Analysis of two imported cases of yellow fever infection from Ivory Coast and The Gambia to Germany and Belgium

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Abstract

Background: yellow fever remains one of the great burdens for public health in the endemic regions in Africa and South America. The under reporting of yellow fever cases in the respective regions and lack of international interest leads to an underestimation of the constant danger in these areas. Non-vaccinated travelers take a high risk without the effective protection of YFV 17D vaccination.

Objectives: Two YF cases were imported to Europe in the last 4 years. We characterized two yellow fever virus (YFV) isolates from severely infected patients coming back from Africa, Ivory Coast and The Gambia, by genome sequencing and phylogenetic analysis.

Study design: The virus infections in different organs were analyzed with pathological, immunohistological, electronmicroscopical and quantitative real-time PCR methods.

Results and Conclusion: High virus loads in spleen and liver (2.4 × 10^6 to 3 × 10^7 GE/mL) demonstrated by real time PCR show massive virus replication leading to extraordinary progression of the disease in these patients. Immunohistological and electronmicroscopical analysis confirms virus particles in liver tissue. In all other organs no virus could be detected. A fast, specific and sensitive virus PCR detection is recommended for diagnostic of acute infections. The further sequence alignments show that the new isolates belong to the type II West African strain with great homology to over 40-year old YF isolates from Senegal and Ghana. The divergence observed was on average 3.3%, ranging from 0.0% to 5.0% in the coding region of Gambia2001 strain and 2.9%, ranging from 0.0% to 4.3% in the coding region of the IvoryC1999 strain. Most mutations (5.0%/4.3%, respectively) occurred in the envelope protein.

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1. Introduction

The yellow fever virus (YFV) is the prototype of the positive-stranded enveloped RNA-flavivirus family, a group of 68 important human and veterinary viral pathogens. The genome contains 10.862 nucleotides with a 5′ cap-structure and a non-polyadenylated 3′- end encoding a polyprotein of 3.411 amino acids, which is cleaved by proteolytic processing into 11 viral polypeptides (Hahn et al., 1987). The flavivirus genome organization is 5′- C, prM, M, E, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 - 3′ (Chambers et al., 1990; Lin et al., 1993).

The WHO estimates that approximately 200,000 yellow fever (YF) cases occur in South America and Africa each year (Tomori, 1999; Mutebi and Barrett, 2002). In the last
decade, epidemics have been regularly reported from Ghana, Gabon, Liberia, Senegal, Benin and Ivory Coast and several South American countries (WHO Disease Outbreak News).

Yellow fever is still one of the great burdens for public health in the endemic regions in Africa and South America. The under reporting of yellow fever cases in the respective regions and lack of international interest leads to an underestimation of the constant danger in these areas. Non-vaccinated travelers take a high risk without the effective protection of yellow fever vaccine. In this study, we present a further characterization of these cases of the recent years.

2. Material and methods

2.1. Immunohistochemistry analysis of the imported German case

A complete examination of the German patient was performed by pathologists 8 h post-mortem. Dissection of the internal organs was carried out in situ and tissue samples from liver, kidneys, heart, lungs, spleen, bone marrow, lymph nodes, stomach and gut were immediately fixed in neutral buffered formalin (4%, AFIP-Grade) over 2 days. The brain was removed and fixed in toto for neuropathological investigations. Additional tissue was sampled for electron microscopy, stained and fixed appropriately. All histological investigations. Tissues were stained with standard H&E, PAS, van Giesson’s elastica stain, Gomorri’s reticulin method, fuchsin-orangeG for the demonstration of fibrin and oil red O for the demonstration of lipids. The removed tissues were analyzed for yellow fever specific antigen using the monoclonal antibody (mab 6330, IgG, 5.5 mg/ml in PBS) directed against yellow fever virus (Gelderblom et al., 1985). For immunohistochemistry, slides of paraffin embedded autopsy material of the liver, heart, spleen, lungs and kidneys were examined. For primary antibody detection, we used the Super Sensitive Immunodetection System (peroxidase-kit, P or alkaline phosphatase-kit, AP, Biogenex, USA) and using either DAB or Fast Red containing 5% levamisole (Sigma, Germany) as chromogens and hematoxylin for counterstain in the final reaction steps. Insect cells (C6-36, ATCC, CRL-1660), infected with the patient’s yellow fever virus strain served as positive reaction controls. Negative controls were prepared by either omitting the primary antibody in the first step or by replacing the yellow fever antibodies with a mouse mab directed against Aspergillus niger glucose oxidase (DAKO X 0931, IgG1, Denmark).

2.2. EM preparation

Biopsy material of the liver was used for performing the electron microscope examination of the liver tissue for presence of yellow fever virus. Insects C6/36 cells infected with YFV served as a control. Cells and tissues were fixed with 2.5% glutaraldehyde, washed, and treated with 1% uranylacetate, 1% osmium tetroxide (Merck, Darmstadt, Germany) and 0.1% tannic acid. After dehydration, the cells were embedded in Epon 512 (Polyscience Inc., Wetzlar, Germany). Ultrathin sections were stained with 2% lead citrate and 2% uranylacetate.

2.3. Virus cultivation and purification of RNA for sequencing

Virus was isolated from serum of the German and Belgian imported cases. Vero E6 cells (ATCC, CRL-1586) were infected with these inoculates and after 4–8 days cell culture supernatant was used for virus purification with the QIAamp viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer’s instruction.

2.4. Serological YF diagnostic

Patient serum was analyzed by immunofluorescence assay, EIA and in plaque reduction assay as described previously (Niedrig et al., 1999; Reinhardt et al., 1998).

2.5. RNA isolation from tissue of the imported Belgian case

Tissues (gullet, lung, heart, suprarenal gland, kidney, uterus, bladder, skin, pectoral muscle, stomach, pancreas, bone marrow, brain tissue) from the Belgian traveler were obtained from the local hospital in Brussels and stored frozen until use. Total RNA was extracted with cesium chloride gradient and isolated with the silica-based membrane Qiagen RNeasy Maxi Kit (Qiagen, Germany) according to the manufacturer’s instruction.
2.6. cDNA synthesis of the extracted RNA

YFV RNA was eluted with 1000 μl of sterile water. Total cDNA was obtained by reverse transcription (RT) of 2 μl of purified RNA, 2 μl of 10x buffer, 2 μl of 10 mM DTT, 5 μl of 2.5 mM dNTP (each), 0.5 μl of random hexamers (50 ng/μl) and 6 μl of sterile water. Samples were heated to 72 °C for 3 min and subsequently chilled on ice for 3 min to allow primer hybridisation. After addition of 1 μl of 20 U RNase inhibitor and 0.5 μl 200 U/μl reverse transcriptase, samples were incubated for 40 min at 42 °C. Subsequently, the RT reaction was terminated by enzyme inactivation at 83 °C for 5 min.

2.7. RT-PCR for determination of virus load in tissues

Virus load was determined by quantitative real-time PCR, which was performed in a Perkin Elmer 7700 Sequence Detection System (Applied Biosystems, USA). Amplifications were done in 50 μl reaction mixtures containing 2 μl template cDNA, 0.5 μM of each primer, 0.1 μM TaqMan probe, 0.2 mM dNTP (each) and 4 mM MgCl2. As passive fluorescent reference dye 1 μM ROX (6-carboxy-X-rhodamine) was added to the mixture. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 45 cycles with 95 °C for 30 s and 60 °C for 1 min (Bae et al., 2003).

2.8. One step RT-PCR

The RNA was used as a template in a one step reverse transcriptase PCR (RT-PCR). The oligonucleotide primers (Table 1) were designed according to general guidelines for PCR primer and were synthesized by Metabion (Planegg-Martinsried, Germany) and TIB Molbiol (Berlin, Germany). The cDNA synthesis for the sequence reaction was performed at 60 °C for 30 min or by 50 °C for 30 min. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 45 cycles with 94 °C for 30 s and 60 °C for 1 min (Bae et al., 2003).

2.9. Sequence analysis

PCR products were sequenced directly (in both directions) without subcloning into plasmid vectors, by using an automated DNA sequencer ABI PRISM 3100 Genetic Analyzer (California, USA). The fragments were analyzed by the cycle-sequencing method with the Big Dye Terminator Cycle Sequencing Ready Reaction kit 2.1 (Applied Biosystems, USA). For each sequence reaction, 3.2 pmol of primer (Table 1) was added to 5–20 ng purified DNA in a reaction mix containing the four dye-labeled dideoxynucleotide terminators. Cycle sequencing parameters were used as described in the manufacturer’s protocol.

2.10. Nucleotide sequencing studies

For the nucleotide sequencing studies we used a 670 bp DNA fragment, which included the 3′-end 108 nucleotides of the pre-membrane (prM) protein gene, the entire 225 nucleotides of the membrane (M) protein gene and the 5′-end 337 nucleotides of the envelope (E) protein-coding gene. This region was used before as a representative sample of the YF genome (Mutebi et al., 2001; Wang et al., 1996).

Nucleotide sequences were imported and edited in EditSeq Software. Phylogenetic analysis was performed using Clustal, SEAL, Phylip (Neighbor-joining) and Treesview with Phylip package software BioEdit was used for plot identities. The tree was rooted using a homologous sequence of Dengue-1 virus (GenBank accession number NC001474).

3. Results and discussion

3.1. Diagnostic of an acute YF case

The presence of YFV could be seen in the liver tissue from autopsy or biopsy either by immunohistological staining or by EM evaluation. The histopathological and immunohistochimical findings were typical for hemorrhagic fever infections including YF infections and were previously described (Klotz and Belt, 1930; de Filippis et al., 2002; MMWR, 2002). The pathological picture was also consistent with the lethal yellow fever infection of the Belgian case (Colebunders et al., 2002). Interestingly, extensive tissue necrosis sparing only lymphocytes around the central arteries also occurred in the spleen.
The wide range of clinically similar diseases impairs the recognition of YF cases in the early stages of illness. A fast diagnostic clearance is desirable for the development of suitable medical treatment and appropriate safety measurements for patients coming from Africa. In the recent imported YF cases, it turned out that the fastest and most sensitive diagnostic method is the RT-PCR (Drosten et al., 2003; Bae et al., 2003). Virus isolation is recommended as additional method but takes more time and requires L-3 laboratory capacity and expertise. Virus isolation on Vero cells leads to a significant cytopathic effect after 1 day. The finding of the massive virus load found in these patients clearly demonstrates that the detection of YF virus genome by RT-PCR should be no problem for a clear and quick diagnosis in a severely diseased YF patient. High amounts of YFV RNA was measured in liver and spleen of 3 × 10^7 GE/mL and 2.4 × 10^6 GE/mL respectively. No YFV nucleic acid could be detected in gullet, lung, heart, suprarenal gland, kidney, uterus, bladder, skin, pectoral muscle, stomach, pancreas, bone marrow and brain tissue.

Immunohistological staining and EM preparation confirms the presence of YF virus particles in the liver (within the cisternae of the endoplasmic reticulum, data not shown), but no virus could be detected in the spleen.

The liver seems to be the primary target for virus propagation visible by the immunohistological staining and EM examination (data not shown) and the measuring of the YF copy numbers. The fast degradation of the organ leads to a fast release of YFV in the blood easily detectable by PCR. The degradation of the liver by the virus results in an enormous increase of the liver enzyme activity found in both patients (Teichmann et al., 1999; Colebunders et al., 2002). The tremendous release of cell debris into the blood can have a negative influence on the results of the diagnostic PCR as described previously (Drosten et al., 2002). To overcome this inhibiting effect, the specimen to be analyzed has to be used in higher dilution.

Other serological diagnostic methods for detection of YF specific IgM or IgG antibodies by immunofluorescence assay, EIA or plaque reduction neutralization assay were not able to show any specific immune response in these patients. Both patients failed to generate any specific humoral immune response before they died.

3.2. Molecular biological analysis of the yellow fever strains

The consensus sequence was determined for the strains IvoryC1999 and Gambia2001 of human origin. The 26 nucleotides sequence at both the 5′- and 3′-ends corresponds to that of the oligonucleotide primers used for cDNA synthesis, e.g. to that of the 17D strain of YFV. No attempt to obtain the sequence of these terminal regions was made during this study. The complete sequences were deposited in GenBank accession number AY572535 (Gambia2001) and AY603338 (IvoryC1999).

We selected a representative fragment of the entire YFV-genome according to Mutebi et al. (2001). This 670 nucleotide fragment includes the coding regions for three proteins: the pre-membrane (prM), membrane (M) and envelope (E). Nucleotide sequences of this region were determined for 38 previously published YFV-amplificates (Mutebi et al., 2001). With these strains we regenerared the phylogenetic tree of Mutebi et al. (2001) and included our two new strains from the imported cases in this phylogram (Fig. 1) to permit identification of the genotypes.

We could confirm that the lineages split up into five genotypes, three in East/Central Africa and two in West Africa (Angola, East/Central Africa, East Africa, West Africa I and West Africa II), based on phylogenetic relationships and variations in nucleotide sequence. The new strains IvoryC1999 and Gambia2001 could be localized in the West African II genotype (Figs. 1 and 2). In this genotype, there were most of the Senegal strains, the two Burkina Faso strains and one Guinea strain. A former Ivory Coast strain (1982) was grouped into the West Africa I genotype, between the Nigeria strains. Fig. 2 shows the nucleotide sequence alignment for the distal 250 nucleotides of the pre-membrane protein gene of 40 YF viruses from Africa (grouped into five genotypes). They showed an extensive nucleotide variation in contrast to the high degree of sequence homology of the amino acid sequence (data not shown). Both new strains could be clearly matched in the West Africa II Genotype and show genotype-specific differences.

The complete consensus sequence of both strains was compared to the Asibi strain sequence on the entire 10,862 nucleotide genome. This comparison revealed nucleotide substitutions scattered throughout the entire genome without any base insertions or deletions. The distribution of nucleotide variation between the sequences is shown in Table 1. We found 394 divergent positions (3.6%) for Gambia2001 and 367 (3.4%) divergent positions for IvoryC1999. There was no significant variation in individual genes, except in that of the envelope E protein gene, which showed a higher variability (5.0%). The 5′-end non-coding region sequence was identical to the Asibi strain.

The analysis of the applied methods provides reliable diagnosis of YFV infections. The molecular approach by PCR and sequencing leads to a deeper comprehension regarding the nucleotide sequence of YFV and its implications for flavivirus gene expression, evolution (Rice et al., 1985; Lepiniec et al., 1994), epidemiology and phylogeny (Bryant and Barrett, 2003; Chang et al., 1995; Deubel et al., 1986; Psan et al., 1997). In conclusion, the molecular phylogenetic analyses could determine the two European imported cases to their genotype. Both strains IvoryC1999 and Gambia2001 clearly belong to the West Africa II genotype whereas the IvoryCS2 strain, which caused the epidemic 1982 in the Ivory Coast belongs to the West Africa I genotype. This suggests that the virus in this region has changed by mutations or IvoryC1999 was imported through travelling. Concerning the stability of this RNA virus (Trent et al., 1981, 1983; Monath et al., 1983),
Fig. 1. Neighbor-joining phylogram derived from the nucleotide sequence of the prM/E region of 40 wild YFV strains from Africa. Bold marked strains are the two imported cases.
Fig. 2. Nucleotide sequence alignment for the distal 250 nucleotides of the prM/M-protein gene of the consensus sequences of five YFV genotypes from Africa. This alignment highlights nucleotide variations among different genotypes and similarities within genotypes. Dots indicate identity with the consensus sequence. Cons WAI: consensus sequence of West Africa I genotype strains, cons WAI: consensus sequence of West Africa II genotype strains, cons ECA: consensus sequence of East/Central Africa genotype strains, cons EA: consensus sequence of East Africa genotype strains.
the latter suggestion is more probable since the homology of the strains IvoryCR2 and IvoryC1999 is only 91%.

Reviewing the risk factors for YF during travel in YF-endemic regions, the use of YF 17D vaccine is highly recommended to prevent infection. Despite the occurrence of some fatal cases after vaccination, the attenuated 17D yellow fever vaccine belongs to the most effective and safest vaccine we have (Month and Cetron, 2002).

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