JOURNAL OF CLINICAL MICROBIOLOGY, June 1999, p. 2061–2063 Vol. 37, No. 6
0095-1137/99/$04.00+0

Detection of Antibodies to Hepatitis C Virus in Dried Blood Spot Samples from Mothers and Their Offspring in Lahore, Pakistan

S. P. PARKER,[1]* H. I. KHAN,[2] AND W. D. CUBITT[1]

Virology Department, Camelia Botnar Laboratories, Great Ormond Street Hospital for Children, London WC1N 3JH, United Kingdom,[1] and Department of Paediatrics, King Edward Medical College & Mayo Hospital, Lahore, Pakistan[2]

Received 2 November 1998/Returned for modification 25 January 1999/Accepted 15 March 1999

Dried blood spot samples from mothers and their offspring attending the obstetric and pediatric departments of two hospitals in Lahore, Pakistan, were tested for antibodies to hepatitis C virus (HCV). The seroprevalence of HCV in the women was 6.7% (95% confidence interval [CI], 4.3 to 9.1), and that in the children was 1.3% (95% CI, 0.34 to 2.26). Four anti-HCV immunoglobulin G (IgG)-positive children had mothers that were anti-HCV IgG negative, which suggested that their infection was community acquired.

Hepatitis C virus (HCV) and HBV are significant causes of morbidity and mortality in Pakistan, where studies of patients with chronic hepatitis and hepatocellular carcinoma, in Rawalpindi and Karachi, showed that between 20 and 30% had antibodies to HCV and that 70 to 80% had evidence of past HBV infection (3, 10).

The seroprevalence of HCV in Pakistan is unclear, and its epidemiology, particularly in women and children, has yet to be established, although a study that used a second-generation antibody assay reported a seroprevalence of 0.44% in a group of 226 apparently healthy children in Karachi (1). Molecular studies have identified HCV type 3 as the predominate strain in Pakistan (9, 10) and also in northern India (5); in contrast, HCV type 1 is the predominate strain in southern India (12).

A cost-effective strategy involving an in-house enzyme immunoassay (EIA) as a screening test (7), an additional test based on a gelatin particle agglutination (GPA) assay (Fujirebio Inc., Tokyo, Japan), and confirmation by a third-generation immunoblot assay (RIBA 3.0; Ortho-Clinical Diagnostics, Amersham, United Kingdom) has been proposed for determination of the seroprevalence of HCV in dried blood spots (DBS) obtained from children and women of childbearing age in Lahore, Pakistan. DBS, which consist of whole blood collected on filter paper, are a highly effective alternative to serum, as they simplify the collection, storage, and shipment of samples for epidemiological surveys (6, 8, 11).

A total of 955 whole-blood samples were obtained from 417 consenting women (aged 17 to 53 years) and 538 children (aged 1 day to 16 years) reporting to the emergency and outpatient sections of the pediatric department of Mayo Hospital and from pregnant women at the obstetric unit of Lady Atchinson Hospital in Lahore. Samples were obtained by heel or finger prick, blotted onto Whatman no. 1 filter paper, and allowed to air dry. Samples were stored at ambient temperature in envelopes with silica gel desiccant and transported to England for testing.

A 5.5-mm-diameter DBS was punched from each filter paper sample, and antibodies were eluted overnight as described previously (7). Within our laboratory DBS derived from Whatman no. 1 filter paper were found to elute consistently (±10%) in comparison to those derived from Schleicher & Scheull 2992 filter paper, the type used for Guthrie card studies in the United Kingdom.

Initially, samples and positive and negative eluate controls were screened by an in-house anti-HCV immunoglobulin G (IgG) EIA (7) in which plates were coated with a combination of the recombinant proteins c22, c200, and NS5 (Chiron Corporation, Emeryville, Calif.). Reactive samples showing an optical density (OD) greater than the cutoff value and with a test result/negative eluate control result ratio (*T*/*N*) of ≥2.0 were subjected to further testing. The assay cutoff was determined by using the mean plus 3 standard deviations of results for a population of nonreactive samples. Assay performance was validated by the Shewart multirule procedure. Initial reactive samples were then retested by the same protocol as for the initial EIA but with each individual recombinant protein. Samples that were repeatedly reactive were subject to confirmation by RIBA 3.0. DBS eluates were diluted 1:20 (50 μl in 1 ml) in reagent buffer; otherwise, the assay was performed according to the manufacturer's instructions. In addition all initial reactive samples were tested in the GPA assay. Lyophilized gelatin particles, sensitized with the HCV recombinant antigens c22-3 and c200, were reconstituted before use to make a 1% suspension of particles. This suspension was further diluted 1:10 in TPHA buffer as described previously (8). A 25-μl aliquot of diluted particles was added to 25 μl of DBS eluate diluted 1:5 in TPHA buffer in a V-well plate, and the plate was shaken briefly and allowed to stand on a vibration-free surface at room temperature for 4 h. Gelatin particles formed a distinct agglutination pattern in the presence of HCV-specific antibody. In the absence of specific antibody, particles settled to form a uniform button at the base of the V well.

Forty-four eluates were reactive in the EIA with an OD at 450 nm (OD_{450}) greater than the cutoff (range, 0.20 to 1.43; *T*/*N*, 2.1 to 13.7) (Table 1). Repeat testing by individual EIAs revealed that although 38 eluates showed reactivity to the core recombinant protein c22, only 29% were reactive to c200 and only 24% were reactive to NS5.

RIBA 3.0 testing confirmed the presence of antibody in 30 of the 38 repeatedly reactive samples. Of the remaining samples, 7 were classified as indeterminate (identification no. [ID] 148,

* Corresponding author. Mailing address: Virology Department, Camelia Botnar Laboratories, Great Ormond Street Hospital for Children, London WC1N 3JH, United Kingdom. Phone: 0171 405 9200, ext. 2420. Fax: 0171 829 8639. E-mail simon.parker@GOSH-TR.NTHAMES.NHS.UK.

TABLE 1. Complete anti-HCV results for all 44 anti-HCV EIA-reactive samples

Sample ID[a]	Mother and/or child[b] (age of child)	Anti-IgG HCV EIA result		Reactivity to anti-IgG HCV protein			RIBA 3.0 result[c]				GPA assay result
		OD_{450}	*T*/*N*	c22	c200	NS5	c100	c33	c22	NS5	
25	M	1.30	13.7	+	+	+	−	2+	4+	−	+
32	M	0.24	2.5	−	−	−	−	+/−	+/−	−	−
43	C (4 yr)	0.56	5.9	+	−	−	1+	1+	3+	−	+
92	M	0.25	2.6	+	−	−	−	+/−	−	−	+
112	M	0.71	7.5	+	−	−	+/−	1+	2+	−	+
148	M	1.00	10.5	+	+	−	−	+/−	3	−	+
159	C (3 mo)	**1.17**	**8.3**	+	+	−	1+	4+	2+	−	+
160	M	**1.17**	**12.3**	+	+	−	2+	4+	4+	−	−
164	M	0.65	6.8	+	−	−	+/−	1+	2+	−	−
181	C (2 mo)	**0.25**	**2.6**	+	−	−	−	+/−	1+	−	+
182	M	**0.25**	**2.6**	+	−	−	−	−	1+	−	+
359	M	0.45	4.7	+	+	−	+/−	2+	1+	−	+
367	M	1.34	9.6	+	+	−	1+	4+	3+	−	+
373	M	1.01	11.6	+	−	−	−	1+	3+	−	+
418	C (2 yr)	0.42	4.4	+	−	−	1+	2+	1+	−	+
422	C (5 yr)	**0.54**	**5.7**	+	−	−	1+	2+	1+	−	+
423	M	**0.44**	**4.6**	+	−	−	1+	2+	1+	−	+
431	C (1 yr)	0.83	5.9	+	−	−	1+	2+	1+	−	+
436	C (17 mo)	0.93	6.7	+	−	−	1+	2+	1+	−	+
437	M	0.94	6.7	+	−	−	1+	2+	1+	−	+
455	M	0.94	6.7	+	−	−	1+	2+	1+	−	+
461	M	0.92	6.6	+	−	−	1+	2+	1+	−	+
468	M	0.97	6.9	+	−	−	2+	3+	2+	−	+
486	M	1.11	11.7	+	−	+	−	+/−	4+	−	−
494	M	1.10	11.6	+	+	+	3+	4+	4+	−	+
543	C (15 mo)	0.21	2.2	−	−	−	−	−	−[c]	−	−
558	M	0.81	5.8	+	−	−	−	+/−	2+	−	+
582	M	0.61	4.4	+	−	−	+/−	+/−	2+	−	+
592	M	0.24	2.5	−	−	−	−	−	−	−	−
599	C (11 yr)	0.67	4.8	+	−	−	−	+/−	2+	−	+
625	M	1.11	11.7	+	+	−	−	1+	4+	−	−
641	M	0.21	2.2	−	−	−	−	−	−	−	−
665	C (4.5 yr)	0.32	3.4	−	−	−	−	−	−	−	−
666	M	0.62	7.8	+	+	+	3+	4+	3+	−	+
696	M	1.45	10.4	+	+	−	3+	4+	4+	−	+
707	M	1.27	9.1	+	−	−	1+	1+	4+	−	+
816	M	0.62	4.4	+	−	−	−	2+	1+	−	+
823	C (9 yr)	0.20	2.1	−	−	−	−	−	−	−	−
840	M	0.70	7.4	+	−	+	−	1+	2+	−	+
846	M	1.18	12.4	+	+	+	+/−	2+	4+	−	+
858	C (3 yr)	1.19	8.5	+	−	−	−	1+	3+	−	+
874	M	1.38	9.8	+	−	+	4+	1+	3+	2+	+
908	M	1.43	10.2	+	−	+	1+	3+	4+	3+	+
943	M	0.55	5.8	+	−	+	−	1+	2+	−	−

[a] Numbers in bold are for mother-child pairs.
[b] M, mother; C, child.
[c] Values 1+ to 4+ are measures of band intensity in relation to high- and low-intensity IgG control bands. +/−, band intensity less than that of the low IgG control band; −, no bands detected. Interpretation: RIBA 3.0 positive, a minimum of two bands of 1+ intensity must be present; RIBA 3.0 indeterminate, at least one band of 1+ intensity must be present.

181, 182, 486, 558, 582, and 599) by virtue of c22 reactivity greater than or equal to 1+ and 1 sample was negative (ID 92). All 30 RIBA 3.0-confirmed samples had antibody to the c22 and c33 protein bands, but only 63.3% had antibody to c100 and 6.7% had antibody to NS5. All 7 RIBA 3.0 indeterminates exhibited reactivity to c22 by EIA, with 2 samples, ID 148 and ID 486, additionally reactive to c200 and NS5, respectively, by EIA. The modified GPA assay detected antibody in 26 EIA-reactive, RIBA 3.0-confirmed samples, in 6 of 7 RIBA 3.0-indeterminate samples (*T*/*N*, 2.6 to 10.5), and also in 1 EIA-reactive, anti-c22 EIA-reactive, RIBA 3.0-negative sample (ID 92). The assay failed to detect antibody in four eluates (IDs 160, 164, 625, and 943) that were reactive by EIA and confirmed by RIBA 3.0. Titration of these eluates in the GPA assay discounted the possibility of any prozone effect. If we assume that the GPA recombinant proteins were derived from a source different from that of the EIA and RIBA 3.0 assays, it is suggested that reactivity in the GPA assay provides evidence that the c22-positive, RIBA 3.0-indeterminate eluates are genuine positives. However, lack of reactivity by the GPA assay in the four RIBA 3.0-confirmed samples suggests that the assay may be an inappropriate screening tool in countries such as Pakistan where there is a higher frequency of HCV type 3.

This study estimates the seroprevalence of HCV in women of childbearing age to be 6.7% (95% confidence interval, 4.3 to 9.1) and that in children (IDs 159 and 181 were neonates born to anti-HCV-positive mothers) to be at least 1.3% (95% confidence interval, 0.34 to 2.26). These rates compare to a rate of

0.44% found in a study of 226 apparently healthy children (1) and to a rate of 0 to 12% in a community in Hafizabad, Pakistan (2). The high rate of seropositivity in Pakistan compared to rates in other countries can be explained by an increased risk of transmission due to therapeutic injection by contaminated needles and syringes (2) or transfusion with unscreened blood. Four children that were anti-HCV positive had mothers who were seronegative and clearly had acquired HCV from elsewhere.

The anti-HCV algorithm proved to be a cost-effective tool for the analysis of DBS due to a low rate of repeat testing. Only six samples (0.63%) failed to be confirmed by supplementary testing. The sensitivity of the screening EIA with DBS from Pakistan (OD range, 0.20 to 1.43) appears similar to that seen when 76 anti-HCV samples from children at Great Ormond Street Hospital for Children (London, United Kingdom) were tested (OD range, 0.27 to 1.66).

The lack of reactivity to c200 and NS5 in these samples has also been seen in a study of patients infected with type 3 virus using these recombinants in samples from England and South Africa (unpublished data) and in a study using NS3 and NS5 recombinants derived from type 3a virus (4). Additionally, lack of reactivity within the Pakistani samples may have been due to emerging variants, such as subtype 3g, which have been reported in the neighboring region of northern India (5).

The high seroprevalence rates in this study highlight the need for increased awareness of HCV and its routes of transmission in a community where transfusion with unscreened blood products and the reuse of syringes and needles are not uncommon, particularly in some rural communities.

H. I. Khan's travel expenses were provided by the British Council.

We are indebted to the Chiron Corporation for the generous donation of HCV recombinant proteins and Michael Rasche of Bayer for donating Serodia-HCV GPA reagents.

REFERENCES

1. **Agboatwalla, M., S. Isomura, K. Miyake, T. Yamashita, T. Morishita, and D. Samin Akram.** 1994. Hepatitis A, B and C seroprevalence in Pakistan. Indian J. Pediatr. **61:**545–549.
2. **Luby, S. P., K. Qamruddin, A. A. Shah, A. Omair, O. Pahsa, A. J. Khan, J. B. McCormick, F. Hoodbhouy, and S. Fisher-Hoch.** 1997. The relationship between therapeutic injections and high prevalence of hepatitis C infection in Hafizabad, Pakistan. Epidemiol. Infect. **119:**349–356.
3. **Mujeeb, S. A., R. Khanani, S. Kaher, Q. Jamal, and N. Iqbal.** 1997. Prevalence of hepatitis B surface antigen and HCV antibodies in hepatocellular carcinoma cases in Karachi, Pakistan. Trop. Dr. **27:**45–46.
4. **Neville, J. A., L. E. Prescott, V. Bhattacherjee, N. Adams, I. Pike, B. Rodgers, A. El-Zayadi, S. Hamid, G. M. Dusheiko, A. A. Saeed, G. H. Haydon, and P. Simmonds.** 1997. Antigenic variation of core, NS3, and NS5 proteins among genotypes of hepatitis C virus. J. Clin. Microbiol. **35:**3062–3070.
5. **Panigrahi, A. K., J. Roca, S. K. Achraya, S. Jameel, and S. K. Panda.** 1996. Genotype determination of hepatitis C virus from northern India: identification of a new subtype. J. Med. Virol. **48:**191–198.
6. **Parker, S. P., and W. D. Cubitt.** The use of the dried blood spot sample (DBS) in epidemiological studies. J. Clin. Pathol., in press.
7. **Parker, S. P., W. D. Cubitt, and A. E. Ades.** 1997. A method for the detection and confirmation of antibodies to hepatitis C virus in dried blood spots. J. Virol. Methods **68:**199–205.
8. **Parker, S. P., M. B. Taylor, A. E. Ades, W. D. Cubitt, and C. S. Peckham.** 1995. Use of dried blood spots for the detection and confirmation of HTLV-I specific antibodies for epidemiological purposes. J. Clin. Pathol. **48:**904–907.
9. **Shah, H., S. W. Jafri, I. A. Malik, L. Prescott, and P. Simmonds.** 1997. Hepatitis C virus (HCV) genotypes and chronic liver disease in Pakistan. J. Gastroenterol. Hepatol. **12:**758–761.
10. **Tong, C. Y. W., R. Khan, N. J. Beeching, W. U. Z. Tariq, C. A. Hart, N. Ahmad, and I. A. Malik.** 1996. The occurrence of hepatitis B and C viruses in Pakistani patients with chronic liver disease and hepatocellular carcinoma. Epidemiol. Infect. **117:**327–332.
11. **Unlinked Anonymous HIV Surveys Steering Group.** 1996. Unlinked Anonymous HIV Prevalence Monitoring Programme: England and Wales, data to the end of 1995. Department of Health, Public Health Laboratory Service, Institute of Child Health, London, United Kingdom.
12. **Valliammai, T., S. Thyagarajan, A. Zuckerman, and T. Harrison.** 1995. Diversity of genotypes of hepatitis-C virus in southern India. J. Gen. Virol. **76:**711–716.