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Date: 03/09/91

Number of pages (including this one): 20

GRO-C

Dear Dr. Rejman,

Dr H. Gunson (National Director, National Blood Transfusion Service) has asked me to send you a copy of the latest draft of our paper intended for publication in *Vox Sanguinis*.

I can be contacted by Fax. on GRO-C

Yours sincerely,

GRO-C

Dr. J.A. Garson.
(Senior Lecturer in Virology)

-3 SEP 1991

HEPATITIS C VIRAEMIA IN UNITED KINGDOM BLOOD DONORS: A MULTICENTRE STUDY

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Short Title: Hepatitis C Viraemia in UK blood donors.

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HEPATITIS C VIRAEMIA IN UNITED KINGDOM BLOOD DONORS: A MULTICENTRE STUDY

ABSTRACT

Of 10,633 blood donations tested in three regional blood transfusion centres with two commercial first generation screening assays for antibodies to the hepatitis C virus (HCV), 65 (0.61%) were found to be repeatedly reactive in one or both assays. Five of the 65 were confirmed positive by recombinant immunoblot assay (Ortho RIBA-2) and a further four were judged indeterminate. All five RIBA-2 positive donations and one of the four RIBA-2 indeterminates were shown to be viraemic by HCV-RNA polymerase chain reaction (PCR) assays performed at three independent reference laboratories. The remaining 56 screen test reactive donations proved negative by both RIBA-2 and PCR. We conclude that while first generation anti-HCV screening assays generate a high proportion of false reactions when screening low prevalence populations, results of the RIBA-2 confirmatory test correlate well with PCR findings and thus indirectly with both hepatitis C viraemia and infectivity.

INTRODUCTION

Following the introduction of blood donor screening for hepatitis B surface antigen in the 1970's it became apparent that most residual cases of post transfusion hepatitis (PTH) were of the non A, non-B (NANB) type. The agent responsible remained unknown until the discovery of hepatitis C virus (HCV) by Choo and colleagues [1] in 1989. Cloning and expression of a segment (designated C100) of the non-structural region of the HCV genome rapidly led to the development of commercially available assays for antibodies to the virus [2]. Although these 'first generation' assays have proved to be relatively reliable when used to test high seroprevalence groups such as haemophiliacs, their specificity in the context of low seroprevalence populations has repeatedly been questioned [3,4,5]. Despite these doubts concerning specificity, blood transfusion services in many countries have introduced the assays for screening donations.

Studies in the United Kingdom and Holland [6,7] have shown that the correlation between screen test reactivity (anti-C100) and infectivity of individual donations is poor, although better than earlier tests for "surrogate" markers of NANB hepatitis. In contrast, polymerase chain reaction (PCR) techniques [5,8] for the detection of circulating HCV-RNA have been shown to be capable of differentiating between infectious and non-infectious donations. However, PCR techniques are not currently suitable for mass donor screening and doubts have been raised concerning the reliability and reproducibility of

PCR results between different laboratories [9]. Although HCV-RNA can be detected by dot blot hybridization [10], in the absence of a culture system or assay for circulating HCV antigen, PCR is the only adequately sensitive means of direct viral detection.

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A multicentre study was set up by the National Directorate of the Blood Transfusion Service in England and Wales and the Scottish Blood Transfusion Service as a prelude to the introduction of donor screening in the U.K. Three Regional Transfusion Centres tested donations with two commercially available first generation anti-HCV assays. Donations that were repeatedly reactive in either or both assays were referred to three independent reference laboratories for confirmatory serology [11] and PCR analysis for the detection of viraemia. In this initial report, we present in detail the results of the PCR analysis from the three reference laboratories, together with an outline of the relevant serological findings. Full data from the first generation anti-HCV screening tests, the second generation screening tests subsequently performed on the same samples, and the results of further confirmatory serology, will be presented in a second report.

MATERIALS AND METHODS

Specimens

Blood donations were screened at three Regional Blood Transfusion Centres (North London, Glasgow and Newcastle) with Ortho and Abbott first generation anti-HCV tests, in accordance with the manufacturer's instructions. Aliquots of each of the repeatedly reactive samples were referred to three reference laboratories (Virus Reference Laboratory at Colindale, VRL; University College and Middlesex School of Medicine, UCMSM; Regional Virus Laboratory, Glasgow, RVL). PCR analysis was performed for RVL by the Department of Medical Microbiology, University of Edinburgh, UE.

Confirmatory Serological Assay

The referred specimens were tested with the Ortho RIBA-2 assay [11]. This assay has 4 recombinant HCV antigens, 5-1-1, C100-3, C33c and C22-3 and a specificity control antigen, superoxide dismutase, SOD. According to the manufacturer's criteria, a sample was judged "positive" if it was reactive with two or more of the HCV antigens and "indeterminate" if it reacted with one HCV antigen only.

Viraemia Detection by Polymerase Chain Reaction

In each of the three independent reference laboratories PCR was performed "blind" by operators who had no knowledge of the results of the confirmatory serological assay. Each laboratory used a different protocol as described below.

Nucleotide numbering of primers and probes are given according to the system of Kato et al [12] unless otherwise stated.

At UCMSM, the methods employed for extraction of viral RNA from plasma and for cDNA synthesis were exactly as previously described [13] but with random hexamers used for priming cDNA synthesis in place of antisense primer. Nested PCR was performed with the NS5 region primer set [5] and also with the 5' noncoding region primer set [14] as previously described.

At VRL, viral RNA was extracted from 50 µl aliquots of plasma by the method of Boom et al [15] and reverse transcribed using MuLV reverse transcriptase and random hexamer primers. PCR was performed with the following four sets of nested primers: 1) the NS3 primer set [16] with inner primers and probe developed at VRL [9]; 2) the NS3 primer set and probe [17] with inner primers 5'-AGA TGC GGT TTC GCG CACT (3089, numbered according to Chiron patent [18]) and 5'-CCA CAT TTG ATC CCA CGAT (3476, numbered according to [18]); 3) the 5' noncoding region primers [14] with the addition of 5'-AGT GGT CTG CGG AAC CGG TGA GTA CAC CGG (130) as an internal probe; 4) a novel set of 5' noncoding region primers, outer 5'-GCG ACA CTC CAC CAT AGA (7) and 5'-CAC GGT CTA CGA GAC CTC CC (325), inner 5'-GTG AGG AAC TAC TGT CTT (35) and 5'-CGC AAG CAC CCT ATC AGG CA (297) with the same internal probe as in 3) above. The specificity of the PCR products was confirmed by oligomer hybridization [19].

At UE, the RNA PCR was performed as described previously [8]. Primers for the 5' noncoding region [14] and new primers in NS3 were used. The sequence of the NS3 primers were based on comparative sequence data obtained in the NS3 region with primers ED1-4 [8]. The sequences are: ED5 (outer, 4991) 5'TCT TGA ATT TTG GGA GGG CGT CTT, ED6 (outer, 5156) 5'CTT CCA CAT CTG GTC CCA CGA TGG, ED7 (inner, 5013) 5'CAT ATA GAT GCC CAC TTC CTA TC and ED8 (inner, 5116) 5'CTA GCG CAC ACG GTG GCT TGG TA. The expected sizes of the PCR bands are 165 and 104 base pairs for the outer and inner products respectively.

RESULTS

A total of 10,633 blood donations were tested by the three (Regional) Blood Transfusion Centres. Sixty five (0.61%) were repeatedly reactive in either or both of the commercial (anti-C100) screening assays and these were referred to each of the three reference laboratories. Five of the 65 were confirmed positive on RIBA-2 testing, 4 gave an indeterminate result and the remaining 56 were RIBA-2 negative (Table 1).

The 65 referred samples were also tested by PCR for the presence of HCV-RNA. The same 6 samples were found to be PCR positive by all three reference laboratories working independently and employing different experimental protocols (Table 1). Five of the 6 PCR positive samples were RIBA-2 positive and the other was RIBA-2 indeterminate. All 6 PCR

positive samples were reactive in both the Abbott and the Ortho screening assays.

Several different sets of nested PCR primers amplifying different regions of the HCV genome were used in the study. The NS3 primers used by VRL (2 sets) detected 4 of the 6 positives whereas the NS3 primer set used by UE detected all 6. The NS5 primer set used by UCMSM detected only 3. The most consistent results were generated by the NS3 primers from UE and the 5' noncoding region primers which detected all 6 of the positives at VRL, UE and UCMSM (Table 1). In addition, a further donation GRO-A Table 1) was found to be PCR positive with the UE NS3 primer set only. Repeat testing at UE of the UE aliquot and of a separate aliquot of the same donation sent from UCMSM, confirmed the NS3 primer set result. Titration experiments demonstrated that the HCV genome titre of the sample was just at the cut-off level of the NS3 PCR assay. Sequencing studies revealed that the NS3 sequence from donation GRO-A was quite distinct from that of other HCV "isolates" present in the UE laboratory at that time. A repeat donation from the same donor given approximately nine months later proved negative by both RIBA-2 and PCR (with 5'NCR and NS3 primer sets) when tested at UCMSM and UE.

DISCUSSION

What does this study tell us about the "true" prevalence of HCV infection in the U.K. blood donor population? The 0.61% (65 of 10,633) "crude" seroprevalence observed here is consistent with a number of other reports of blood donor screening using the first generation Ortho anti-C100 ELISA [6,7]. It is apparent however that the majority (56 of 65; 86%) of screen reactive donations identified by this test were false since they showed no reactivity in the RIBA-2 assay and were negative by PCR. If the calculation is based exclusively on confirmed RIBA-2 positive donations a seroprevalence of 0.047% (5 of 10,633) is obtained. Although this figure is likely to be more accurate than the 'crude' 0.65% seroprevalence it is probably an underestimate because at least some RIBA-indeterminate donations (e.g. GRO-A of Table 1) may be from HCV-infected individuals [20]. Furthermore, the anti-C100 based first generation tests will miss those donors whose serum contains antibodies to structural HCV proteins only. Antibody assays will also fail to identify those donors in the acute phase of HCV infection prior to seroconversion. The existence of infectious but anti-C100 negative donations has been reported previously [21,22].

The pattern of RIBA-2 reactivity (C22 band only) exhibited by the PCR positive donation 247021NL, suggests that the RIBA-2 assay may not be sufficiently sensitive to confirm all genuine anti-HCV positive donations. Antibodies against C100 were

almost certainly present in this serum because it was repeatedly reactive in both the Abbott and the Ortho anti-C100 screening assays, and yet the RIBA-2 C100 band was scored as non-reactive by all three reference laboratories. Similar observations relating to inadequate sensitivity of the RIBA-2 test have been made previously [23]. RIBA-2 "indeterminate" donations, especially those with C22 only or C33 only patterns, should therefore be regarded with due caution.

The most striking finding of the present study is the clear association between RIBA-2 positivity and PCR positivity. The association implies that most if not all RIBA-2 confirmed anti-HCV positive donors are viraemic and therefore potentially infectious. This knowledge will undoubtedly facilitate the donor counselling that will be necessitated by the introduction of anti-HCV screening. A similar association between confirmed anti-HCV positivity and viraemia has also been observed in patients with community acquired chronic NANB hepatitis [24] and in patients with haemophilia [25]. If further blood donor studies confirm that RIBA-2 positivity is invariably or almost invariably accompanied by viraemia, then the requirement for PCR analysis of blood donations should decline greatly. Studies are underway to determine whether the close association between RIBA-2 positivity and viraemia also exists when donations are tested by second generation screening assays, i.e. ELISAs incorporating both structural and non-structural HCV proteins.

In view of the complexity of the technique and doubts about the feasibility of avoiding contamination, it is both remarkable and reassuring to note that the three independent reference laboratories, using different PCR protocols, agreed on the same 6 PCR positive samples from a total of 65 tested. These results suggest that PCR for HCV-RNA is a reliable and reproducible diagnostic technique particularly when primers from the highly conserved 5' noncoding region [26] are used.

However, one donation GRO-A) was found to be repeatedly PCR positive (on two separate aliquots) at UE when tested with the NS3 primer set, although, in agreement with VRL and UCMSM, it proved to be PCR negative with 5' noncoding region primers. Since the titre of HCV-RNA in this sample was just at the cut-off level of the NS3 PCR it seems likely that minor sensitivity differences between the NS3 primer set and the other primer sets may have been responsible for the discordant result. Sequence analysis of this sample appears to exclude the possibility of contamination from previously or concurrently amplified PCR products in the UE laboratory. The absence of both HCV antibody and HCV-RNA in a later donation from the same donor suggests either that the donor had a transient low level viraemia at the time of the first donation, or that the first donation had received low level contamination with HCV at the screening laboratory before or during separation of the aliquots for despatch to the reference laboratories.

Follow up studies are planned to determine whether the HCV-PCR positive donors identified here have evidence of hepatic dysfunction or evidence of other blood borne viral infections. Attempts will also be made to discover how these donors became infected with HCV and whether or not they have transmitted the infection to other family members.

We conclude that first generation anti-HCV tests generate a high proportion of false reactions when used to screen U.K. blood donations. Fortunately, the RIBA-2 confirmatory test appears to differentiate accurately between true and false reactivity as judged by the results of PCR analysis for HCV-RNA. Finally, from the data presented here it would seem prudent to regard RIBA-2 reactive donations, whether "positive" or "indeterminate", as likely to be viraemic and therefore capable of transmitting HCV infection.

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REFERENCES

1. CHOO QL, KUO G, WEINER AJ, OVERBY LR, BRADLEY DW, HOUGHTON M: Isolation af a cDNA clone derived from a blood borne non-A, non-B viral hepatitis genome. Science 1989;244:359-362.

2. KUO G, CHOO QL, ALTER HJ, GITNICK GL, REDEKER AG, PURCELL RH, MIYAMURA T, DIENSTAG JL, ALTER MJ, STEVENS CE, TEGTMEIER GE, BONINO F, COLOMBO M, LEE WS, KUO C, BERGER K, SHUSTER JR, OVERBY LR, BRADLEY DW, HOUGHTON M: An assay for circulating antibodies to a major aetiologic virus of human non-A, non-B hepatitis. Science 1989; 244:362-364.

3. CONTRERAS M, BARBARA JAJ: Screening for hepatitis C virus antibody. Lancet 1989;ii:505.

4. FLEGG PJ: Ethics of screening for hepatitis C virus. Lancet 1989;ii:1221.

5. GARSON JA, TEDDER RS, BRIGGS M, TUKE P, GLAZEBROOK JA, TRUTE A, PARKER D, BARBARA JAJ, CONTRERAS M, ALOYSIUS S: Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity Lancet 1990;335:1419-1422.

6. VAN DER POEL CL, REESINK HW, SCHAASBERG W, LEENTVAAR-KUYPERS A, BAKKER E, EXEL-OEHLERS PJ, LELIE PN: Infectivity of blood seropositive for hepatitis C virus antibodies. Lancet 1990; 335:558-560.

7. CONTRERAS M, BARBARA JAJ, ANDERSON CC, RANASINGHE E, MOORE C, BRENNAN MT, HOWELL DR, ALOYSIUS S, YARDUMIAN A: Low incidence of non-A, non-B post-transfusion hepatitis in London confirmed by hepatitis C virus serology. Lancet 1991;337:753-757.

8. SIMMONDS P, ZHANG LQ, WATSON HG, REBUS S, FERGUSON ED, BALFE P, LEADBETTER GH, YAP PL, PEUTHERER JF, LUDLAM CA: Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users. Lancet 1990;336:1469-1472.

9. CLEWLEY JP: Detection of hepatitis C virus RNA in serum. Lancet 1990;336:309-310.

10. MAENO M, KAMINAKA K, SUGIMOTO H, ESUMI M, HAYASHI N, KOMATSU K, ABE K, SEKIGUCHI S, YANO M, MIZUNO K, SHIKATA T: A cDNA clone closely associated with non-A, non-B hepatitis. Nucleic Acids Res 1990;18(9):2685-2689.

11. VAN DER POEL CL, CUYPERS HTM, REESINK HW, WEINER AJ, QUAN S, DI NELLO R, VAN BOVEN JJP, WINKEL I, MULDER-FOLKERTS D, EXEL OEHLERS PJ, SCHASSBERG W, LEENTVAAR-KUYPERS A, POLITO A, HOUGHTON M, LELIE PN: Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet 1991;337:317-319.

12. KATO N, HIJIKATA M, OOTSUYAMA Y, NAKAGAWA M, OHKOSHI S, SUGIMURA T, SHIMOTOHNO K: Molecular cloning of a human hepatitis C genome from Japanese patients with non-A, non-B hepatitis. Proc Nat Acad Sci USA 1990;87:9524-9528.

13. GARSON JA, TUKE PW, MAKRIS M, BRIGGS M, MACHIN SJ, PRESTON FE, TEDDER RS: Demonstration of viraemia patterns in haemophiliacs treated with hepatitis C virus contaminated factor VIII concentrates. Lancet 1990;336:1022-1025.

14. GARSON JA, RING C, TUKE P, TEDDER RS: Enhanced detection by PCR of hepatitis C virus RNA. Lancet 1990;336:878-879.

15. BOOM R, SOL CJA, SALIMANS MM, JANSEN CL, WERTHEIM VAN DILLEN PME, VAN DER NOORDAA J: Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990;28(3):495-503.

16. WEINER AJ, KUO G, BRADLEY DW, BONINO F, SARACCO G, LEE C, ROSENBLATT J, CHOO QL, HOUGHTON M: Detection of hepatitis C viral sequences in non-A, non-B hepatitis. Lancet 1990;335:1-3.

17. KUBO Y, TAKEUCHI K, BOONMAR S, KATAYAMA T, CHOO QL, KUO G, WEINER AJ, BRADLEY DW, HOUGHTON M, SAITO I, MIYAMURA T: A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. Nucleic Acids Res 1989;17:10367-10372.

18. Chiron Corporation: European Patent Application 88310922.5.

19. KELLOGG DE, KWOK S: Detection of human immunodeficiency virus; in INNIS MA, GELFAND DH, SNINSKY JJ, WHITE TJ (eds): PCR protocols: a guide to methods and applications. Academic Press Inc, San Diego 1990, pp 337-347.

20. WEINER AJ, TRUETT MA, ROSENBLATT J, HAN J, QUAN S, POLITO AJ, KUO G, CHOO QL, HOUGHTON M, AGIUS C, PAGE E, NELLES MJ: HCV testing in low-risk population. Lancet 1990;336:695.

21. ESTEBAN JI, GONZALEZ A, HERNANDEZ JM, VILADOMIU L, SANCHEZ C, LOPEZ-TALAVERA JC, LUCEA D, MARTIN-VEGA C, VIDAL X, ESTEBAN R, GUARDIA J: Evaluation of antibodies to hepatitis C virus in a study of transfusion-associated hepatitis. N Engl J Med 1990;323:1107-1112.

22. INABA S, FUKUDA M, OKOCHI K, IRITA Y, TOKUNAGA K, KIYOKAWA H, MAEDA Y: HCV transmission after receiving anti-c100-negative blood units. Lancet 1991;337:1354.

23. CRAXI A, FIORENTINO G, DI MARCO V, MARINO L, MAGRIN S, FABRIANO C, PAGLIARO L: Second generation tests in diagnosis of chronic hepatitis C. Lancet 1991;337:1354.

24. BRILLANTI S, GARSON JA, TUKE PW, RING C, BRIGGS M, MASCI C, MIGLIOLI M, BARBARA L, TEDDER RS: The effect of alpha interferon therapy on hepatitis C viraemia in community acquired chronic non-A, non-B hepatitis: a quantitative PCR study. J Med Virol 1991;34:136-141.

25. TEDDER RS, BRIGGS M, KING C, TUKE PW, JONES P, SAVIDGE GF, RODGERS B, GARSON JA: Hepatitis C antibody profile and viraemia prevalence in adults with severe haemophilia. Brit J Haem 1991; (in press)

26. HAN JH, SHYAMALA V, RICHMAN KH, BRAUER MJ, IRVINE B, URDEA MS, TEKAMP-OLSON P, KUO G, CHOO QL, HOUGHTON M: Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. Proc Nat Acad Sci USA 1991;88:1711-1715.

TABLE 1

PCR ANALYSIS OF RIBA-2 POSITIVE AND INDETERMINATE DONATIONS

DONATION	ORTHO RIBA-2						PCR					
							VRL		UCMSM		UE	
	5-1-1	C100	C33	C22	SOD	RESULT	NS3	5'	NS5	5'	NS3	5'
GRO-A	R	R	R	R	NR	P	P	P	P	P	P	P
	R	R	R	R	NR	P	P	P	P	P	P	P
	R	R	R	R	NR	P	P	P	P	P	P	P
	NR	R	R	R	NR	P	E	P	N	P	P	P
	R	R	R	R	NR	P	P	P	N	P	P	P
	NR	NR	NR	R	NR	I	N	P	N	P	P	P
	R[1,2]	NR	NR	NR	NR	I	N	N	N	N	N	N
	R[2]	NR	NR	NR	NR	I	N	N	N	N	N	N
	NR	R[1,2]	NR	NR	NR	I	N	NT	N	N	N	N
	NR	E	NR	NR	NR	N	N	N	N	N	P	N

R = Reactive
NR = Non-reactive
P = Positive
I = Indeterminate
N = Negative
E = Equivocal; band visible but less intense than manufacturer's recommended cut-off in the RIBA-2 test, or an ambiguous result by PCR
NT = Not tested
[1] = Result when tested at RVL
[2] = Result when tested at VRL
5' = 5' noncoding region