Journal of Viral Hepatitis, 2007, **14,** 413–418 doi:10.1111/j.1365-2893.2006.00812.x

High incidence of hepatitis C virus reinfection within a cohort of injecting drug users

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Received June 2006; accepted for publication July 2006

SUMMARY. A retrospective cohort study was established of injecting drug users (IDUs) to assess evidence for hepatitis C virus (HCV) protective immunity through a comparison of incidence of initial HCV infection and HCV reinfection. Incidence of initial HCV infection was determined among HCV seronegative IDUs, and HCV reinfection determined among IDUs with newly acquired HCV infection, HCV viraemia and subsequent HCV RNA clearance. Serum was available for HCV RNA analysis from stored samples taken at the time of prior blood-borne virus screening. Potential HCV reinfection was defined as a positive HCV RNA following at least one negative HCV RNA. Incidence of initial HCV infection was 17/100 person-years (95% CI, 14–20/100 person-years). The incidence of potential HCV reinfection was 42/100 person-years (95% CI, 25–61/100 person-years), and after excluding cases without a change in HCV genotype and less than three consecutive HCV RNA negative assessment, incidence of reinfection was 31/100 person-years (95% CI, 17–62/100 person-years). Following adjustment for HCV risk behaviour variables the incidence rate ratio of HCV reinfection to initial infection was 1.11 ($P = 0.8$). Several cases of HCV reinfection appear to have developed persistent infection.

Keywords: hepatitis C virus; injecting drug users; protective immunity; reinfection.

INTRODUCTION

Incidence of hepatitis C virus (HCV) infection among injecting drug users (IDUs) ranges from 5–40% per year [1–9]. Following acute HCV infection an estimated 25% of cases undergo viral clearance, generally within the initial 6 months of infection [10]. Factors associated with HCV clearance include female gender, ethnicity, symptomatic presentation, absence of HIV infection, rapid decline in HCV RNA, and the strength and pattern of HCV-specific CD4 and CD8 cell responses [11].

Hepatitis C virus reinfection following natural viral clearance has been demonstrated among IDUs [12,13], thalassemic children [14], and in chimpanzee studies [15]. In chimpanzees, HCV reinfection with both homologous and heterologous strains is possible [15,16]. Although HCV reinfection is consistent with a lack of complete HCV protective immunity, chimpanzee studies and a previous study among IDUs in Baltimore suggested that prior HCV infection may provide partial HCV protective immunity [12]. In the Baltimore IDU cohort, those with previous HCV infection and viral clearance had a lower risk of HCV reinfection than the risk of initial HCV infection among the HCV seronegative group. However, there have been no further studies among IDUs to confirm these findings.

In Australia, over 80% of new HCV infections are attributed to injection drug use, and estimated HCV incidence increased from 11 000 in 1997 to 16 000 in 2001 [17]. The ongoing collection and storage of sera obtained from a large cohort of IDUs attending a primary health care clinic in Sydney provided the opportunity to conduct an investigation of the risk of HCV reinfection. Specific aims were to determine the incidence and predictors of HCV reinfection and to compare these to initial HCV infection. Studies of HCV reinfection both inform HCV vaccine development and provide public health guidance for preventive strategies in at-risk populations.

Abbreviations: IDUs, injecting drug users; HCV, hepatitis C virus; KRC, Kirketon Road Centre.

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METHODS

Study participants

A retrospective cohort study was established at Kirketon Road Centre (KRC). The KRC is a government funded, primary health care facility located in inner Sydney that focuses on prevention, care and treatment of HIV and other transmissible infections among IDUs, street youth, and sex workers. Since 1991, testing for anti-HCV antibodies at the KRC has been available in the context of clinical care and is offered to all clients who report a history of injecting drug use. KRC serological testing (including for HIV, HBV and HCV) is undertaken at a single laboratory (South-Eastern Area Laboratory Service – SEALS, Prince of Wales Hospital), with results for HIV, HBV, and HCV entered on the KRC database and a serum sample stored at −20 °C at SEALS.

The KRC cohort has been previously described, including findings on HCV incidence [8,18] and viral clearance [19]. In brief, eligible KRC cohort subjects were anti-HCV antibody negative IDUs on initial testing from July 1993 who underwent repeat anti-HCV antibody testing prior to March 2002 (HCV seronegative cohort) (Fig. 1). Subjects from the HCV seronegative cohort who seroconverted to anti-HCV antibody positive within 2 years of their most recent anti-HCV antibody negative result were considered to have newly acquired HCV infection and longitudinal HCV RNA testing on stored serum was undertaken to determine viral clearance (HCV newly acquired cohort) [19] (Fig. 1).

Hepatitis C virus reinfection

To determine incidence and risk factors for HCV reinfection, subjects in the HCV newly acquired cohort with possible HCV RNA clearance (at least one negative HCV RNA following an initial HCV RNA positive result) formed a further sub-cohort (HCV clearance cohort). Potential reinfection cases were identified within this clearance cohort as having a positive HCV RNA following at least one negative HCV RNA assessment (Fig. 1).

Stored serological specimens from subjects within the HCV clearance cohort were retrieved and tested for the presence of HCV RNA. All available specimens following clearance were included. To investigate whether the reappearance of viraemia among the potential reinfection cases was reinfection or relapse from low level viraemia, genotyping was performed on the positive HCV RNA specimens either side of the negative HCV RNA specimens (i.e. before clearance and at 'reinfection').

Probable reinfection was defined as a change in HCV genotype following a negative HCV RNA or no change in genotype but at least three consecutive negative HCV RNA results.

Virological methods

The presence of HCV RNA was determined retrospectively for all specimens from the newly acquired HCV cohort by the VERSANT HCV RNA 3.0 Assay (HCV 3.0 bDNA Assay; Bayer Diagnostics, Leverkusen, Germany) [19]. Specimens that were below the assay detection limit of 3.2×10^3 copies/mL were tested for HCV RNA by nested amplification of the 5-untranslated region (5′-UTR) of the HCV genome, as described previously [20]. RT and PCR were performed in a one-step reaction and utilized the following first round oligonucleotide primer pairs [21]: KY80, sense (5′-GCAGAAAGCGTCTAGCCATGGCGT-3′) and KY78, antisense (5′-CTCGCAAGCACCCTATCAGGCAGT-3′). Second round primers [20] were: hep21b (5′-GAGTGYGTRCAGCCTCCAGG-3′) and hep22 (5′-GCRACCCAACRCTACTCGGCT-3′).

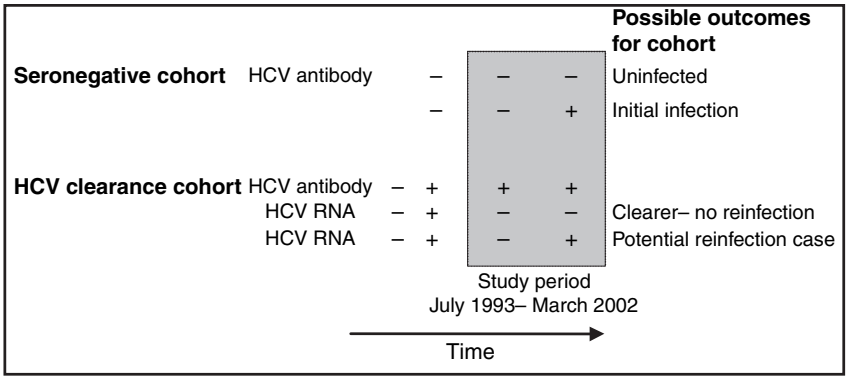

Fig. 1 Schematic outline of the seronegative cohort and hepatitis C virus (HCV) clearance cohort, and the outcomes. The HCV antibody negative cohort of injecting drug users was at risk for HCV infection during the study period (July 1993–March 2002). Initial HCV infection within this cohort was defined as HCV antibody seroconversion within the study period. The HCV clearance cohort included subjects with documented HCV RNA clearance, defined as at least one negative HCV RNA assessment following a positive HCV RNA. Potential reinfection cases were identified within this clearance cohort as having a positive HCV RNA assessment following clearance.

The standard single-letter code was used for degenerate bases: Y is C or T; and R is A or G. HCV genotyping was carried out by sequencing the 5′-UTR as described elsewhere [20].

Statistical methods

Incidence of HCV reinfection was calculated using the person-years method among subjects in the HCV clearance cohort. Person-years were calculated from the date of HCV clearance, estimated as the midpoint between the last positive HCV RNA test and first negative HCV RNA test. The censoring date was either the date of reinfection, defined as the midpoint between the last negative HCV RNA test and the subsequent positive HCV RNA test, or the last negative HCV RNA test during the study period.

Incidence of HCV reinfection was compared with incidence of initial HCV infection within the HCV seronegative cohort. Time at risk for the HCV seronegative cohort was calculated from the date of first negative anti-HCV antibody test within the study period. The censoring date was either the date of HCV infection, estimated as the midpoint between the date of the last negative antibody test and the first positive antibody test, or the date of last negative anti-HCV antibody test. We calculated incidence rate ratios to compare the incidence rate and examine predictors of initial HCV infection and HCV reinfection using the Poisson random effects model. HCV reinfection incidence was adjusted for demographic and risk behaviour characteristics, which differed between the HCV seronegative and HCV clearance cohort, including main drug injected, sharing of injecting equipment and history of incarceration. The chi-square test was used to test differences in proportions in characteristics between groups. Statistical analyses were performed using STATA (version 8.0; Stata-Corp) software. Statistical tests were two-sided. $P < 0.05$ was considered to be statistically significant. To examine any effect, the length of serum storage had on the HCV RNA viral load, linear regression was performed using GraphPad Prism (version 4.0 for Windows; GraphPad) software.

Ethical approval for the study was obtained from the South Eastern Sydney Area Health Service Research and Ethics Committee.

RESULTS

During the study period, 423 IDUs from the KRC met the inclusion criteria for the HCV seronegative cohort and were followed up for a median of 1.0 years (676 years total; range <0.1–8.6). Based on anti-HCV antibody seroconversion within a 2-year window period, 99 cases of newly acquired HCV infection occurred during follow-up and 18 subjects from this group were initially HCV viraemic with subsequent loss of HCV RNA (HCV clearance cohort). The HCV clearance cohort was followed for a median of 1.2 years (29 years total; range 0.3–3.6). Subjects in the HCV seronegative cohort were of similar age and gender as those in the HCV clearance cohort (Table 1). However, subjects in the HCV clearance cohort were more likely to report a history of incarceration (72% *vs* 31%, $P < 0.001$), sharing of injecting equipment (94% *vs* 55%, $P = 0.01$), and heroin as their major drug injected (67% *vs* 42%, $P < 0.001$).

Table 1 Characteristics of injecting drug users in the seronegative cohort compared with the clearance cohort

	Seronegative cohort ($n = 423$)	Clearance cohort ($n = 18$)	P value
Sex			
Male (%)	166 (39%)	7 (39%)	0.19
Female	253 (60%)	10 (56%)	
Transgender	4 (1%)	1 (5%)	
Age at baseline (years)			
Median	23	23	
Range	14.5–54.0	16.3–31.6	
Under 20 years	90 (21%)	4 (22%)	0.87*
20–24 years	160 (38%)	7 (39%)	
25 and over	173 (41%)	7 (39%)	
History of imprisonment			
Yes	132 (31%)	13 (72%)	<0.001
No	291 (69%)	5 (28%)	
Share injecting equipment			
Yes	234 (55%)	17 (94%)	0.001
No	189 (45%)	1 (6%)	
Main drug injected	(55 missing)		
Heroin	176 (42%)	12 (67%)	<0.001
Cocaine	50 (12%)	1 (5%)	
Methamphetamine	119 (18%)	0	
Other	23 (6%)	5 (28%)	

*P test for trend.

The incidence of initial HCV infection within the HCV seronegative cohort was 17/100 person-years (95% CI, 14–20/100 person-years), with 114 of 423 (27%) developing anti-HCV antibodies during the study period. Initial HCV infection was more likely among subjects <20 years at baseline ($P = 0.005$), <17 years at first injection ($P < 0.001$), those reporting ever sharing injecting equipment ($P < 0.001$), or reporting a history of incarceration ($P < 0.001$) (Table 2).

Of the 18 subjects with newly acquired HCV infection and HCV RNA loss, 13 had a subsequent HCV RNA positive result and were identified as potential reinfection cases. The incidence of potential HCV reinfection was 42/100 person-years (95% CI, 25–61/100 person-years). There was no significant association between potential HCV reinfection and gender, age at baseline, age at first injection, major drug injected, sharing of injecting equipment and history of incarceration (Table 2).

Table 2 Univariate predictors of initial hepatitis C virus (HCV) infection and HCV reinfection

	Initial HCV infection ($n = 114/423$)				HCV reinfection ($n = 13/18$)			
Variable	Incidence per 100 person-years (95% confidence interval)	IRR	*P* value	Overall *P* value	Incidence per 100 person-years (95% confidence interval)	IRR	*P* value	Overall *P* value
Sex							NS	
Male	19.0 (14.4–25.0)	1		0.28	42.0 (17.47–100.9)	1		0.12
Female	15.3 (11.9–19.6)	0.716	0.17		45.7 (21.77–95.79)	1.088	0.886	
Transgender	58.4 (8.2–414)	2.081	0.54		372.7 (52.5–2645.9)	8.878	0.046	
Age at baseline								
<20 years	28.8 (20.8–39.9)	1		0.005*	79.6 (25.7–246.9)	1		0.81*
20–24 years	15.1 (11.1–20.6)	0.498	0.01		34.4 (14.3–82.7)	0.433	0.251	
>25 years	13.2 (9.6–18.2)	0.431	0.003		54.2 (22.6–130.2)	0.681	0.598	
Age at 1st injection								
<17 years	33.4 (25.6–43.5)	1		<0.001*	50.4 (26.2–96.9)	1		0.83*
17–20 years	13.2 (9.2–19.0)	0.325	<0.001		32.7 (4.6–232.1)	0.649	0.682	
>21 years	10.6 (7.2–15.6)	0.270	<0.001		45.5 (14.7–141.1)	0.903	0.879	
Major drug injected								
Heroin	27.8 (22.1–35.0)	1		<0.001	38.5 (19.2–76.9)	1		0.25
Other	10.1 (7.3–14.1)	0.305	<0.001		74.6 (31.1–179.2)	1.940		
Missing	8.7 (3.9–19.4)	0.250	0.003		–			
Shared ever								
No	8.1 (5.4–12.1)	1		<0.001	232.6 (32.7–1651.6)	1		0.11
Yes	23.6 (19.2–29.1)	3.559			44.3 (25.2–78.0)	0.19		
Incarceration history								
No	11.4 (8.7–14.9)	1		<0.001	34.3 (11.1–106.3)	1		0.50
Yes	28.3 (22.1–36.4)	3.157			53.3 (28.7–99.1)	1.556		

IRR, incidence rate ratio.
**P* test for trend.

Incidence of potential HCV reinfection among subjects with HCV clearance was higher than incidence of initial HCV infection among HCV seronegative subjects, although non-significant (incidence rate ratio, 2.5; $P = 0.12$). After adjustment for major drug injected, sharing of injecting equipment and history of incarceration, the incidence rate for HCV reinfection was similar to the initial HCV infection rate (incidence rate ratio, 1.11; $P = 0.80$).

Of the 13 potential reinfection cases, 12 had serum specimens available for HCV genotyping either side of the HCV RNA negative assessment to distinguish reinfection from relapse. Five subjects (4, 6, 8, 10 and 11) had no change in HCV genotype between the two time points. The genotypes of these subjects were 3a ($n = 3$) and 1 ($n = 2$). However, seven subjects had a change in HCV genotype between their positive HCV RNA specimen before and following clearance (Fig. 2). HCV genotype changed from genotype 3a to genotype 1 in one subject (12), genotype 1 to genotype 3b in one subject (3) and genotype 1 to genotype 3a in five subjects (1, 2, 5, 7 and 9). The change in HCV genotype in these seven subjects indicates a higher likelihood of true HCV reinfection (probable reinfection). Of the five subjects with no change in genotype, two subjects had five consecutive negative HCV RNA results between viraemic episodes including them as probable reinfection cases.

Thus, HCV reinfection incidence was re-estimated including probable reinfection cases ($n = 9$), and was 31/100 person-years (95% CI, 17–62/100 person-years).

Among nine subjects with probable HCV reinfection, seven subjects had at least one further HCV RNA assessment following documented HCV RNA recurrence (Fig. 2). HCV RNA remained persistently positive in four of these subjects (1, 2, 4 and 9), including two subjects (2, 4) for four further HCV RNA assessments over a period more than 2 years.

DISCUSSION

Among a cohort of IDUs with previously documented newly acquired HCV infection and viral clearance, we found a high risk of HCV reinfection. Following adjustment for demographic and behavioural HCV risk characteristics, incidence of potential HCV reinfection was similar to incidence of initial HCV infection among seronegative IDUs. These findings argue against significant protective HCV immunity, at least for heterologous HCV strains. Persistent HCV viraemia in some cases of HCV reinfection further supports limited protective

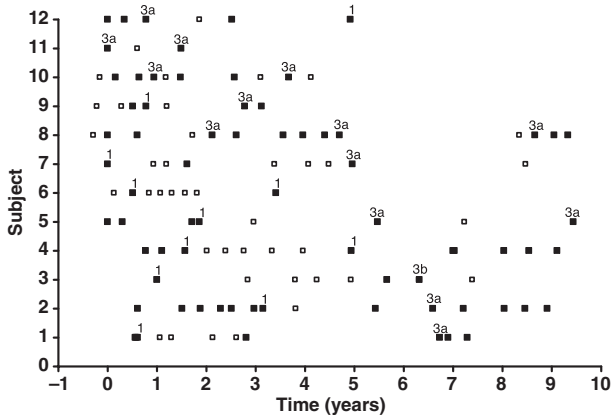

Fig. 2 Follow up of the 12 potential reinfection cases (subjects 1–12) who had positive hepatitis C virus (HCV) RNA specimens either side of the negative HCV RNA specimen from the estimated time of infection (time = 0 years). Positive (■) and negative (□) HCV RNA results of all available specimens from these subjects are shown. The HCV genotypes of specimens are above the time point examined. Nine subjects were identified as probable reinfection cases with either a change in genotype (subjects 1, 2, 3, 5, 7, 9 and 12) or at least three consecutive negative tests following initial infection (subjects 4 and 6).

immunity, and suggests that protective HCV vaccines should be based on epitopes from multiple genotypes.

Several study limitations should be considered in the interpretation of these findings. We relied on retrospective HCV RNA testing of serum specimens stored at −20 °C which is known to result in degradation and influence the stability of HCV RNA [22,23]. This may have resulted in specimens with low HCV viral load being reported as HCV RNA negative. However, a validation of HCV RNA testing performed on 22 stored serum found 95% concordance between retrospective HCV RNA testing and clinical HCV RNA results for tests carried out at the time of sampling, as detailed previously [19]. An inability to undertake further HCV genomic sequencing prevented us from examining protection against homologous HCV genotypes, and may have led to an underestimate of incidence of HCV reinfection. The retrospective study design, use of serum taken for clinic-based blood-borne virus screening, and IDU-based study population led to marked heterogeneity in terms of number and interval of longitudinal samples for evaluation. Some cases of HCV reinfection may have been undetected because of rapid HCV RNA clearance, although this would bias the study towards a lower incidence of HCV reinfection.

Important differences were evident between the HCV seronegative and HCV clearance cohort, including a higher rate of incarceration, more frequent sharing of injection equipment, and greater heroin use in the HCV clearance cohort. These factors were associated with risk of initial HCV infection, consistent with several previous studies [5,7,8,24]. Although we have adjusted for these differences between the two cohorts in estimation of our incidence rate ratio, further confounding by HCV risk behaviour is a potential explanation for the lack of demonstrated protective immunity.

Evidence for partial HCV protective immunity derives largely from chimpanzee studies, in which re-challenge with homologous HCV genotypes leads to lower level and shorter duration HCV viraemia and self-limited infection [16,25–27]. Although re-challenge with heterologous HCV genotypes is also associated with lower level HCV viraemia in chimpanzee studies, persistent infection is often documented [16]. Lower incidence of HCV reinfection compared with initial HCV infection, and lower level HCV viraemia and frequent self-limited infection in HCV reinfection cases in a prospective cohort of Baltimore IDUs provided the initial human evidence for partial protective HCV immunity [12]. Although our study demonstrated contrasting findings to Mehta *et al.* [12] in terms of relative risks of initial HCV infection *vs* HCV reinfection, other findings are similar: current IDUs are at high risk of both HCV infection and reinfection; and some cases of HCV reinfection develop persistent infection. The latter finding is important in terms of public health messages for current IDUs with documented HCV clearance. Studies of HCV reinfection also provide valuable insights in relation to HCV vaccine development. The presence of high incidence of HCV reinfection, persistent infection in some reinfection cases, including evidence from chimpanzee studies, suggests that protective HCV vaccines will need to be based on epitopes from multiple HCV genotypes. Such protective HCV vaccines are unlikely to provide sterilizing immunity, with the objective being to limit development of persistent infection.

Public health education messages for current IDUs with newly acquired HCV infection should incorporate evidence of HCV reinfection. In particular, ongoing risk of infection needs to be highlighted for those with spontaneous HCV clearance. The high incidence of HCV reinfection also indicates ongoing high levels of injecting risk behaviour and potential broader HCV spread despite HCV diagnosis and harm-reduction based counselling.

Our study highlights the challenges that need to be overcome in HCV prevention, both in terms of reduction of HCV exposure among current IDUs and development of protective HCV vaccines. Prospective studies of newly acquired HCV infection and reinfection, including virological and immunological characterization are required to further advance understanding of HCV natural history and guide prevention initiatives.

ACKNOWLEDGEMENTS

The National Centre in HIV Epidemiology and Clinical Research is funded by the Australian Government Department of Health and Ageing. Bayer Diagnostics provided quantitative hepatitis C virus RNA assays for the study.

REFERENCES

1 Crofts N, Aitken CK. Incidence of blood borne virus infection and risk behaviours in a cohort of injecting drug users in Victoria, 1990–1995. *Med J Aust* 1997; 167: 17–20.

2 Crofts N, Hopper JL, Bowden DS, Breschkin AM, Milner R, Locarnini SA. Hepatitis C virus infection among a cohort of Victorian injecting drug users. *Med J Aust* 1993; 159: 237–241.

3 Garfein RS, Doherty MC, Monterroso ER, Thomas DL, Nelson KE, Vlahov D. Prevalence and incidence of hepatitis C virus infection among young adult injection drug users. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998; 18: S11–S19.

4 Hagan H, Thiede H, Des Jarlais DC. Hepatitis C virus infection among injection drug users: survival analysis of time to seroconversion. *Epidemiology* 2004; 15: 543–549.

5 Hahn JA, Page-Shafer K, Lum PJ *et al.* Hepatitis C virus seroconversion among young injection drug users: relationships and risks. *J Infect Dis* 2002; 186: 1558–1564.

6 Miller CL, Johnston C, Spittal PM *et al.* Opportunities for prevention: hepatitis C prevalence and incidence in a cohort of young injection drug users. *Hepatology* 2002; 36: 737–742.

7 Thorpe LE, Ouellet LJ, Hershow R *et al.* Risk of hepatitis C virus infection among young adult injection drug users who share injection equipment. *Am J Epidemiol* 2002; 155: 645–653.

8 van Beek I, Dwyer R, Dore GJ, Luo K, Kaldor JM. Infection with HIV and hepatitis C virus among injecting drug users in a prevention setting: retrospective cohort study. *Br Med J* 1998; 317: 433–437.

9 Villano SA, Vlahov D, Nelson KE, Lyles CM, Cohn S, Thomas DL. Incidence and risk factors for hepatitis C among injecting drug users on Baltimore, Maryland. *J Clin Microbiol* 1997; 35: 3274–3277.

10 Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J Viral Hepat* 2006; 13: 34–41.

11 Alberti A, Boccato S, Vario A, Benvegnu L. Therapy of acute hepatitis C. *Hepatology* 2002; 36: S195–200.

12 Mehta SH, Cox A, Hoover DR *et al.* Protection against persistence of hepatitis C. *Lancet* 2002; 359: 1478–1483.

13 Proust B, Dubois F, Bacq Y *et al.* Two successive hepatitis C virus infections in an intravenous drug user. *J Clin Microbiol* 2000; 38: 3125–3127.

14 Lai ME, Mazzoleni AP, Argiolu F *et al.* Hepatitis C virus in multiple episodes of acute hepatitis in polytransfused thalassaemic children. *Lancet* 1994; 343: 388–390.

15 Farci P, Alter HJ, Govindarajan S *et al.* Lack of protective immunity against reinfection with hepatitis C virus. *Science* 1992; 258: 135–140.

16 Prince AM, Brotman B, Lee DH *et al.* Protection against chronic hepatitis C virus infection after rechallenge with homologous, but not heterologous, genotypes in a chimpanzee model. *J Infect Dis* 2005; 192: 1701–1709.

17 Law MG, Dore GJ, Bath N *et al.* Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. *Int J Epidemiol* 2003; 32: 717–724.

18 NCHECR. HIV/AIDS viral hepatitis and sexually transmitted infections in Australia Annual Surveillance Report 2005. National Centre in HIV Epidemiology and Clinical Research (NCHECR): The University of New South Wales, Sydney, NSW.

19 Jauncey M, Micallef JM, Gilmour S *et al.* Clearance of hepatitis C virus after newly acquired infection in injection drug users. *J Infect Dis* 2004; 190: 1270–1274.

20 White PA, Zhai X, Carter I, Zhao Y, Rawlinson WD. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. *J Clin Microbiol* 2000; 38: 477–482.

21 Young KK, Resnick RM, Myers TW. Detection of hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. *J Clin Microbiol* 1993; 31: 882–886.

22 Halfon P, Khiri H, Gerolami V *et al.* Impact of various handling and storage conditions on quantitative detection of hepatitis C virus RNA. *J Hepatol* 1996; 25: 307–311.

23 Lagging LM, Garcia CE, Westin J *et al.* Comparison of serum hepatitis C virus RNA and core antigen concentrations and determination of whether levels are associated with liver histology or affected by specimen storage time. *J Clin Microbiol* 2002; 40: 4224–4229.

24 Villano SA, Vlahov D, Nelson KE, Cohn S, Thomas DL. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. *Hepatology* 1999; 29: 908–914.

25 Nascimbeni M, Mizukoshi E, Bosmann M *et al.* Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. *J Virol* 2003; 77: 4781–4793.

26 Major ME, Mihalik K, Puig M *et al.* Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. *J Virol* 2002; 76: 6586–6595.

27 Weiner AJ, Paliard X, Selby MJ *et al.* Intrahepatic genetic inoculation of hepatitis C virus RNA confers cross-protective immunity. *J Virol* 2001; 75: 7142–7148.