Antiviral Therapy 2012; **17:**1415–1422 (doi: 10.3851/IMP2472)

Review

The challenges of hepatitis C diagnosis and the potential role of Stimmunology™ to address them

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The diagnosis of HCV infection is hindered by the long seronegative window period, the high rate of false-positives and the need to differentiate between current and cleared infection. Stimmunology™ is a technology by which antibody production can be stimulated, even in a whole blood sample, *in vitro*. Such stimulation leads to an increase in HCV antibody levels in the blood sample, enabling the detection of HCV infection prior to seroconversion. This increase in the levels of the HCV antibodies, which can be achieved within days of infection, practically resolves the window period problem. The detection of the infection, even at its seronegative stage, translates to increased diagnostic sensitivity and the concomitant dilution of 'noise' in the sample leads to a >96% reduction in the false-positive rate. The stimulation step acts upon HCV-primed lymphocytes in the blood sample; therefore, only in the presence of infection would the increased antibody levels be detected, thus differentiating between current and cleared infection. Clinical diagnostic data have been collected to provide insight as to how the diagnosis of HCV infection may be improved using this technology.

Challenges in the diagnosis of HCV infection

The hepatitis C epidemic is not a new one, yet it has been ignored for some time. Although HIV has been the epidemic dealt with on global and national levels, the silent epidemic of hepatitis C [1–3], which kills more people than AIDS, is now rising in the public's awareness [4–6]. Such awareness is fostered by several factors such as HIV–HCV-coinfection [7–9], the spread of HCV among HIV-monoinfected people [10–12] and among other at risk populations, and the recent availability of new drugs for treating hepatitis C [13,14]. These drugs, and hopefully others in the pipeline, have lower toxicity and a shorter duration of treatment [15,16], thus making the argument for early treatment [17–19] stronger, thereby offering potential for, not only a curtailing of the epidemic, but also an eradication of it. We have gained much insight into the combat against blood-borne diseases while working with HIV, and the lessons learnt there should be applicable to HCV with regard to treatment and the successes achieved, providing impetus to identify carriers of infection with a view to offering them antiviral treatment [20–22]. The HCV epidemic presents us with new and different challenges regarding diagnosis, which in turn affect treatment decisions.

Long seronegative window period

Unlike most infections, antibodies against HCV do not appear within 7–10 days of the infection. This seronegative window period can last several months [20,23,24] and thus a negative antibody test does not necessarily suggest no HCV infection. Since the virus resides mainly in the liver, its absence from the blood, at detectable levels, also does not connote a clean bill of health [25–27]. In individuals with immune suppression (for example, pregnant women, men who have sex with men, haemodialysis patients), the window period is even longer [28–30]. The current tests for HCV antibodies detect only immunoglobulin G, which delays the diagnosis even further [31,32], requiring repeat testing several weeks or months later [33]. Resolving the problem of the long seronegative window period is important for tracking a potential HCV outbreak. This seronegative window period is an immunological enigma as HCV antigens are

very immunogenic, and antibodies should have been detectable within days of infection.

High (and variable) rates of false-positive results

Current HCV diagnostic assays have relatively high rates of false-positivity [34–36], the extent of which varies from population to population, and from country to country. This is a major concern as it means, on the epidemiological level, that we would be over-estimating prevalence (and in a varying, unknown degree) in different populations around the world. On another level, the low specificity of the antibody assays affects the blood supply, as it causes a loss of safe blood, with temporary deferral of the donor leading to additional potential loss of blood donations.

Lack of differentiation between current and resolved infection

This is important in HCV infection as 15–25% of new infections spontaneously resolve [37–41]. Those who resolve the infection may still test positive in the antibody assays for many years [42,43] following clearance of the virus [44]. Using molecular assays for the detection of viral genome offers only a partial solution to identification of resolution [43] as the lack of detection of the HCV viral genome in measurable levels in the blood is not a clear indication of the state of the infection in the liver [25,27]. Thus, current assays do not provide a clear diagnosis of current HCV infection.

Stimmunology as proposed solution to the challenges of hepatitis C diagnosis

We propose a different approach for improving HCV-antibody-based diagnosis, so as to meet the challenges outlined above. The approach is based on examination of, not only the levels of HCV antibodies in plasma, but also levels produced *in vitro*, in a whole blood sample, thus measuring not only what antibody has been produced sometime in the past but also the current capability and potential for antibody production at the time of testing. Stimmunology™ is a technology that enables the production of antibodies, in a whole blood sample, even in the face of peripheral immune suppression. Overcoming the suppression in a whole blood sample holds the key for closing the window period of the HCV infection. The ability to drive forward HCV antibody production in a whole blood sample is dependent on the presence of HCV-primed B-cells in the blood sample. Such primed B-cells would be present in the blood within days of infection, and newly produced HCV-naive B-cells will be primed by HCV viral antigens for as long as the viral infection will persist [9,45]. Once the infection is cleared (spontaneously, or following antiviral treatment), there will be no further priming of HCV B-cells (or T-cells). At that time, while the already produced antibody levels in the blood will remain high for years, the ability to enhance further antibody production *in vitro* by newly primed B-cells would be gone within days.

The application of Stimmunology for the detection and diagnosis of HCV infection is the SMARTube™. By incubating a small volume of blood (1 ml) in the SMARTube, for several days, antibodies could be produced to detectable levels, even prior to seroconversion [46–48]. This means that very early infection can be detected using currently available diagnostic kits by using SMARTplasma™ (the plasma-supernatant in the SMARTube following incubation of blood in the SMARTube) instead of standard plasma obtained from unstimulated blood.

In essence, a whole-blood sample is collected in an anticoagulant, and then sent to the laboratory. A total of 1 ml of blood is transferred to the SMARTube and incubated for 3–5 days at 37°C with 5% CO_2. During incubation, the red cells in the blood sample settle at the bottom of the SMARTube, and the mononuclear cells settle above them, exposing lymphocytes to the stimulating culture media in the SMARTube. The stimulated lymphocytes lead to antibody production by the B-cells which have been primed *in vivo*. These antibodies are secreted into the culture supernatant fluid (SMARTplasma), which now contains both the original plasma antibodies, and additional antibodies produced during incubation. The SMARTplasma is collected from the top of the SMARTube for testing (and for storage for future uses). The antibody levels found in SMARTplasma can be high enough to reach detectable levels, even in samples from infected individuals who are still in the seronegative window period.

Using the SMARTube as a pre-analytic device can thus overcome the challenge of the long seronegative window period during HCV infection. The ability to detect very early infection, independently from where the virus is residing at those early stages, and without having to wait for the production of antibodies *in vivo* to reach detectable levels, would be important for curtailing the spread of HCV. A tool which enables early detection of HCV infection also could provide an opportunity for early treatment, at stages which might require, in the future, lower doses, and shorter treatment duration (maybe even with monotherapy).

Field validation of sensitivity and specificity

Clinical laboratory trials have been conducted in several countries to determine how the seronegative window period may be shortened through the use of the SMARTube [49]. Among blood samples from 2,722 high-risk individuals originating from populations representing

different regions of the world, there were 252 people found to be seropositive as determined by regular serology. All those 252 and 13 additional samples were antibody-positive using the same diagnostic kits and confirmation algorithms after pre-treatment in the SMARTube. Thus, the use of SMARTplasma for testing increased diagnostic sensitivity by 5.2%. It is important to note that these additional positive individuals were at the early stages of infection.

In a diagnostic assay, increased diagnostic sensitivity usually comes at the cost of reduced specificity, and vice versa. Since the incubation step in the SMARTube increases the levels of antibodies in the sample and thus the signal measured/seen in the diagnostic kit, it is necessary to evaluate its effect on the specificity of anti-HCV antibody detection. During testing for hepatitis C in routine settings, 3,840 blood samples were tested using both plasma and SMARTplasma. Of these, 31 were plasma-positive at initial screening, and only 4 of them were also positive in their corresponding SMARTplasma. The other 27 tested negative in SMARTplasma; as their plasma tested negative upon repeat testing, that is, their reactivity found at initial testing, was false-positive. Thus, not only was no adverse effect on specificity observed, but increased diagnostic specificity was achieved, with a 96% reduction in the false-positive rate when using the SMARTube. This observation is probably due to the fact that while the SMARTube process increases the levels of the specific HCV antibodies (and thus the 'signal'), it reduces the 'noise' by the 1:4 dilution of the plasma in the SMARTube. This dilution of plasma does not affect the ability to detect even early stages of seroconversion, when the antibody levels in plasma are low, as there is an increase in antibody levels following the stimulation step. To further support this point, it has been shown that persons sampled in the seronegative window period, with antibodies below detection limits of the assay in the plasma, have detectable levels in the SMARTplasma following the activation of antibody production *in vitro*.

The stimulation index using the SMARTube for more informed diagnosis

Based on the concepts outlined, a model was developed for distinguishing current infection from a cleared one. In this model, the levels of antibodies are measured in both plasma and SMARTplasma. Figure 1A is a heuristic graph depicting the expected change in antibody levels in both types of samples over time during the course of an HCV infection. As can be seen from this graph, the reduction in antibody levels in SMARTplasma happens shortly after clearance of the HCV infection. This is due to the fact that the level of antibodies in the SMARTplasma is dependent on both the antibodies already present in the blood, and the newly produced antibodies, in culture, by HCV-primed B-cells. With primed cells gone from the blood shortly after the clearance of the virus from the circulation, *in vitro* production ceases too. Thus, the decrease of antibody levels in SMARTplasma precedes the one observed in the plasma. However, if the HCV infection is chronic, there is a small set of newly primed B-cells in the blood, which maintains antibody levels in plasma and, following stimulation in culture, produces antibody into SMARTplasma. Based on these two different patterns, the ratio between the antibody levels in the SMARTplasma and the antibody levels in the plasma, termed stimulation index (SI), differs according to the stage of infection. In Figure 1B, arrows have been added onto the previous graph to highlight the comparative levels of antibodies in SMARTplasma and in plasma (and thus the SI values). As can be seen there are four different possible SI values, and each one could serve as an indicator of a different stage in the HCV infection:

SI=∞ indicates very early infection, when antibodies in the SMARTplasma appear prior to seroconversion. Dividing a positive reading of the SMARTplasma by zero, as the plasma is negative for HCV antibodies, yields an infinitesimal (∞) value.

SI>>1 indicates recent seroconversion due to HCV infection. The antibody production *in vivo* has not yet reached its full capacity, and thus it can be further stimulated *in vitro*. This leads to higher levels of antibodies in SMARTplasma than in the concomitant plasma.

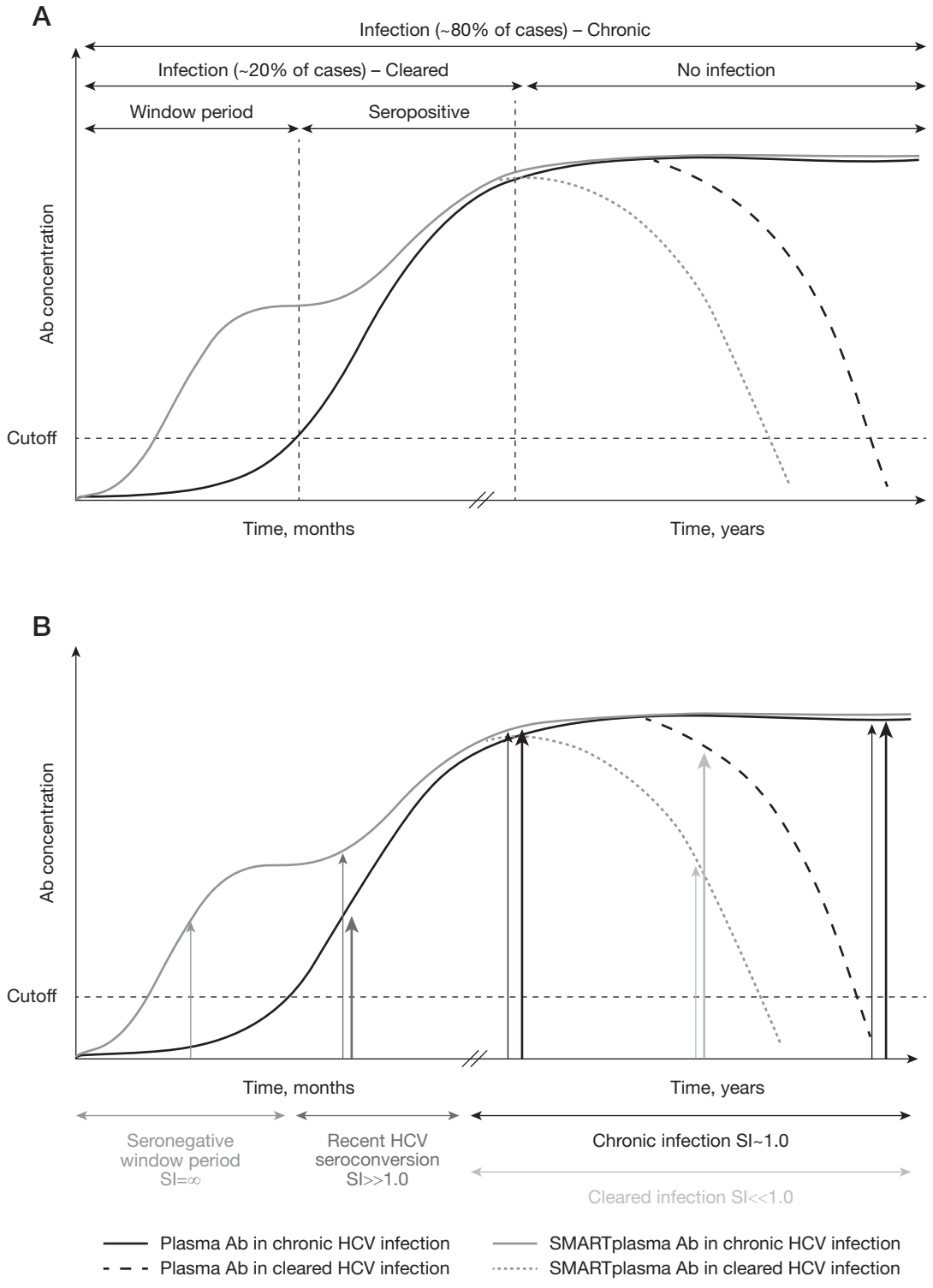
SI~=1 indicates long term infection which has not cleared. The antibody production *in vivo* is at its full capacity, and no further stimulation is detected in the SMARTplasma.

SI<<1 indicates cleared infection. Although there are still high levels of antibodies in the plasma, there is no further priming of B-cells, and thus no production of antibodies *in vitro*. This leads to a decrease in antibody levels in SMARTplasma following the incubation, when compared to the level in the concomitant plasma.

Clinical laboratory results analysed with SI

A clinical study with the SMARTube was based on 500 consecutive blood samples referred to the clinical laboratory in Hacettepe University, Ankara, Turkey, for HCV testing [50]. From each sample, 1 ml of blood was transferred to the SMARTube which was then incubated. HCV antibodies in the sample plasma and the post-incubation SMARTplasma were tested using the same assay for immunoglobulin G anti-HCV (Diagnostik Bioprobes, Milan, Italy). Plasma in 29 of the 500 samples was positive. Of these, 22 of their corresponding SMARTplasma tested positive. Further

Figure 1. A heuristic graph, depicting the expected change in the SI with the SMARTube, over the course of the HCV infection



(A&B) The SMARTube stimulation index (SI) in different stages of HCV infection. The curves are drawn based on expected SI values. Time, months, estimated to be 4–8 months. Time to loss of HCV antibodies (Ab), following spontaneous clearance of virus, has been reported to be 4–12 years. The arrows in (B) point to the level of Ab in plasma (bold arrows) and in SMARTplasma (non-bold arrows) at a given time point; the difference in their height represents the SI values at that stage of the infection.

Table 1. Testing for HCV antibodies using both plasma and SMARTplasma from a field sample

Patient number	Standard plasma anti-HCV signal/ cutoff value	SMARTplasma anti-HCV signal/ cutoff value	Anti-HCV immunoblot reactivity	HCV RNA detectability	Stimulation index	Stage of HCV infection as inferred from the stimulation index
9227	9.53	3.48	-	-	0.37	Cleared infection ?
9226	10.29	4.4	-	-	0.43	Cleared infection ?
5228	14.16	13.97	+	-	0.99	Long-term infection
0074	13.35	7.91	+	+	0.59	?
5493	11.15	8.95	+	-	0.80	Cleared infection
9137	9.16	9.16	NA	NA	1.00	Long-term infection
5277	13.58	13.41	NA	NA	0.99	Long-term infection
0434	12.97	13.76	+	+	1.06	Long-term infection
0247	15.28	10.11	+	+	0.66	?
9228	13.27	9.93	+	+	0.75	Long-term infection
7815	11.85	6.05	+	-[a]	0.51	Cleared infection
0225	11.82	15.47	+	-[a]	1.31	Recent infection
9337	10.37	3.79	+	-	0.37	Cleared infection
8211	14.41	14.65	+	+	1.02	Long-term infection
1593	13.24	15.93	+	-[a]	1.20	Recent infection
6479	13.47	14.48	+	+	1.07	Long-term infection
9087	12.34	14.34	+	-[a]	1.16	Long-term infection
1656	12.84	13.93	+	-[a]	1.08	Long-term infection
5288	14.09	14.12	+	NT	1.00	Long-term infection
9848	7.22	13.38	+	+	1.85	Recent infection
0452	12.41	12.14	+	+	0.98	Long-term infection
5171	12.36	12.36	+	+	1.00	Long-term infection

[a]HCV RNA tested positive in a blood sample taken in the past. NA, data not available; NT, not tested; ?, needs further investigation.

testing revealed that anti-HCV reactivity in plasma of the seven plasma-positive/SMARTplasma-negative samples were falsely positive. Further examination of the data, taking into consideration the SI, showed that the 22 truly-positive samples originated from people at different stages of HCV infection (Table 1). Most (12) of the samples had SI=1 (±0.1), connoting chronic infection. Five had values <1, connoting cleared infection. Two additional samples had virus detected (marked '?' in the interpretation column of Table 1); their status needs to be further investigated. Three of the seropositives had SI values >1, indicating recent infection.

Overall data from this study are summarized in Figure 2, which shows how the resolution of hepatitis C endemicity may be refined as antibody-positive individuals segmented based on SI values. Testing of SMARTplasma from cultured cells of the whole-blood sample enabled infections, even prior to seroconversion, to be detectable using a currently available HCV diagnostic assay. Plasma kept from the unstimulated blood samples were then tested for HCV antibody. Testing of SMARTplasma and plasma enabled identification of early (seronegative) infections. Thus, using the SMARTube for early detection of antibodies (and thus infection), and for calculating the SI value, led to an informative epidemiological picture of the character of HCV endemicity in the population studied (Figure 2).

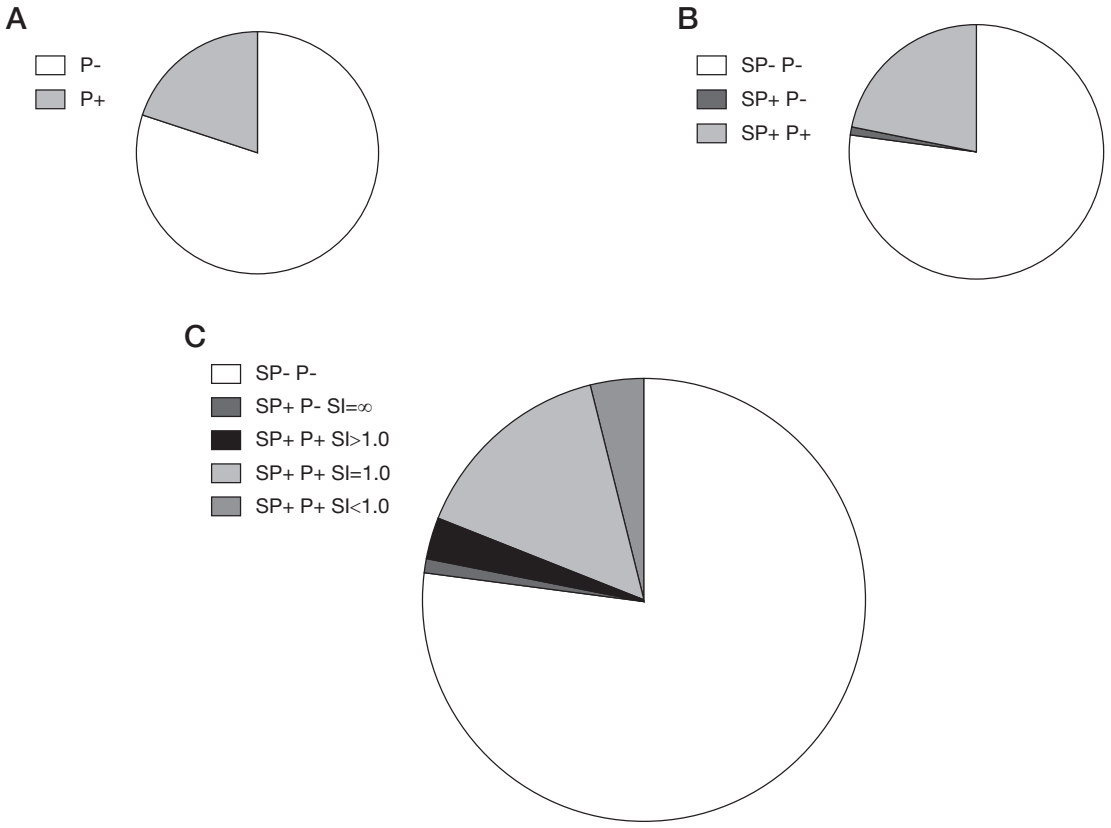
The future

Further research using samples from individuals at known stages of the infection should be carried out to substantiate further the efficacy of the approach to hepatitis C diagnosis as proposed here. It is important to note that in order to evaluate the SMARTube's contribution to hepatitis C diagnosis (including staging), the SMARTube should be incorporated into existing, running studies in both high-risk groups and in people who are known to be seropositive with a recorded history of HCV infection; this is due to the fact that the SMARTube requires a fresh blood sample (within 24 h, kept at room temperature). With additional data collected and analysed, the potential role of the SMARTube and the SI for surveillance of HCV epidemic infection in different high risk populations and for investigating HCV outbreaks can be realized.

Acknowledgements

We are extremely grateful for the professional, scientific and technical help of Svetlana Gorodin.

Figure 2. An epidemiological picture of the HCV epidemic in a clinical hospital setting, as derived by using the pre-analytic step in the SMARTube for both early HCV diagnosis, and for calculating the SI of HCV antibody levels, using current EIA antibody assays



(A) Seroprevalence, based on testing only the plasma for HCV antibodies. **(B)** Seroprevalence, based on testing the SMARTplasma for HCV antibodies. Those in the seronegative window period were also antibody-positive, providing a more true measure of prevalence. Testing the plasma of all the antibody-positive samples enables differentiation between those in the window period and those who have seroconverted already. **(C)** The distribution of the stimulation index (SI; that is, the ratio of the HCV antibody levels in SMARTplasma and those in plasma) among those who tested positive for HCV antibodies in the SMARTplasma. Those in the seronegative window period have SI=∞ as their positive optical density (OD) readings are divided by zero, as the plasma is still negative for antibodies (SP+ P-). Those positive in both SMARTplasma and plasma (SP+ P+) are divided to three groups according to their SI values. SI>1.0 indicates a recent infection, SI=1.0 indicates a long term chronic infection, and SI<1.0 indicates a cleared infection. See text for additional information. P-, plasma negative for HCV antibodies; P+, plasma positive for HCV antibodies; SP-, SMARTplasma negative for HCV antibodies; SP+, SMARTplasma positive for HCV antibodies. Both plasma and SMARTplasma were tested for HCV antibodies using Ortho HCV antibody test. The ratio of ODs was recorded as the SI.

Disclosure statement

TJ-C has shares in SMART Biotech Ltd. SU declares no competing interests.

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Accepted 23 May 2012; published online 7 December 2012