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DETECTION OF HEPATITIS C VIRAL SEQUENCES BY "NESTED" PCR PREDICTS INFECTIVITY OF ANTI-C100 POSITIVE BLOOD DONORS.

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DETECTION OF HEPATITIS C VIRAL SEQUENCES BY "NESTED" PCR PREDICTS INFECTIVITY OF ANTI-C100 POSITIVE BLOOD DONORS.

Abstract

Of 1100 blood donations tested during a prospective study of post-transfusion non-A, non-B hepatitis (PTNANBH), we identified 6 (0.55%) which were repeatedly reactive in a commercial assay for antibodies to the C100 protein of hepatitis C virus (HCV). Only 1 of these 6 donations (17%) transmitted PTNANBH to a recipient. HCV RNA sequences were detected in the serum of the implicated donor by a novel, non-isotopic, PCR-based assay. HCV sequences were not detected in any of the remaining 5 anti-C100 positive donors. Stored sera from blood donors who had been involved in 3 episodes of PTNANBH which occurred in 1981 were also found to contain HCV sequences. Although we do not propose that the PCR assay in its present form is suitable for mass donor screening, we consider that the detection by PCR of HCV sequences in blood donations is a better predictor of infectivity than the detection of anti-C100 alone.

INTRODUCTION

Since the introduction of reliable serological tests for hepatitis A (HAV) and hepatitis B (HBV) viruses in the 1970's, it has become apparent that the majority of cases of post-transfusion hepatitis are now caused by neither HAV nor HBV 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 recent cloning[2] of a sequence from a small, positive-stranded RNA virus, tentatively designated hepatitis C virus (HCV).

Sero-epidemiological studies based on the detection of antibodies against C100, a recombinant non-structural HCV protein, suggest that HCV causes most cases of post-transfusion non-A, non-B hepatitis (PTNANBH) worldwide[3]. In the absence of a test for viraemia, the screening of blood donations for antibodies to C100 (anti-C100) is being considered by many blood transfusion services, although the precise relationship between the presence of anti-C100 and the infectivity of donated blood is unknown.

Aspects of this relationship are currently being investigated as part of a large prospective study of blood donors and recipients at the North London Blood Transfusion Centre (NLBTC). We have examined anti-C100 positive donations from this prospective study using a newly developed assay for the detection of HCV RNA sequences. The assay, which is based upon a modification of the polymerase chain reaction[4] (PCR), has also been used to investigate frozen donor and recipient sera from 3 incidents of PTNANBH which occurred in 1981.

MATERIALS AND METHODS

Sera

Serum samples from 1100 donors and 300 recipients enrolled in the continuing NLBTC prospective study of PTNANBH (commenced July 1987), were stored at -20°C. Sera frozen since 1981 from 10 donors associated with 3 cases of PTNANBH and from 2 of the jaundiced recipients were also available for study. The diagnosis of PTNANBH was based on a rise in serum alanine aminotransferase (ALT) 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.

Immunoassay

Serum samples were tested retrospectively for the presence of anti-C100 with the Ortho Diagnostics ELISA kit used in accordance with the manufacturer's instructions. Repeatedly reactive sera were titrated to end points in human serum negative for anti-C100.

Detection of HCV Sequences

Serum or plasma RNA was extracted, reverse transcribed and amplified as described below. The reverse transcription/PCR primer sequences shown in Table 1 were derived from a cDNA clone[6] (designated pDX100) of HCV isolated from a UK blood donor[7] implicated in PTNANBH. Figure 1 shows the position of these primers on the HCV genome.

RNA Extraction

Volumes of 5 - 50µl of serum (or plasma) were made up to 200µl by adding sterile distilled water. The 200µl sample was added to an equal volume of 2 x PK buffer (0.2M TrisCl pH7.5, 25mM EDTA, 0.3M NaCl, 2% w/v SDS, proteinase K 200µg/ml), mixed and incubated at 37°C for 40 minutes. Proteins were removed by extracting twice with phenol/chloroform and once with chloroform alone. 20µg glycogen were added to the aqueous phase and the RNA then precipitated by addition of 3 volumes of ice-cold absolute ethanol. After storage at -70°C for 1 hour the RNA was pelleted in an Eppendorf centrifuge (15 minutes, 14000 rpm, 4°C). The pellet was washed once in 95% ethanol, vacuum desiccated and dissolved in 10µl of sterile distilled water. RNA solutions were stored at -70°C.

cDNA Synthesis

A 10µl mixture was prepared containing 2µl of the RNA solution, 50ng of the synthetic oligonucleotide d95, 10mM Hepes-HCl pH6.9 and 0.2mM EDTA pH8.0. This 10µl mix was overlayed with 2 drops of mineral oil, heated for 2 minutes in a water bath at 90°C and cooled rapidly on ice. cDNA synthesis was performed after adjusting the reaction to contain 50mM Tris-HCl pH7.5, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 0.5mM each of dATP, dCTP, dGTP and dTTP, 20 units of RNase inhibitor (Pharmacia) and 15 units of cloned MLV reverse transcriptase (Pharmacia) in a final volume of 20µl. The 20µl mix was incubated at 37°C for 90 minutes. Following synthesis the cDNA was stored at -20°C.

"Nested" PCR

Throughout this study false-positive PCR results were avoided by strict application of the contamination prevention measures of Kwok and Higuchi[8].

a) Round 1

The polymerase chain reaction was performed in a 50µl mixture containing 10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% w/v gelatin, 1 unit recombinant Taq DNA polymerase (Perkin Elmer Cetus), 200µM each dNTP, 30ng of each 'outer' primer (d94 and d95) and 5µl of the cDNA solution. After an initial 5 minute denaturation at 94°C, 35 cycles of 95°C for 1.2 minutes, 56°C for 1 minute, 72°C for 1 minute were carried out, followed by a final 7 minute extension at 72°C (Techne PHC-1 Automated Thermal Cycler).

b) Round 2

The reaction mixture was as described above for Round 1 but 125ng of each 'inner' primer, N1 and N2, were used instead of the 'outer' primers d94 and d95. A 1µl aliquot of the Round 1 PCR products was transferred to the Round 2 50µl reaction mix. 25 cycles of 95°C for 1.2 minutes, 46°C for 1 minute, 72°C for 1 minute were performed followed by a 7 minute extension at 72°C.

c) Analysis

A 20µl aliquot of the Round 1 and Round 2 PCR products were analysed by electrophoresis on a 2% agarose gel. Bands were visualized by ethidium bromide staining and photographed at 302 nm.

RESULTS

Performance of the "Nested" PCR with pDX100 Plasmid DNA

The amplified products of the first and second rounds of the PCR were 729 and 402 bp respectively, as predicted from the prototype HCV sequence[9] and from the *sequence* of plasmid pDX100. Figure 2 illustrates the dramatic improvement in sensitivity obtained by performing the second round reaction, enabling a single molecule of the pDX100 plasmid to be detected by ethidium bromide staining without the use of a radioactive probe.

Predictive Value of Anti-C100 Serology and PCR in the Prospective Study

Sera from six of the 1100 donors (0.55%) enrolled into the prospective study were repeatedly reactive in the anti-C100 assay. Of these six donors only one (donor D6) proved to be infectious as judged by the development of PTNANBH and seroconversion in a recipient (recipient R6) - see Table 2. HCV sequences were detected by PCR in the serum of this donor (D6, Figure 3) but not in any of the other five anti-C100 positive donor sera. It is of interest to note that the PCR products generated by donor D6 were only rendered visible by the extra amplification provided by the second round reaction. The recipient, R6, who developed PTNANBH had also received blood from seven other donors (D7 to D13). Sera from these donors were tested and found to be both antibody negative and PCR negative

Investigation of Three Further Incidents of PTNANBH

Samples of sera which had been stored since the initial investigation of three incidents of PTNANBH which occurred in 1981, were also tested for anti-C100 and HCV sequences. In each incident, one of the donors was shown to be viraemic by PCR, (Table 3 and Figure 3). Donor D19 was implicated in two separate incidents; in the second of these incidents HCV sequences were also detected by PCR in the recipient (R9) during the acute icteric phase. Sera from both viraemic donors (D14 and D19) were found to contain anti-C100.

Duration of the Carrier State in Viraemic Donors

Donors D14 and D19 remain viraemic nine years after their implication in incidents of PTNANBH in 1981. Donor D19 was also shown by PCR to be viraemic on two separate occasions in 1982.

Surrogate Markers and Anti-C100 Titres in the Implicated Donors

Two of the three implicated donors (D6 and D14) reported here had normal ALT levels at the time of recall following their implication. The third (D19) had marginally elevated ALT levels on recall. Antibody to hepatitis B core antigen was detected in each implicated donor. On close questioning all three admitted to having used recreational intravenous drugs in the past. The titre of anti-C100 in the three viraemic donors (GMT 1:10, range 1:4 - 1:32) did not differ significantly from that observed in the non-transmitting donors (GMT 1:5 , range 1:2 - 1:8)

DISCUSSION

Although the development and availability of the test for antibody to HCV based upon the single recombinant protein C100 has considerably advanced our knowledge of the epidemiology of NANBH, more fundamental insights into the biology of HCV infection have been hampered by the lack of a practical assay for viraemia. The PCR based technique described here represents such as assay. Application of the nested primer principle[10] has enhanced sensitivity to a degree that obviates any requirement for *potentially* hazardous isotopically-labelled probes. The time consuming ultracentrifugation, blotting, washing and autoradiographic steps of the protocol recently described by Weiner *et al*[11] are also avoided. Furthermore, the procedure confers increased specificity because a product of the correct size will only be produced if four separate fluid-phase hybridisations occur.

It has been estimated[12] that transfusion-associated NANBH gives rise to approximately 75,000 cases of chronic hepatitis per year in the USA, 20% of which progress to cirrhosis. Although comparable figures are not yet available in the UK, it is generally accepted that there is a need to test donated blood for the presence of the agent or agents which cause PTNANBH.

While the development of the Ortho test for anti-C100 represents a significant advance over the use of surrogate markers such as anti-HB core and serum ALT[13], its introduction as a routine screening test for blood donors would not be achieved without creating certain problems. Firstly, from the findings of the present study it appears that only a small proportion (1 of 6, 17%) of anti-C100 positive donations constitute a risk of transmitting PTNANBH. This finding is supported by a recently published prospective study of blood donors in Amsterdam[14], in which only 17% (6 of 35) of anti-HCV positive blood products were found to be associated with

PTNANBH. Based on an anti-C100 seroprevalence of 0.55% - 0.7% (the present study and van der Poel[14]) and a figure of 2.5×10^6 donations annually in the UK, it follows that approximately 10,000 donations per year would be unnecessarily discarded. Secondly, in the absence of any confirmatory tests, counselling of the large numbers of donors found to be reactive in the anti-C100 assay would pose considerable problems, both ethical and logistic. Thirdly, the anti-C100 assay will not detect all potentially infectious donors because there is a prolonged delay (mean 22 weeks) between infection and seroconversion and because 40% of acute NANBH cases fail to develop antibody to this protein at all[13]. Finally, the financial implications of introducing the anti-C100 assay for total donor screening must be considered. At £2.50 per test the reagent costs alone would amount to £6.25 million per year and the additional costs resulting from donor counselling, withdrawal of blood and its products, replacement of donors and confirmatory testing would be considerable.

Addressing the problem of differentiating between infectious and non-infectious donors, van der Poel *et al*[14] suggest that an ELISA ratio (mean test OD/cut off OD) in excess of 2 and a raised serum ALT 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 HCV (D6, D14, D19) and those who did not, nor was there an association with elevated ALT.

What accounts for Ortho test reactivity in non-transmitting donors? There are several possible explanations. The reactivity might be due to specific "convalescent" antibody in donors who have had a self limiting HCV infection in the past. Alternatively, the reactivity might be non-specific due to antibodies directed against yeast cell contaminants of the C100 antigen preparation or due to antibodies 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 upon

recombinant proteins other than C100. The possibility that the reactivity may be false, as a result of testing stored frozen samples, is considered unlikely because the seroprevalence observed in the NLBTC prospective study (0.55%) is very similar to that reported in a recent study[15] using fresh samples.

From the longitudinal studies performed on donors D14 and D19, it appears that once established the viraemic carrier state may persist for many years. Such carriers are likely to be continuing source of transfusion-associated hepatitis and numerically may constitue a greater risk of infection than donors acutely infected with HCV. In order to minimize 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, the PCR-based technique described here represents a significant step forwards since it promises the accurate identification of the small proportion of anti-C100 positive donors who are carriers of the virus. Although the PCR assay in its present form is not suited to the mass-screening needs of a blood transfusion laboratory, recently described modifications of PCR technology[16] indicate its potential for large scale testing. Preliminary studies in this laboratory suggest that PCR may also prove invaluable in defining the time course of the viraemia in infected individuals and allow rapid diagnosis of acute hepatitis C weeks or months before diagnosis by C100 serology is possible.

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FIGURE LEGENDS

Figure 1

Title: Diagram of HCV genome showing primer locations.

Legend: Schematic illustration of the relative positions of the C100 encoding region and the pDX100 sequence on the HCV genome. The location of the PCR oligonucleotide primers within the pDX100 sequence is also shown. The heavier line towards the 5' end of the HCV genome denotes the region thought to encode structural proteins.

Figure 2

Title: Single molecule detection by "Nested" PCR.

Legend: a) Ethidium bromide staining of 1st round PCR products run on a 2% agarose gel. The number of target copies of the HCV derived pDX100 plasmid is shown above each lane. The detection limit after the 1st round is approximately 50 copies. MW = molecular weight marker, ϕx174/Hae III digest.

b) Ethidium bromide staining of 2nd round PCR products. Note that the sensitivity has been enhanced to a level that permits single copy detection of the pDX100 plasmid.

NB. The ϕx174/Hae III digest has run a shorter distance into the gel than in a).

Figure 3

Title: Detection of HCV sequences in donors implicated in PTNANBH.

Legend: The 3 lanes on the left show the 402bp 2nd round PCR products generated by sera from the three implicated donors D14, D6 and D19 (see Tables 2 and 3). The 3 lanes on the right show the 729bp 1st round PCR products from the same donors. Note that donor D6 only generates a visible signal in round 2. MW = ϕx174/Hae III digest.

Table 1

Synthetic Oligonucleotide Sequences#

Designation	Sequence	Nucleotide Positions*	Product Size
d94 sense PT**	5 'ATGGGGCAAAGGACGTCCG 3' ----------A---------	6220-6238	729bp
d95 antisense PT	5 'TACCTAGTCATAGCCTCCGTGAAG 3' -----G------------------	6949-6926	
N1 sense PT	5 'GAGGTTTTCTGCGTCCA 3' --------------T--	6339-6355	402bp
N2 antisense PT	5 'GCGATAGCCGCAGTTCT 3' ----------------	6743-6727	

Oligonucleotides prepared on an Applied Biosystems 381A DNA synthesiser.

** PT = Prototype HCV sequence[9].

* Nucleotide positions are numbered according to the prototype HCV sequence[9]. The d94, d95 and N1 sequences each differ from the prototype HCV sequence at one nucleotide position as shown. The N2 sequence is identical to that of the prototype.

Table 2

Donor/Recipient Data Summary : Prospective Study

DONORS			RECIPIENTS		
Donor	anti-C100	PCR	Recipient	PTNANBH	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*	Yes#
D10	-	-			
D11	-	-			
D12	-	-			
D13	-	-			

* Incubation period 1 month
\# Seroconversion occurred at 5 months post-transfusion

Table 3

Donor/Recipient Data Summary: 1981 PTNANBH Incidents

DONORS			RECIPIENTS		
Donor	anti-C100	PCR	Recipient	PTNANBH	anti-C100 seroconversion
D14	+	+			
D15	-	-			
D16	-	-	R7	Yes*	NA**
D17	-	-			
D18	-	-			
D19	+	+	R8	Yes#	Yes
D20	-	-			
D19	+	+			
D21	-	-	R9$	Yes	Yes##
D22	-	-			
D23	-	-			

* Incubation period 1 month
** NA = Specimen not available
\# Incubation period 7 weeks
Seroconversion occurred by 12 weeks post-transfusion
$ HCV viral sequences were detected by PCR in serum from recipient R9, taken during the acute icteric phase

Fig. 1

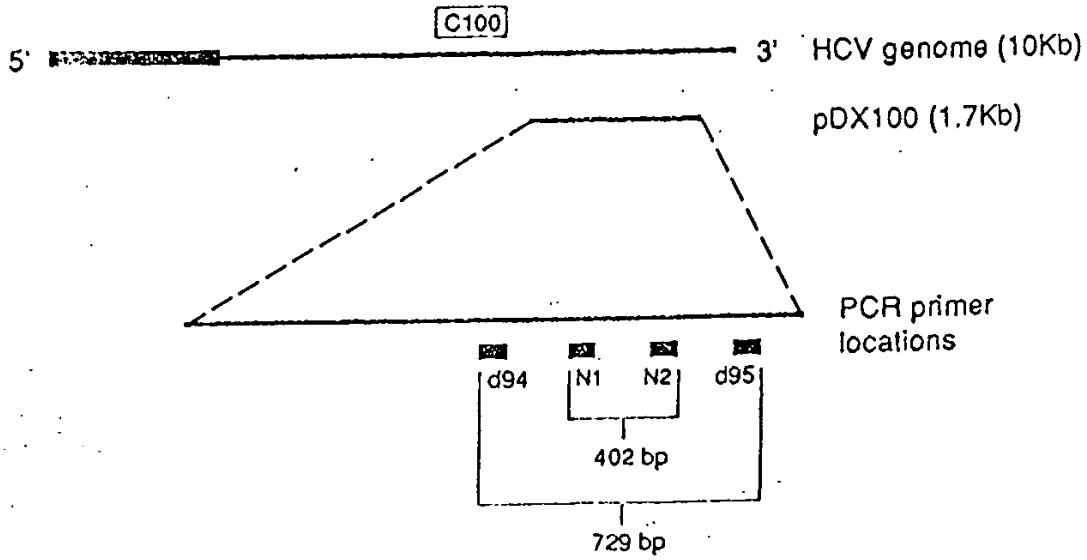
C100
5'
3'
HCV genome (10Kb)
pDX100 (1.7Kb)
PCR primer locations
d94
N1
N2
d95
402 bp
729 bp

Fig. 2

A

B

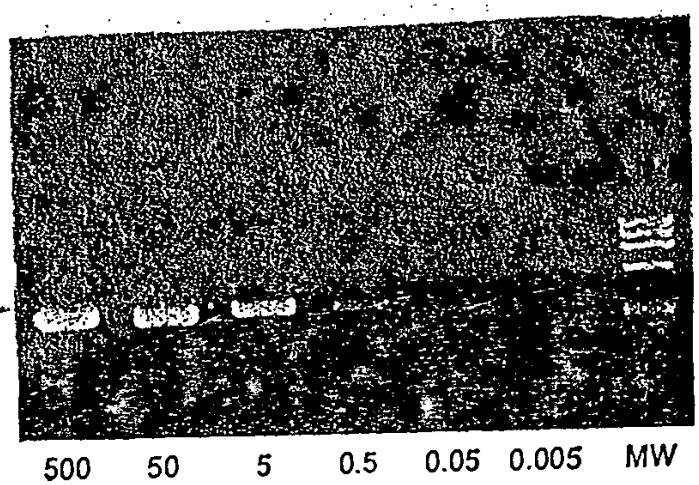

Fig. 3