JOURNAL OF CLINICAL MICROBIOLOGY, June 2009, p. 1931–1933 Vol. 47, No. 6
0095-1137/09/$08.00+0 doi:10.1128/JCM.02245-08

Outbreak of Hepatitis E Virus Infection in Darfur, Sudan: Effectiveness of Real-Time Reverse Transcription-PCR Analysis of Dried Blood Spots▽

Audrey Mérens,[1]* Philippe Jean Guérin,[2] Jean-Paul Guthmann,[2] and Elisabeth Nicand[1]

National Reference Laboratory for Hepatitis E, Hospital Val-de-Grâce, Paris, France,[1] and Epicentre, Paris, France[2]

Received 21 November 2008/Returned for modification 3 February 2009/Accepted 23 March 2009

Biological samples collected in refugee camps during an outbreak of hepatitis E were used to compare the accuracy of hepatitis E virus RNA amplification by real-time reverse transcription-PCR (RT-PCR) for sera and dried blood spots (concordance of 90.6%). Biological profiles (RT-PCR and serology) of asymptomatic individuals were also analyzed.

Hepatitis E virus (HEV) is a spherical, nonenveloped, single-stranded RNA virus (2, 16) that belongs to the new genus, *Hepevirus* (14). This pathogen is responsible for at least 50% of acute non-A non-B hepatitis in developing countries, where it causes sporadic infection but also large epidemics usually associated with fecal contamination of water (4–6, 10, 11, 15). A large outbreak of hepatitis E was reported in June 2004 in the internally displaced population camps of Darfur, in West Sudan, and across the border in Chad: at least 5,000 HEV infections were recorded from June to December 2004 (9). A task force, set up by the nongovernmental organizations Médecins Sans Frontières (Doctors Without Borders) and Epicentre in refugee camps in Darfur and by the World Health Organization and Centers for Disease Control in refugee camps in Chad, conducted a field study of this large epidemic. The samples were drawn from both clinical patients and asymptomatic individuals (3, 9, 13). The French National Reference Laboratory (CNR) for hepatitis E monitored the biological and virological investigations. The aims of this study were to assess the feasibility of amplifying HEV RNA from dried blood spots (DBS) in comparison with amplification from serum and to describe the biological profiles of patients and asymptomatic individuals.

Eighty-nine displaced persons living in the Chad Goz Amer camp (median age, 28 years; range, 7 to 65 years) and 92 persons in the Sudanese Mornay camp (median age, 25 years; range, 3 to 75 years) were investigated in August and September 2004. Two groups were defined in each camp. The first was composed of patients considered to be HEV infected, i.e., patients presenting or having presented jaundice since 1 July 2004 and with negative test results for a malarial diagnosis (n = 36 in Goz Amer; n = 56 in Mornay). The time before onset of jaundice was reported by the physicians of medical teams after investigation of patient histories. The second group included individuals that were asymptomatic from the start of the outbreak (n = 53 in Goz Amer; n = 36 in Mornay). Two types of biological samples were collected for each individual. First, whole-blood samples were collected by venipuncture and were centrifuged locally to isolate sera. Aliquots were conserved between +4 and +8°C in the field and at −80°C at CNR. Second, finger-prick whole-blood samples were collected on filter paper. The filter papers were thoroughly air dried and were stored at ambient local temperatures (28 to 40°C) in individual paper bags to prevent contamination and preserve long-term stability. As various medical teams were involved, two types of filter paper were used: Whatman paper in Goz Amer and Isocode Stix in Mornay (Schleicher and Schuell Bioscience, Inc.).

Samples were shipped to France, and serum samples were tested for anti-HEV immunoglobulin G (IgG) and IgM with enzyme-linked immunosorbent assay HEV IgG and HEV IgM kits (Genelabs Diagnostics and Abbott). HEV RNA was amplified from DBS and serum samples from each refugee. Paper sample areas were eluted in 220 μl of phosphate-buffered saline–Tween buffer. A final elution or serum volume of 200 μl was used for nucleic acid extraction with a MagnA pure RNA isolation kit (Roche Diagnostics). After a retrotranscription step, HEV RNA was amplified and detected by consensus TaqMan real-time reverse transcription-PCR (RT-PCR) performed on a LightCycler as described previously (8). Samples with a crossing point inferior to 43, without amplification of the negative control, were considered positive (8). Samples with discordant results for serum and DBS were tested in duplicate in two independent real-time PCR assays (retrotranscription and PCR). HEV RNA amplification results for sera and DBS were concordant in 90.6% of cases (Table 1), with no statistically significant differences (McNemar chi-square test). The kappa correlation coefficient was calculated as 0.808. The results of amplification from DBS and sera were in agreement at 86.5% for Whatman paper and 94.6% for Isocode Stix. No significant difference between the two papers was observed in this study (P = 0.1). Thus, DBS are easy to collect and store and have been successfully used for the amplification of HEV RNA by real-time RT-PCR. However, in nine cases, HEV RNA was amplified in serum samples but not in DBS samples. Six of the nine samples were stored on Whatman paper, and the amount of blood on the filter was less than 50 μl. Three of the nine samples were collected on Isocode Stix in suitable

* Corresponding author. Mailing address: Hôpital d'Instruction des Armées Bégin, 69 Avenue de Paris, 94 160 Saint-Mandé Cedex, France. Phone: 00 (33) 1-40-51-46-30. Fax: 00 (33) 1-43-98-53-36. E-mail: merens-a@wanadoo.fr.

▽ Published ahead of print on 1 April 2009.

TABLE 1. Comparison of results of real-time HEV RNA RT-PCR amplification from serum and from whole blood collected on filter paper[a]

RT-PCR result for serum samples (n = 181)	No. of DBS samples (n = 181) with RT-PCR result on:			
	Whatman paper (n = 89)		Isocode Stix (n = 92)	
	Positive	Negative	Positive	Negative
Positive	31	6	39	3
Negative	6	46	2	48

[a] Samples were collected from 181 refugees in the Goz Amer and Mornay internally displaced population camps.

volumes, and discrepancies may be explained by persistence of natural PCR inhibitors in the paper matrix or by RNases introduced by finger contact when handling the papers during collection. HEV amplification sensitivity is dependent on standardizing the quantity of blood and on the careful handling of filter papers with gloves, especially for this RNA virus. In eight cases, DBS samples were positive and serum samples were negative for HEV RNA amplification; these cases occurred in samples from patients with clinical and serological evidence of acute hepatitis (n = 3) and in samples from asymptomatic refugees in which anti-HEV IgM was detected in the serum (n = 4). These cases, which provided the best results for HEV amplification from DBS samples, may be explained by the degradation of RNA in sera due to an absence of cooler conditions during transport (less than −20°C).

The second objective of this study was to describe the various biological profiles identified. Among the group defined as hepatitis E cases, all the specimens were positive for at least one of the HEV markers HEV RNA, anti-HEV IgG, and anti-HEV IgM (Table 2). HEV RNA was detected in 65.1% of cases; among these, 51 of 58 had detectable anti-HEV IgM. The Abbott IgM anti-HEV assay had a sensitivity of 86% in comparison with the results of RT-PCR. These results are consistent with data reported under outbreak conditions (7, 12). Interestingly, HEV RNA amplification was positive in 7 of 12 (58.3%) DBS specimens with negative anti-HEV IgM results, allowing the diagnosis of HEV infection. Conversely, among the cases in which HEV RNA amplification was negative (34.9%), 26 of 31 (83.9%) had detectable anti-HEV IgM. These observations underline the importance of using different biological tests for the HEV diagnosis, as none of the tests are sufficiently sensitive for use alone. Interestingly, HEV viremia was detectable with TaqMan HEV RNA real-time RT-PCR for more than 39 days after the onset of jaundice. Another point to underline is the large proportion of asymptomatic individuals displaying a biological profile consistent with acute hepatitis E infection (43.5%), with a detectable viremia for 31.5% of cases (Table 3). No statistically significant differences were observed when comparing the ages of individuals with icteric infections and those with asymptomatic HEV infections; however, clinical observations have documented the frequency of symptoms among older populations (1). An accurate evaluation of the spread of disease and the infection attack rate also requires biological samples from a cross section of asymptomatic individuals.

TABLE 2. Results of real-time HEV RT-PCR and detection of specific anti-HEV antibodies for individuals with jaundice[a]

Antibody results	No. (%) of samples with indicated RT-PCR result		Total no. (%)
	Positive	Negative	
IgG and IgM positive	51 (57.2)	24 (27)	75 (84.2)
IgG positive, IgM negative	7 (7.8)	5 (5.6)	12 (13.4)
IgG negative, IgM positive	0	2 (2.2)	2 (2.2)
IgG negative, IgM negative	0	0	0
Total	58 (65)	31 (35)	89 (100)

[a] Serum samples were collected from 89 individuals in the Goz Amer and Mornay camps.

TABLE 3. Results of real-time HEV RT-PCR and detection of specific anti-HEV antibodies for asymptomatic individuals[a]

Antibody results	No. (%) of samples with indicated RT-PCR result		Total no. (%)
	Positive	Negative	
IgG and IgM positive	9 (9.8)	9 (9.8)	18 (19.6)
IgG positive, IgM negative	10 (10.9)	27 (29.3)	37 (40.2)
IgG negative, IgM positive	1 (1.1)	2 (2.2)	3 (3.3)
IgG negative, IgM negative	9 (9.8)	25 (27.2)	34 (37)
Total	29 (31.5)	63 (68.5)	92 (100)

[a] Serum samples were collected from 92 asymptomatic individuals in the Goz Amer and Mornay camps.

DBS have already been used for PCR detection of hepatotropic viruses, but this study is the first report using real-time nucleic acid amplification of DBS collected under tropical conditions in refugee camps during an outbreak. DBS requires only 50 μl of blood, which is an advantage for children. DBS samples do not require a centrifuge and can be mailed unrefrigerated with a low biohazard risk. Thus, RT-PCR of DBS samples may provide an accurate and reliable tool for conducting field studies of outbreaks in developing countries.

We thank the local medical teams for collecting specimens and Gregory Armstrong from the Centers for Disease Control and Prevention, Atlanta, GA. We also thank Vincent Enouf and Mélanie Caron for their technical contributions to this study.

Declaration of interest: we do not have a financial interest in any organization that could be perceived as a real or apparent conflict of interest in the context of the material submitted here. There was no grant support for the work.

REFERENCES

1. **Aggarwal, R., H. Shahi, and K. Nai.** 1997. Evidence in favour of high infection rate with hepatitis E among young children in India. J. Hepatol. **26:**1425–1426.
2. **Balayan, M. S., A. G. Andjaparidze, S. S. Savinskaya, E. S. Ketiladze, D. M. Braginsky, A. P. Savinov, and V. F. Poleschuk.** 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology **20:**23–31.
3. **Boccia, D., J. P. Guthmann, H. Klovstad, N. Hamid, M. Tatay, I. Ciglenecki, J. Y. Nizou, E. Nicand, and P. J. Guerin.** 2006. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clin. Infect. Dis. **42:**1679–1684.
4. **Bryan, J. P., M. Iqbal, S. Tsarev, I. A. Malik, J. F. Duncan, A. Ahmed, A. Khan, A. Khan, A. R. Rafiqui, R. H. Purcell, and L. J. Legters.** 2002. Epidemic of hepatitis E in a military unit in Abbotrabad, Pakistan. Am. J. Trop. Med. Hyg. **67:**662–668.

5. **Bryan, J. P., S. A. Tsarev, M. Iqbal, J. Ticehurst, S. Emerson, A. Ahmed, J. Duncan, A. R. Rafiqui, I. A. Malik, R. H. Purcell, et al.** 1994. Epidemic hepatitis E in Pakistan: patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. J. Infect. Dis. **170:**517–521.
6. **Buisson, Y., M. Grandadam, E. Nicand, P. Cheval, H. van Cuyck-Gandre, B. Innis, P. Rehel, P. Coursaget, R. Teyssou, and S. Tsarev.** 2000. Identification of a novel hepatitis E virus in Nigeria. J. Gen. Virol. **81:**903–909.
7. **El-Sayed Zaki, M., M. H. El-Deen Zaghloul, and O. El Sayed.** 2006. Acute sporadic hepatitis E in children: diagnostic relevance of specific immunoglobulin M and immunoglobulin G compared with nested reverse transcriptase PCR. FEMS Immunol. Med. Microbiol. **48:**16–20.
8. **Enouf, V., G. Dos Reis, J. P. Guthmann, P. J. Guerin, M. Caron, V. Marechal, and E. Nicand.** 2006. Validation of single real-time TaqMan PCR assay for the detection and quantitation of four major genotypes of hepatitis E virus in clinical specimens. J. Med. Virol. **78:**1076–1082.
9. **Guthmann, J. P., H. Klovstad, D. Boccia, N. Hamid, L. Pinoges, J. Y. Nizou, M. Tatay, F. Diaz, A. Moren, R. F. Grais, I. Ciglenecki, E. Nicand, and P. J. Guerin.** 2006. A large outbreak of hepatitis E among a displaced population in Darfur, Sudan, 2004: the role of water treatment methods. Clin. Infect. Dis. **42:**1685–1691.
10. **Iqbal, M., A. Ahmed, A. Qamar, K. Dixon, J. F. Duncan, N. U. Islam, A. Rauf, J. P. Bryan, I. A. Malik, and L. J. Legters.** 1989. An outbreak of enterically transmitted non-A, non-B hepatitis in Pakistan. Am. J. Trop. Med. Hyg. **40:**438–443.
11. **Isaacson, M., J. Frean, J. He, J. Seriwatana, and B. L. Innis.** 2000. An outbreak of hepatitis E in northern Namibia, 1983. Am. J. Trop. Med. Hyg. **62:**619–625.
12. **Myint, K. S. A., T. P. Endy, R. V. Gibbons, K. Laras, M. P. Mammen, Jr., E. R. Sedyaningsih, J. Seriwatana, J. S. Glass, S. Narupiti, and A. L. Corwin.** 2006. Evaluation of diagnostic assays for hepatitis E virus in outbreak settings. J. Clin. Microbiol. **44:**1581–1583.
13. **Nicand, E., G. L. Armstrong, V. Enouf, J. P. Guthmann, J. P. Guerin, M. Caron, J. Y. Nizou, and R. Andraghetti.** 2005. Genetic heterogeneity of hepatitis E virus in Darfur, Sudan, and neighboring Chad. J. Med. Virol. **77:**519–521.
14. **Okamoto, H.** 2007. Genetic variability and evolution of hepatitis E virus. Virus Res. **127:**216–228.
15. **Schlauder, G. G., G. J. Dawson, I. K. Mushahwar, A. Ritter, R. Sutherland, A. Moaness, and M. A. Kamel.** 1993. Viraemia in Egyptian children with hepatitis E virus infection. Lancet **341:**378.
16. **Tam, A. W., M. M. Smith, M. E. Guerra, C. C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120–131.