shorter (1–9 days) than after transmission via infected animals (5–13 days). Like other hemorrhagic fevers, the symptoms of the initial phase are rather unspecific: fever, myalgia, headache, nausea, vomiting, and diarrhea. During the further course of the disease encephalopathy, bleeding, and organ failure may develop, which are associated with high mortality.

Case reports suggest that treatment with ribavirin may be beneficial (Fisher-Hoch et al., 1995a; Papa et al., 2002b), although its efficacy has not been confirmed by larger clinical studies. A vaccine against CCHF virus is not available.

2.7. Crimean-Congo hemorrhagic fever in Europe

2.7.1. Clinical description

Case (Drosten et al., 2002b): A farmer's wife living near Pristina (Kosovo) was bitten by a tick on May 23, 2000 while working in her garden. The disease started on May 28 with chills, myalgia, nausea, vomiting, headache, and backache. She visited an outpatient clinic in Prizren, where broad-range antibiotic therapy was initiated. On day 2, she developed massive hemorrhage with hematemesis (7–8 times per day), melena, hematuria, metrorrhagia and petechiae. She was hospitalized without special isolation measures in a critical condition on day 3, with a temperature of 39.7 °C and still severe hemorrhage. The platelet count was reduced to 30,000 μl^{-1}, and coagulation was impaired (clotting time: 7 min and 17 s; normal <6 min). On day 4, epistaxis and gingival bleeding were additionally observed and the high fever continued (40.1 °C). Large ecchymoses appeared at the sites of venipuncture. The patient was fully orientated without neurological symptoms. Liver enzymes were elevated (AST 547 U/l and ALT 90 U/l). Diuresis was 1500 ml per day and

creatinine was increased (480 μmol/l). On day 5, she developed a polyuria (4500 ml per day). The next day, the hemorrhagic diathesis had almost disappeared; blood pressure was 100/70 mmHg. She recovered completely. Supportive therapy during the course of the disease consisted in hydration and control of temperature. No blood transfusions were administered. No secondary cases occurred in the hospital.

2.7.2. Diagnostic procedure and phylogenetic analysis

This case again underlines that liver enzyme elevations with AST being higher than ALT are a diagnostic hallmark of VHF. As in Lassa fever, antibody detection is not reliable to diagnose CCHF, at least early during disease. In the described case, antibodies to CCHF virus were not detected by immunofluorescence assay on day 3, but a viral RNA concentration of 7.7×10^5 genome equivalents per ml was found by real-time RT-PCR in plasma. On day 16, RT-PCR was negative, and now high titers of specific IgM and IgG antibodies were detectable (Drosten et al., 2002b).

There have been early reports on CCHF cases in former Yugoslavia (Gligic et al., 1977; Vesenjak-Hirjan et al., 1991). In 2001, an outbreak occurred in Kosovo. As of June 2001, WHO has reported 69 suspected cases (15 laboratory-confirmed), out of which 6 died (Anonymous, 2001). Since CCHF virus strains from this area had not been characterized genetically, the amplified viral cDNA was sequenced and subjected to phylogenetic analysis (Fig. 8). CCHF virus consists of seven major genetic lineages. The Kosovo isolate constitutes a distinct lineage that includes only one further strain isolated from the Black Sea region 33 years ago (Butenko et al., 1968). Phylogenetic analysis of additional CCHF viruses isolated in the Kosovo region

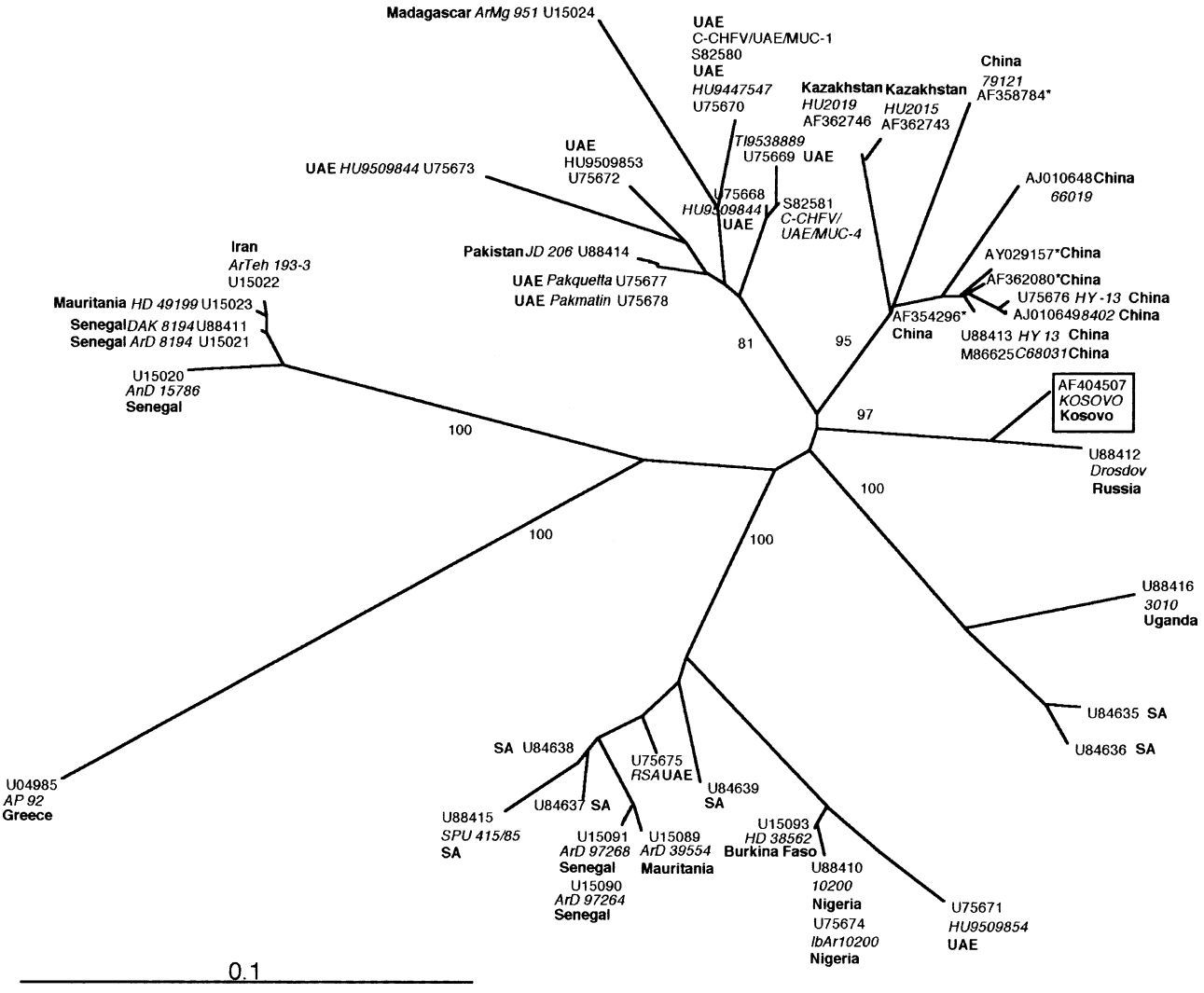

Fig. 8. Phylogenetic analysis of CCHF virus, including the strain from Kosovo. A total of 46 sequences (219 nucleotides) of the S-segment were analyzed with the PHYLIP software package (J. Felsenstein, University of Washington), using the neighbor-joining and maximum-likelihood (not shown) algorithms. Both methods identified the same seven major genetic groups. The bootstrap support values for each group are shown at the respective branches. Geographic origin (bold), GenBank accession number (standard font) and strain denomination (if available, in italics) are given for each isolate (modified from Drosten et al., 2002b).

and in Albania in 2001 confirmed this relationship (Papa et al., 2002a,b). Therefore, it seems that currently a distinct lineage of CCHF virus is circulating in the Kosovo and Albania, which may have originated from the Black Sea region of the former Soviet Union.

3. Part III

3.1. Laboratory diagnosis of pathogens causing syndromes similar to VHF

Important pathogens which can cause symptoms similar to VHF are *Plasmodium falciparum*, *Neisseria meningitidis*, *Leptospira interrogans*, and viruses causing fulminant hepatitis. Hence, a close collaboration between laboratories specialized in VHF diagnostics and facilities able to diagnose these pathogens is desirable.

Especially malaria tropica can resemble a VHF and should always be excluded in travelers with fever who are returning from endemic areas, even if prophylaxis was taken. Malaria is commonly diagnosed by thin/thick smears. PCR is available and has been found slightly more sensitive than blood smears (Rubio et al., 1999, 2002). In post-mortem examinations PCR has also proven a useful tool (Becker et al., 1999). Real-time PCR is now available for plasmodia (Hermsen et al., 2001; Witney et al., 2001).

Neisseria meningitidis can cause a systemic inflammation syndrome with disseminated intravascular coagulation and bleeding (Waterhouse–Friedrichsen's syndrome). The pathogen is endemic all over the world, with a focus in sub-Saharan Africa ("meningitis belt"). The diagnosis is established by culture and microscopy which may fail if blood or CSF samples have been inadequately stored or drawn after initiation of antibiotic therapy. In the year 2000, a person developed a Waterhouse–Friedrichsen's syndrome while returning by aircraft from Ethiopia to Germany (Preiser and Just-Nübling, 2000). A VHF was suspected, but PCR (Newcombe et al., 1996) rapidly disclosed meningococcus sepsis. This assay has recently been replaced by more specific real-time PCR methods (Guiver et al., 2000; Corless et al., 2001).

The typical symptoms of Leptospirosis or Weil's disease are sudden onset of high fever, with myalgia, conjunctivitis, and rash followed by jaundice, hepatitis, and renal failure. In the acute febrile period, the bacteria can be cultured from blood in special media, which may take more than a week. In the late phase, *Leptospira* can be detected in urine by microscopy or culture. Conventional and real-time PCR assays have been developed for rapid detection of *Leptospira* (Woo et al., 1997, 1998).

Hepatitis is a common symptom of VHF. Besides of the "professional" hepatitis viruses A, B, D, and E, a variety of other viruses can cause severe acute hepatitis. Fulminant hepatic failure due to infection with herpes simplex virus type 1 was seen in 2000 in a traveler who returned from Kenya to Germany. Initially, a VHF was suspected. The diagnosis was established post-mortem by electron microscopy, virus culture, and PCR (ter Meulen, 2001). Several further reports exist on fulminant hepatitis caused by herpes viruses (Farr et al., 1997; Velasco et al., 1999; Fahy et al., 2000; Pellise and Miquel, 2000; Peters et al., 2000; Fehr et al., 2002; Pinna et al., 2002).

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