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Detection of Hepatitis A Virus RNA in Saliva

Vincent Mackiewicz, Elisabeth Dussaix, Marie-France Le Petitcorps, and Anne Marie Roque-Afonso*

Centre National de Référence pour les Virus à Transmission Entérique (HAV), Laboratoire de Virologie, Hôpital Paul Brousse, Villejuif, France

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Hepatitis A virus (HAV) is shed in feces but also in saliva. HAV RNA was detected in saliva in five out of six acutely infected patients with HAV viremia. Serum and saliva sequences were identical. The simplicity of obtaining material allows the recommendation of the use of saliva for investigation of outbreaks.

Hepatitis A virus (HAV) is the most common agent of acute viral hepatitis in industrialized countries. HAV is a small, particularly resistant, nonenveloped RNA virus that survives on human hands and inanimate objects. Infection is spread chiefly via the fecal-oral route through contact with an infected person. Ingestion of contaminated food or water may also be responsible for outbreaks (4). Investigation of outbreaks often relies on epidemiological and serological studies, but the molecular investigation of HAV cases and contacts is precious since only sequence information will be able to link apparently sporadic cases or apparently distinct outbreaks (9), allowing the implementation of corrective measures. Usually, HAV sequences are isolated from feces or serum. Infected people can excrete HAV in feces for 3 months or longer, and viremia is detectable by reverse transcription-PCR (RT-PCR) in the majority of patients at the onset of symptoms and can persist several weeks after aminotransferases peak (3). Serological investigation of HAV outbreaks has often relied on saliva testing, particularly of children (5, 10), due to the simplicity of obtaining material. Early studies have shown that the saliva of acutely infected patients may be infectious (7); we thus examined the excretion of HAV in saliva and its reliability for sequence investigation.

From November 2002 to November 2003, six patients were referred to our hospital for acute hepatitis with jaundice. Acute HAV infection was diagnosed on the positivity of anti-HAV immunoglobulin M (Biomérieux, Marcy l'Etoile, France). Risk factors and laboratory findings are shown on Table 1. The median age was 31 years (range, 15 to 47 years). Sera were collected at admission and stored at −20°C. Saliva samples were collected 1 to 11 days after serum sampling with a disposable Salivette (Sarstedt, Mercey le Grand, France) and stored at −20°C until use. Viral RNA was extracted from 140 μl of serum or saliva by using the QIAmp viral RNA kit (QIAGEN, Les Ulis, France). Ten microliters of extracted RNA was subjected to RT and PCR amplification by using the One-Step RT-PCR kit (QIAGEN). A 512-bp fragment encompassing the VP1/2A junction was amplified with previously described primers (3). The sensitivity of this RT-PCR assay was 43 IU/ml, as assessed with serial dilutions of the World Health Organization HAV RNA standard purchased from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom). HAV RNA was quantified by using a real-time RT-PCR assay on the LightCycler instrument with an HAV quantification kit (Roche Diagnostics, Mannheim, Germany) and 10 μl of extracted RNA per reaction. The sensitivity of this quantification assay was assessed with the National Institute for Biological Standards and Control HAV RNA working reagent and was found to be 600 genome equivalents/ml. Nucleotide sequencing was carried out by Genome Express (Meylan, France), with a a BigDye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 3730 automatic sequencer, according to the manufacturer's protocol. Sequences were aligned with Clustal X software. Phylogenetic trees were constructed with the MEGA software by the Neighbor-Joining method from a Kimura two-parameter distance matrix, and bootstrap values were determined from 1,000 bootstrap resamplings of the original data. HAV genotypes were determined by the phylogenetic analysis of a 168-nucleotide fragment of the VP1/2A junction, including reference sequences belonging to HAV genotypes IA, IB, II, IIIA, and VII (8).

HAV RNA was detected in and quantified from the serum samples of all six patients. Serum viral loads ranged from 7.3×10^3 to 2.3×10^7 copies/ml. HAV RNA was detected in the saliva from five of six patients, but the viral loads in only four samples, ranging from 9.3×10^2 to 1.9×10^4 copies/ml, fell within the linearity range of the real-time PCR assay. Phylogenetic analysis of patients' sequences is shown on Fig. 1. Five out of six sequences amplified from the serum were assigned to genotype IA. Three of these showed 100% identity within the 168-bp fragment studied. The corresponding three patients (patients 1 to 3) lived in or near Paris and had a common behavioral risk factor for HAV infection (Table 1). It was not possible to identify a risk factor for patient 4, whose sequence differed from the previous sequences by just 1 nucleotide. The fifth IA HAV strain was presumably acquired in Morocco by patient 5, while the only IB sequence was isolated from a patient who had traveled to Cape Verde (patient 6). The sequences isolated from the saliva samples of the five available patients were homologous to the corresponding serum sequence within the studied fragment.

* Corresponding author. Mailing address: Centre National de Référence pour les Virus à Transmission Entérique (HAV), Laboratoire de Virologie, Hôpital Paul Brousse, 94804 Villejuif, France. Phone: 33 1 45596956. Fax: 33 1 45593724. E-mail: anne-marie.roque@pbr.ap-hop-paris.fr.

TABLE 1. Features of HAV infection for six patients[a]

Parameter	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (yr)/sex	34/M	47/M	25/M	34/M	28/M	15/F
Date of symptom onset (day/mo/yr)	10/11/2002	7/4/2003	20/4/2003	4/5/2003	3/8/2003	28/9/2003
Place of contamination	Paris	Paris	Paris	Paris	Cape Verde	Morocco
Risk factor	MSM HIV	MSM HIV	MSM HIV	Unknown	International travel	International travel
Date of serum sampling (day/mo/yr)	14/11/2002	15/4/2003	22/4/2003	8/5/2003	7/8/2003	8/10/2003
Date of saliva sampling (day/mo/yr)	21/11/2002	17/4/2003	25/4/2003	9/5/2003	8/5/2003	8/10/2003
ALT (IU/ml)	632	238	2,800	6,150	3,600	4,000
Serum VP1/2A RNA	+	+	+	+	+	+
Serum RNA quantification	4.9×10^6	7.3×10^3	2.3×10^7	3.6×10^5	2.2×10^4	4.1×10^4
Saliva VP1/2A RNA	+	−	+	+	+	+
Saliva RNA quantification	9.3×10^2	/	1.9×10^4	3.5×10^3	$<6.0 \times 10^2$	1.1×10^3
Copies in serum/copies in saliva (log units)	2.25	/	1.72	1.29	>1.6	1.52
HAV genotype	IA	IA	IA	IA	IB	IA

[a] M, male; F, female; ALT, alanine aminotransferase level; MSM, men having sex with men; +, presence; −, absence; /, not performed.

In the 1980s, titers of infectious virus in saliva were found to be 2 to 3 logs lower than titers of virus in serum and 5 to 8 logs lower than titers in stool, as determined with intravenously inoculated chimpanzees (7). Animal experiments have further shown that infectious virus is shed in saliva during the incubation period and in the early acute phase (2) and that HAV RNA may be detected in saliva from 6 h postinoculation until several weeks after hepatitis onset (6). We show here the frequent presence of HAV RNA in the saliva of acutely infected patients and we confirm by real-time PCR quantification a difference of about 2 logs between serum and saliva viral loads. Furthermore, RT-PCR of this noninvasive clinical sample may allow the molecular investigation of HAV cases and contacts similar to that previously shown for measles or mumps

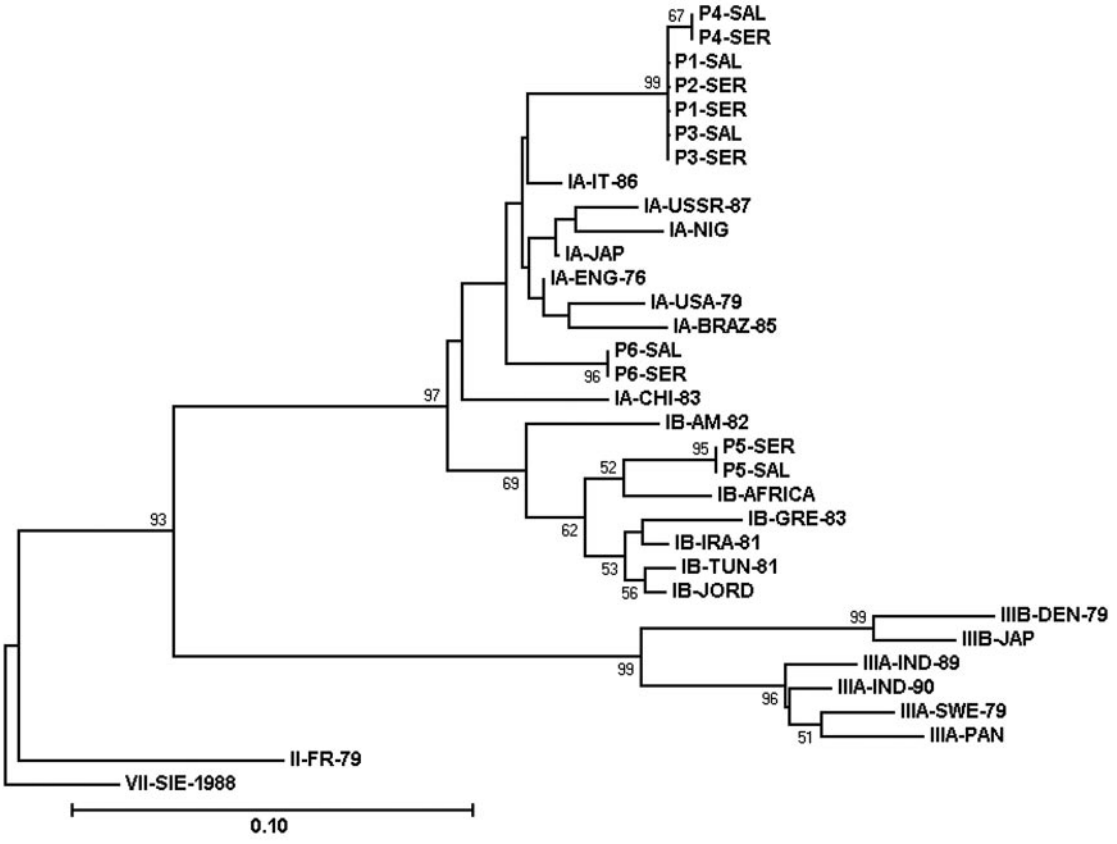

FIG. 1. Phylogenetic analysis of HAV VP1/2A sequences recovered from serum and saliva.

outbreaks (1). In this small series of apparently unrelated cases, phylogenetic analysis revealed a cluster of identical sequences corresponding to three human immunodeficiency virus (HIV)-positive men having sex with men in Paris. Each year, patients sharing this risk factor are referred for acute HAV infection, as were the three patients studied in 2003 for this report. A retrospective phylogenetic analysis of these cases has evidenced an annual shift of the epidemic strain, with all strains belonging to genotype IA (unpublished data).

In conclusion, molecular investigation of HAV cases from saliva samples appears to be a promising tool for a larger understanding of HAV epidemiology.

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