PARTIAL GENOMIC SEQUENCE DETERMINATION OF YELLOW FEVER VIRUS STRAIN ASSOCIATED WITH A RECENT EPIDEMIC IN GABON

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Summary. – A limited epidemic, with important mortality among the initial human cases, occured in a forest region in the north of Gabon by the end of 1994. It was identified as yellow fever according to first serological and reverse transcription/polymerase chain reaction (RT-PCR) results, but no virus was isolated. We received 37 sera from people who lived in the same region and presented symptoms of the disease during the period of concern. In ten of them, we were able to detect and identify yellow fever virus (YFV) RNA by RT-PCR and endonuclease digestion of the RT-PCR products. Nucleotide (nt) sequence of two regions (242 and 161 nt long) of YFV RNA was determined for three sera. As it differed from that of the Asibi strain of YFV, the presence of a new topotype (strain) of YFV is presumed.

Key words: yellow fever virus; topotype; epidemic; reverse transcription/polymerase chain reaction; sequencing

Introduction

Several cases of a fatal illness occurred among a population of gold diggers in the north of Gabon in November and December 1994 (WHO, 1995). Yellow fever was suspected soon on the basis of epidemiological observations and first laboratory results. The evolution of the epidemic was biphasic, with a first sylvatic period by the end of 1994, and a limited propagation in urban mode during the following weeks. Mortality reached 57% and 66% for the two phases, respectively, with 28 fatal cases in total. Vaccination of the local population with YFV 17D strain interrupted the progression of the epidemic. However, no virus was isolated from human sera.

In May 1995, we received 37 sera from people living in the same region as the first cases and having presented symptoms of atypical viral infection during the related period. Serological assays confirmed the results from other laboratories which have analyzed samples from the same epidemic. Of 37 sera 11 were positive for anti-YFV IgG and only 1 was positive for anti-YFV IgM. No virus was isolated in sensitive mosquito C6/36 cells, even after treatment of the sera with dithiotreitol for dissociation of possible immune complex. Although the sera were transported and preserved in poor conditions since their collection, 10 of them were assayed by RT-PCR with primers specific for YFV RNA and all of them were found positive for amplification of a DNA fragment of the expected size. Homology with YFV RNA sequence was unambiguously demonstrated for 6 sera by restriction endonuclease digestion at two specific sites. Furthermore, nucleotide sequence of two cDNA regions was determined for three sera and allowed us to identify a new topotype (strain) of YFV, perhaps responsible for the epidemic.

Materials and Methods

Sera collected just before vaccination underwent long transport at uncontrolled temperature before freezing at -80°C. They

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Abbreviations: nt = nucleotide; RT-PCR = reverse transcriptase/polymerase chain reaction; SDS = sodium dodecyl sulphate; YFV = yellow fever virus

Table 1. Results of RT-PCR amplification of a 369 nt long fragment and specific endonuclease digestion

			Serum No.										Positive control	Negative control
			1	2	3	4	5	6	7	8	9	10		
RT-PCR		369 nt	+++	++	++	++	+	++	+	+++	++	++	+++	-
Restriction endonuclease digestion	*Alu*I	213 nt	+++	++	+++	++	+	+	+++	+++	+	+++	++	-
		369 nt	+++	++	+++	++	+	++	+++	+++	+	+++	++	-
	*Xba*I	351 nt	+++	+	++	+	+	+	++	+	+	++	++	-
		369 nt	+	-	-	-	-	-	-	-	-	-	-	-
Anti-YFV IgG			+	-	+	-	-	+	-	+	-			
Anti-YFV IgM			-	-	-	-	-	-	-	-	-			

Crosses number indicates the intensity of the fluorescent PCR fragments (arbitrary units).

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CTACGGATGGAGAACCGGACTCCACACATTGAGACAGAAGAAGTTGTCAGCCCAGAACTCCACA
------------GT-----------G----------------------------C-----

CGAGTTTTGCCACTGCTAAGCTGTGAGGCAGTGCAGGCTGGGACAGCCGACCTCCAGGTTGCGA
----------G--A-----------------------A----A----------------

AAAACCTGGTTTCTGGGACCTCCCACCCCAGAGT
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Fig. 1
Sequence of the 161 nt long region of YFV cDNA of strains Asibi and Gabon
Strains Asibi (first line) and Gabon (second line). Homology (—), deletion (*).

had been imperfectly decanted and some of them were found contaminated with bacteria.

RT-PCR analysis. Ten sera were taken at random for the analysis. Upon thawing, RNase inhibitor isolated from human placenta (Perkin-Elmer) was added to each serum. Sera were clarified by centrifugation at 10,000 x g for 5 mins. Five µl aliquots were treated with proteinase K in 2% SDS, then extracted with commercial solution of phenol-guanidin thiocyanat RNA BTM (Bioprobe). RNA was finally precipitated with isopropanol. Reverse transcription was performed with MoMLV reverse transcriptase (BRL) and 20-mer primer (5'-GGGTCTCCTCTAACCTC-3') complementary to a conserved sequence at the 3'-end of the RNA of mosquito-borne flaviviruseds, as described previously (Hahn *et al.*, 1987a; Pierre *et al.*, 1988). For PCR, a second primer, fluorescent and specific for YFV RNA (5'-CCATCTAACAGGAATAACCG-3'), giving a 369 bp cDNA fragment, was added. PCR was conducted in 25 cycles with Taq polymerase (Perkin Elmer).

Identification of PCR products was done using fluorescent detection with Genescan system in Applied Biosystems DNA sequencer. After separation of cDNA fragments on a polyacrylamide gel with 6% urea single-stranded fluorescent molecules were detected. This system allowed very sensitive detection and accurate size determination of cDNA. The identity of the detected fragments was further investigated by restriction endonuclease digestion at sites published for the YFV-17D or Asibi RNA sequences (Rice *et al.*, 1985; Hahn *et al.*, 1987b).

DNA sequencing. The products of reverse transcription from three sera were subjected to PCR with a non-fluorescent primer 5'-CCACATCCATTTAGTCATCCATCGT-3') defining a 498 bp fragment. The PCR products were reacted with the 3'-primer, the four fluorescent dideoxynucleotides and Taq polymerase (25 cycles of elongation).

Results and Discussion

Ten of the 37 sera collected were submitted to RT-PCR assay of viral RNA. Five of them were positive for anti-YFV IgG and all of them were positive in RT-PCR (Table 1), giving a cDNA fragment of the expected size (369 nt). YFV RNA and a serum from an European man were used as positive and negative controls, respectively. When a PCR primer specific for dengue virus (another type of negative control) was used there was no amplification product for all the ten sera tested. Digestion with *Alu*I at nt 10574 and *Xba*I at nt 10708 generated unambiguously the expected fluorescent fragments, 213 and 351 bp long, respectively, for 6 sera. None or incomplete digestion with *Alu*I, attested by the persistence of the 369 bp fragment, could be due to non-optimal reaction conditions or to the absence of the recognition palindrome on the amplified sequence. Such heterogeneity is known in some wild-type YFV strains from West Africa (Deubel *et al.*, Lepiniec *et al.*, 1994).

Nucleotide sequence of two regions of the 498 bp fragment amplified was determined for 3 sera and compared with that of the Asibi strain. The first one (242 nt long) is located in the NS5 coding region of viral RNA (data not shown). The second one (161 nt long, nt 10492-10653), located in the more variable 3'-non-coding region of the RNA (Fig. 1), exhibited 94.4% homology with the corresponding fragment of the Asibi strain (Hahn *et al.,* 1987b). Besides, eight mutations (transversions) and one base deletion were detected that have not been reported for Asibi and other West African strains until now. Thus the YFV strain identified in our samples from Gabon seems to be an original topotype.

We report here the feasibility of the identification of YFV in human sera using the Genescan procedure. This strategy allowed us to detect YFV RNA in poorly preserved samples, where no cultivable virus was found. The specificity of the detection was established first by restriction endonuclease digestion of the amplified cDNA and emphasized by its sequence determination. This step unambiguously ruled out the possibility of a contamination of tested materials with other viral strains from the laboratory. The Genescan detection procedure appears as a very sensitive and useful technique, although needing sophisticated equipment.

Sera in our study were from people with imprecise clinical episode. Specific IgG antibody titers were low and IgM were found only in one of 37 sera. Although this result may just reflect poor preservation of the samples, it could also indicate an infection of relatively long history. In this regard, detection of persisting YFV RNA rises interesting questions (worth of further) studies.

We can conclude from our results that a new topotype (Gabon) of YFV has circulated in Gabon by the end of 1994, concomitantly with the occurence of several fatal cases in the local human population. However, the exact role of this virus in that epidemic remains is unknown.

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References

Debuel V, Digoutte JP, Monath TP, Girard M (1986): Genetic heterogeneity of yellow fever virus strains from Africa and the Americas. *J. Gen. Virol.* **67**, 209–213.

Hahn CS, Hahn YS, Rice CM, Lee E, Dalgarno L, Strauss EG, Strauss JH (1987a): Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclisation sequences. *J. Mol. Biol.* **198**, 33–41.

Hahn CS, Dalrymple JM, Strauss JH, Rice CM (1987b): Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proc. Natl. Acad. sci. USA* **84**, 2019–2023.

Lepiniec L, Dalgarno L, Huong VTQ Monath TP, Digoutte JP, Deubel V (1994): Geographic distribution and evolution of yellow fever viruses based on direct sequenciug of genomic c PVTQDNA fragments. *J. Gen. Virol.* **75**, 417–423.

Pierre V, Drouet M-T, Deubel V (1988): Identification of mosquito-borne flavivirus sequences using universal primers and reverse transcription/polymerase chain reaction. *Res. Virol.* **145**, 93–104.

Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH (1985): Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**, 726–733.

WHO, Weekly Epidemiological Record, No. 23, 9 June 1995, **70**, 163–164.