Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity

J. A. GARSON   R. S. TEDDER   M. BRIGGS   P. TUKE
J. A. GLAZEBROOK   A. TRUTE   D. PARKER   J. A. J. BARBARA
M. CONTRERAS   S. ALOYSIUS

Of 1100 blood donations tested during a prospective study of post-transfusion non-A, non-B hepatitis (NANBH), 6 (0·55%) were repeatedly reactive in a commercial assay for antibodies to the C100 protein of hepatitis C virus. Only 1 of the 6 donations (17%) transmitted NANBH to a recipient. Hepatitis C virus RNA sequences were detected in the serum of the transmitting donor by an assay which used the polymerase chain reaction (PCR) and non-radioisotopic detection. No such sequences were detected in the other 5 donors positive for anti-C100. Stored serum samples from blood donors who had been involved in three episodes of post-transfusion NANBH in 1981 also contained hepatitis C viral sequences. Although the PCR assay in its present form is not suitable for mass donor screening, the presence of hepatitis C viral sequences detected by PCR in blood donations seems a better predictor of infectivity than the presence of anti-C100 alone.

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Introduction

Since the introduction of reliable serological tests for hepatitis A and hepatitis B viruses in the 1970s, it has become apparent that most cases of post-transfusion hepatitis are now caused by neither of those viruses but by unknown agents unrelated to any of the previously described hepatotropic viruses.[1] Attempts to identify the non-A, non-B hepatitis (NANBH) agents were unsuccessful until the cloning[2] of a sequence from a small, positive-stranded RNA virus, designated hepatitis C virus.

Seroepidemiological studies based on the detection of antibodies against C100, a recombinant non-structural protein from hepatitis C virus, suggest that this virus is the cause of most cases of post-transfusion NANBH world wide.[3] In the absence of a test for viraemia, many blood transfusion services are considering the screening of donated blood for antibodies to C100 (anti-C100), although the precise relation between the presence of anti-C100 and the infectivity of the blood is unknown. Features of this relation are being investigated as part of a large prospective study of blood donors and recipients at the North London Blood Transfusion Centre. We have examined anti-C100-positive donations from that prospective study by means of a new assay for the detection of hepatitis C virus RNA sequences. The assay, which is based on a modification of the polymerase chain reaction (PCR),[4] has also been used to investigate frozen serum samples from donors and recipients involved in three episodes of post-transfusion NANBH in 1981.

Subjects and methods

Serum samples from 1100 donors and 300 recipients enrolled in the prospective study of post-transfusion NANBH (started July 1987) were stored at −20°C. Blood was obtained regularly from the recipients for at least 6 months. Serum samples frozen since 1981 from 10 donors associated with three cases of post-transfusion NANBH and from 2 of the jaundiced recipients were also available for study. The diagnosis of post-transfusion NANBH was based on a rise in serum alanine aminotransferase exceeding 2·5 times the upper limit of normal in at least two separate post-transfusion samples.[5] Other hepatotropic viruses were excluded by serological testing. Non-viral causes of hepatocellular injury were excluded by conventional clinical and laboratory studies.

Serum samples were tested retrospectively for the presence of anti-C100 with the Ortho Diagnostics enzyme-linked immunosorbent assay kit according to the manufacturer's instructions. Repeatedly reactive samples were titrated to endpoints in human serum negative for anti-C100.

TABLE I—SYNTHETIC OLIGONUCLEOTIDE SEQUENCES*

—	Sequence (5'→3')	Nucleotide positions	Product size
d94 sense PT†	ATGGGGCAAAGGACGTCCG – – – – – – – – – – A – – – – – – – –	6220–6238	729 bp
d95 antisense PT	TACCTAGTCATAGCCTCCGTGAAG – – – – – G – – – – – – – – – – – – – – –	6949–6926	
N1 sense PT	GAGGTTTTCTGCGTCCA – – – – – – – – – – – – – – – T – –	6339–6355	402 bp
N2 antisense	GCGATAGCCGCAGTTCT	6743–6727	

*Oligonucleotides prepared on an Applied Biosystems 381A DNA synthesiser.
†PT = prototype hepatitis C virus sequence (Chiron Corporation: European Patent Application 88310922·5): nucleotide positions numbered according to prototype sequence. d94, d95, and N1 sequences differ from prototype sequence at one nucleotide position as shown. N2 sequence is identical to prototype.

For the detection of hepatitis C viral sequences serum or plasma RNA was extracted, reverse-transcribed, and amplified. The reverse-transcription/PCR primer sequences (table I) were derived from a cDNA clone (designated pDX100; P. Highfield and colleagues, unpublished) of hepatitis C virus isolated from a United Kingdom blood donor[6] implicated in post-transfusion NANBH. Fig 1 shows the position of these primers on the hepatitis C virus genome.

To extract RNA 5–50 µl serum or plasma was made up to 200 µl with sterile distilled water then added to an equal volume of buffer

ADDRESSES: **Section of Virology, Department of Medical Microbiology, University College and Middlesex School of Medicine, London** (J. A. Garson, MD, R. S. Tedder, MRCPath, M. Briggs, BSc, P. Tuke, BSc); **Wellcome Diagnostics, Beckenham, Kent** (J. A. Glazebrook, PhD, A. Trute, BSc, D. Parker, PhD); **and North London Blood Transfusion Centre, Colindale, London, UK** (J. A. J. Barbara, PhD, M. Contreras, MD, S. Aloysius, PhD). Correspondence to Dr J. A. Garson, Section of Virology, Department of Medical Microbiology, UCMSM, School of Pathology, 67 Ridinghouse Street, London W1P 7PN, UK.
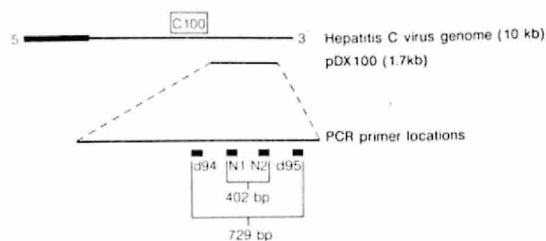
Fig 1—Hepatitis C virus genome showing primer locations.

Heavier line at 5′ end of genome = region encoding structural proteins.

containing 0·2 mol/l "tris"-hydrochloric acid (HCl) pH 7·5, 25 mmol/l ethylenediaminetetra-acetic acid (EDTA), 0·3 mol/l sodium chloride, 2% (weight/volume) sodium dodecyl sulphate, and 200 µg/ml proteinase K, mixed, and incubated at 37°C for 40 min. Proteins were removed by two extractions with phenol/chloroform and one with chloroform alone. 20 µg glycogen was added to the aqueous phase and the RNA precipitated by addition of three volumes of ice-cold absolute ethanol. After storage at −70°C for 1 h the RNA was pelleted in an Eppendorf centrifuge (15 min, 15 800 g, 4°C). The pellet was washed once in 95% ethanol, vacuum dried, and dissolved in 10 µl sterile distilled water. RNA solutions were stored at −70°C.

For cDNA synthesis a 10 µl mixture was prepared containing 2 µl sample RNA solution, 50 ng synthetic oligonucleotide d95, 10 mmol/l Hepes-HCl pH 6·9, and 0·2 mmol/l EDTA pH 8·0. This mixture was covered with 2 drops of mineral oil, heated for 2 min in a water bath at 90°C, and cooled rapidly on ice. cDNA synthesis was carried out after adjustment of the mixture to contain 50 mmol/l "tris"-HCl pH 7·5, 75 mmol/l potassium chloride, 3 mmol/l magnesium chloride, 10 mmol/l dithiothreitol, 0·5 mmol/l of each dNTP, 20 units of ribonuclease inhibitor (Pharmacia), and 15 units of cloned Moloney murine leukaemia virus reverse transcriptase (Pharmacia) in a final volume of 20 µl, which was incubated at 37°C for 90 min. The cDNA was stored at −20°C.

Throughout this study false-positive PCR results were avoided by strict application of the contamination prevention measures of Kwok and Higuchi.[7] Round 1 of the PCR was carried out in a 50 µl mixture containing 10 mmol/l "tris"-HCl pH 8·3, 50 mmol/l potassium chloride, 1·5 mmol/l magnesium chloride, 0·01% (weight/volume) gelatin, 1 unit recombinant Taq DNA polymerase (Perkin Elmer Cetus), 200 µmol/l of each dNTP, 30 ng of each "outer" primer (d94 and d95), and 5 µl of the cDNA sample. After an initial 5 min denaturation at 94°C, 35 cycles of 95°C for 80 s, 56°C

Fig 2—Detection of plasmid pDX100 by nested PCR.

MW = molecular weight marker, φx174/Hae III digest.

TABLE II—SUMMARY OF DATA ON DONORS AND RECIPIENTS IN PROSPECTIVE STUDY

Donors			Recipients		
No	Anti-C100	PCR	No	Post-transfusion NANBH	Anti-C100 seroconversion
D1	+	−	R1	No	No
D2	+	−	R2	No	No
D3	+	−	R3	No	No
D4	+	−	R4	No	No
D5	+	−	R5	No	No
D6	+	+			
D7	−	−			
D8	−	−			
D9	−	−	R6*	Yes (1 mo after transfusion)	Yes (5 mo after transfusion)
D10	−	−			
D11	−	−−			
D12	−	−			
D13	−	−			

*Hepatitis C viral sequences were detected by PCR in serum from this recipient, taken while liver enzymes were high.

for 60 s, 72°C for 60 s were carried out, followed by a 7 min extension at 72°C (Techne PHC-1 automated thermal cycler, Cambridge, UK). The reaction mixture for the second round was the same as for the first with 125 ng of each "inner" primer, N1 and N2, instead of the outer primers d94 and d95. 1 µl of the round 1 PCR products was transferred to the round 2 50 µl reaction mix. 25 cycles of 95°C for 80 s, 46°C for 60 s, 72°C for 60 s were carried out followed by a 7 min extension at 72°C. 20 µl samples of the round 1 and round 2 PCR products were analysed by electrophoresis on a 2% agarose gel. Bands were visualised by ethidium bromide staining and photographed at 302 nm.

Results

The amplified products of the first and second rounds of the PCR with pDX100 plasmid DNA were 729 bp and 402 bp respectively, as predicted from the prototype hepatitis C virus sequence and from the sequence of plasmid pDX100. Fig 2 shows the great improvement in sensitivity obtained with the second round reaction, allowing a single molecule of the pDX100 plasmid to be detected by ethidium bromide staining without the use of a radioactive probe.

Serum samples from 6 of the 1100 donors (0·55%) enrolled in the prospective study were repeatedly reactive in the anti-C100 assay. Of these, only 1 (donor D6) proved to be infectious as shown by the development of post-transfusion NANBH and seroconversion in a recipient (recipient R6; table II). Hepatitis C viral sequences were
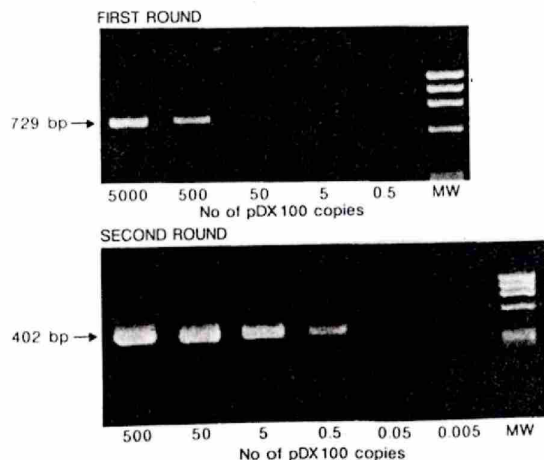
TABLE III—DATA FROM 1981 POST-TRANSFUSION HEPATITIS EPISODES

Donors			Recipients		
No	Anti-C100	PCR	No	Post-transfusion NANBH	Anti-C100 seroconversion
D14	+	+			
D15	−	−		Yes (1 mo after transfusion)	Not available
D16	−	−	R7		
D17	−	−			
D18	−	−			
D19	+	+	R8	Yes (7 wk after transfusion)	Yes
D20	−	−			
D19	+	+			
D21	−	−	R9	Yes	Yes*
D22	−	−			
D23	−	−			

*By 12 wk after transfusion; hepatitis C viral sequences were detected by PCR in serum from this recipient, taken during acute icteric phase.
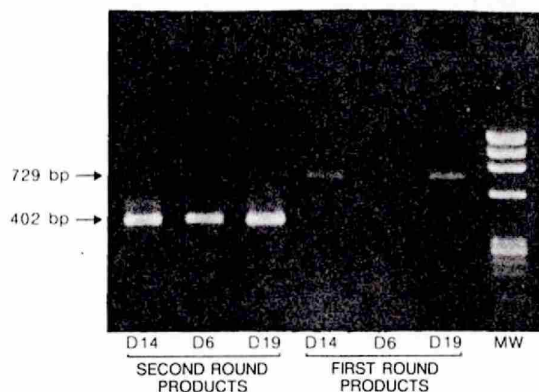
Fig 3—Detection of HCV sequences in donors implicated in post-transfusion NANBH.

detected by PCR in the serum of this donor (D6, fig 3) but not in samples from the other 5 anti-C100-positive donors. It is important that the PCR products from donor D6 were rendered visible only by the extra amplification provided by the second round reaction. The recipient (R6) in whom post-transfusion NANBH developed had received blood from seven other donors (D7 to D13), who were all antibody negative and PCR negative (table II).

Samples of serum stored since the initial investigation of three incidents of post-transfusion NANBH in 1981 were also tested for anti-C100 and hepatitis C viral sequences. In each incident, 1 of the donors was shown to be viraemic by PCR (table III, fig 3). Donor D19 was implicated in two separate incidents; hepatitis C viral sequences were also detected by PCR in 1 of the recipients (R9) during the acute icteric phase. Serum from both viraemic donors (D14 and D19) contained anti-C100.

Testing of recent samples shows that donors D14 and D19 remain viraemic 9 years after their implication in incidents of post-transfusion NANBH. Donor D19 was shown by PCR on stored samples to be viraemic on two occasions in 1982.

2 of the 3 implicated donors (D6 and D14) had normal alanine aminotransferase levels when recalled after their implication. The third (D19) had slightly raised levels on recall. Antibody to hepatitis B core antigen was detected in each implicated donor. When questioned all 3 admitted to intravenous drug abuse in the past. The reciprocal titre of anti-C100 in the three viraemic donors (geometric mean 10, range 4–32) did not differ significantly from that observed in the anti-C100-positive non-transmitting donors (5, 2–8).

Discussion

Although the development and availability of the test for antibody to hepatitis C virus based on the single recombinant protein C100 has greatly advanced knowledge of the epidemiology of NANBH, deeper insights into the biology of this virus infection have been hampered by the lack of a practical assay for viraemia. The PCR-based technique described here is such an assay. Application of the nested primer principle[8] enhanced sensitivity to a degree that makes potentially hazardous radiolabelled probes unnecessary. The time-consuming ultracentrifugation, blotting, washing, and autoradiographic steps of the protocol described by Weiner and colleagues[9] are also avoided. Furthermore, the procedure has greater specificity,

because a product of the correct size will be produced only if four separate fluid-phase hybridisations occur.

Davis and colleagues estimated[10] that transfusion-associated NANBH gives rise to approximately 75 000 cases of chronic hepatitis per year in the United States, 20% of which progress to cirrhosis. Although corresponding figures are not yet available in the United Kingdom, it is generally accepted that there is a need to test donated blood for the presence of the causative agent or agents of post-transfusion NANBH.

The development of the Ortho test for anti-C100 is a substantial advance over the use of surrogate markers such as antibody to hepatitis B core and serum alanine aminotransferase,[11] but its introduction as a routine screening test for blood donors would create some difficulties. First, from our findings only a small proportion (1 of 6 [17%]) of anti-C100-positive donations carry a risk of transmitting NANBH. This finding is supported by a prospective study of blood donors in Amsterdam,[12] in which only 17% (6 of 35) of blood products positive for antibodies to hepatitis C virus were associated with post-transfusion NANBH. Based on an anti-C100 seroprevalence of 0·55–0·70% (our study and that of van der Poel et al[12]) and a total of $2·5 \times 10^6$ donations annually in the United Kingdom, about 10 000 donations per year would be unnecessarily discarded. Secondly, in the absence of any confirmatory tests, counselling of the large numbers of donors reactive in the anti-C100 assay would cause both ethical and logistic difficulties. Thirdly, the anti-C100 assay will not detect all potentially infectious donors because there is a long delay (mean 22 weeks) between infection and serconversion and because in 40% of acute NANBH cases no antibody to this protein ever develops.[11] Finally, the financial implications must be considered: at £2.50 per test the reagent costs alone of introducing the anti-C100 assay for screening all donors would amount to £6·25 million per year and the additional costs of donor counselling, withdrawal of blood and its products, replacement of donors, and confirmatory testing would be substantial.

In an attempt to separate infectious and non-infectious donors, van der Poel et al[12] suggested that a ratio in the enzyme-linked immunoassay (mean test optical density/cutoff optical density) greater than 2 and a higher than normal serum alanine aminotransferase level in anti-C100-positive donations are both significantly associated with infectivity. However, we found no significant difference in titre of anti-C100 between donors who transmitted hepatitis C virus and those who did not, nor any association with high alanine aminotransferase.

What accounts for the reactivity in the anti-C100 assay in non-transmitting donors? There are several possible explanations. There might be specific "convalescent" antibody in donors who have had a self-limiting hepatitis C virus infection in the past. Alternatively, there may be non-specific reactivity due to antibodies against yeast cell contaminants of the C100 antigen preparation or against an unrelated protein which happens to share an epitope or epitopes with C100. Clearly, there is an urgent need for confirmatory serological tests based on recombinant proteins other than C100. We think it unlikely that the reactivity may be false, as a result of testing stored frozen samples, because the seroprevalence observed in the North London Blood Transfusion Centre prospective study (0·55%) is very similar to that found in fresh samples by Kühnl et al.[13]

From the longitudinal studies on donors D14 and D19, it seems that the viraemic carrier state may persist for many years. Such carriers are likely to be a continuing source of transfusion-associated hepatitis and numerically may constitute a greater risk of infection than donors acutely infected with hepatitis C virus. To minimise unnecessary loss of donors and to facilitate donor counselling it is important to identify such infectious carriers. In the absence of a test for antigenaemia, our PCR-based technique is a substantial step forwards, since it allows the accurate identification of the small proportion of anti-C100-positive donors who are carriers of the virus. Although the PCR assay is not yet suited to the mass-screening needs of a blood transfusion laboratory, modifications of PCR technology[14] have shown its potential for large-scale testing. Our preliminary studies suggest that PCR may also be valuable in defining the time course of the viraemia in infected subjects and may allow rapid diagnosis of acute hepatitis C weeks or months before diagnosis by C100 serology is possible.

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Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*

CLAIRE POYART-SALMERON   CECILE CARLIER   PATRICK TRIEU-CUOT
ANDRE-LOUIS COURTIEU   PATRICE COURVALIN

A strain of *Listeria monocytogenes*, isolated from a patient with meningoencephalitis, was resistant to chloramphenicol, erythromycin, streptomycin, and tetracycline. The genes conferring resistance to these antibiotics were carried by a 37-kb plasmid, pIP811, that was self-transferable to other *L monocytogenes* cells, to enterococci-streptococci, and to *Staphylococcus aureus*. The efficacy of transfer and the stability of pIP811 were higher in enterococci-streptococci than in the other gram-positive bacteria. As indicated by nucleic acid hybridisation, the genes in pIP811 conferring resistance to chloramphenicol, erythromycin, and streptomycin were closely related to plasmid-borne determinants that are common in enterococci-streptococci. Plasmid pIP811 shared extensive sequence homology with pAMβ1, the prototype broad host range resistance plasmid in these two groups of gram-positive cocci. These results suggest that emergence of multiple antibiotic resistance in *Listeria* spp is due to acquisition of a replicon originating in enterococci-streptococci. The dissemination of resistance to other strains of *L monocytogenes* is likely.

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Introduction

Severe infections in man due to *Listeria monocytogenes* include abortion, septicaemia, meningitis, and meningoencephalitis; pregnant women, newborn babies, people over 40 years old, and immunocompromised patients are at greatest risk of getting listeriosis.[1] Treatment of choice is benzylpenicillin or ampicillin combined with an aminoglycoside, but tetracycline, erythromycin, or chloramphenicol (alone or in combination), are alternative antibiotics, especially for patients allergic to the penicillins.[3] Most clinical isolates of *L monocytogenes* are uniformly

ADDRESSES: Unité des Agents Antibactériens, Institut Pasteur, Paris (C. Poyart-Salmeron, MD, C. Carlier, P. Trieu-Cuot, PhD, P. Courvalin, MD); and Laboratoire de Bactériologie, Centre National de Référence des *Listeria*, Hôpital Hotel-Dieu Nantes, France (Prof A-L. Courtieu, MD). Correspondence to Dr P. Courvalin, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093, USA.