MAJOR ARTICLE

Survival of Hepatitis C Virus in Syringes: Implication for Transmission among Injection Drug Users

Elijah Paintsil,[1,2] Huijie He,[3] Christopher Peters,[4] Brett D. Lindenbach,[4] and Robert Heimer[3]

Departments of [1]Pediatrics, [2]Pharmacology, [3]Epidemiology and Public Health, and [4]Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut

(See the editorial commentary by Rich and Taylor, on pages XXX–XXX.)

Background. We hypothesized that the high prevalence of hepatitis C virus (HCV) among injection drug users might be due to prolonged virus survival in contaminated syringes.

Methods. We developed a microculture assay to examine the viability of HCV. Syringes were loaded with blood spiked with HCV reporter virus (Jc1/GLuc2A) to simulate 2 scenarios of residual volumes: low void volume (2 μL) for 1-mL insulin syringes and high void volume (32 μL) for 1-mL tuberculin syringes. Syringes were stored at 4°C, 22°C, and 37°C for up to 63 days before testing for HCV infectivity by using luciferase activity.

Results. The virus decay rate was biphasic ($t_{1/2}\alpha = 0.4$ h and $t_{1/2}\beta = 28$ h). Insulin syringes failed to yield viable HCV beyond day 1 at all storage temperatures except 4°, in which 5% of syringes yielded viable virus on day 7. Tuberculin syringes yielded viable virus from 96%, 71%, and 52% of syringes after storage at 4°, 22°, and 37° for 7 days, respectively, and yielded viable virus up to day 63.

Conclusions. The high prevalence of HCV among injection drug users may be partly due to the resilience of the virus and the syringe type. Our findings may be used to guide prevention strategies.

The global burden of morbidity and mortality from hepatitis C virus (HCV) infection is truly pandemic [1]. There is no vaccine for the prevention of HCV infection, and current therapeutic regimens for HCV infection are limited by efficacy, cost, and treatment adverse effects. Therefore, reduction of risk associated with HCV transmission remains the primary strategy for curbing the HCV epidemic. HCV is transmitted primarily through percutaneous exposure to blood contaminated with HCV. The prevalence of HCV is disproportionately high among injection drug users (IDUs), with seroprevalence as high as 95% [2–11]. The transmission of HCV and human immunodeficiency virus (HIV) among IDUs has been associated with the sharing of equipment used to prepare and administer drugs [12–14]. The prevalence of HCV among IDUs exceeds that of HIV across all seroprevalence studies in many countries. Even in locations in the United States where HIV seroprevalence among IDUs is low (1%–10%), HCV seroprevalence among IDUs is high (30%–85%) [15–18]

HCV incident infections continue to occur at a startling high rate in IDU populations worldwide. It is estimated that the probability of transmission of HCV per exposure to a contaminated syringe is 5-fold to 20-fold higher than that of HIV transmission [19–23]. Although harm reduction programs have effectively reduced the incidence of HIV among IDUs, such reductions in incidence have rarely been observed for HCV [8, 24–26]. The difference in transmission be-

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Reprints or correspondence: Dr Elijah Paintsil, Departments of Pediatrics and Pharmacology, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06520 (elijah.paintsil@yale.edu).

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tween HCV and HIV may be attributed to a higher infectivity of HCV compared with HIV. The biology of HCV transmission, however, has not been well characterized because of the lack of an efficient cell culture and small animal model for assessing HCV replication and infectivity. To date, polymerase chain reaction (PCR)–based assay for detecting viral RNA has been used as a surrogate for infectivity; there is no direct correlation between nucleic acid concentration and viable virus [27].

We hypothesized that the efficient transmission of HCV among IDUs may be partly due to the ability of the virus to remain viable in contaminated syringes for prolonged periods. To test this hypothesis, we developed a microculture assay that allowed us to propagate HCV from small residual volumes contained in the dead space of syringes used by IDUs, and to determine the effects of storage at different temperatures for prolonged periods on the viability of HCV in syringes. We report the results of the first study, to our knowledge, that simulates HCV transmission among IDUs by directly assaying HCV infectivity in syringes.

MATERIALS AND METHODS

Virus and cells. The construction of the Jc1/GLuc2A reporter virus was similar to that of J6/JFH(p7-Rluc2A) [28] and has been reported elsewhere [29]. Jc1/GLuc2A is a derivative of the chimeric genotype 2a FL-J6/JFH [30, 31], with a luciferase gene from *Gaussia princeps* inserted between the p7 and NS2 genes. Viral stocks of Jc1/GLuc2A reporter virus were prepared by RNA transfection of Huh-7.5 cells. Four days after transfection, the viral culture media was harvested, clarified by centrifugation at 2000*g* for 10 min, filtered through 0.2-μm pore size filters, and stored at −80°C until use. The titer of HCV was quantified by infecting cells with serial dilutions of the stock virus and determining the dilution that will infect 50% (median tissue culture infective dose [$TCID_{50}$]) of the wells by using the method of Reed and Muench [32].

Highly permissive human heptoma cells (Huh-7.5 subline) [33] were maintained as subconfluent, adherent monolayers in Dulbecco modified Eagle medium, supplemented with 10% heat-inactivated fetal calf serum and 1 mmol/L nonessential amino acids (Invitrogen) at 37°C and 5% CO_2.

Establishing a standard curve. To test the range of the assay sensitivity, we introduced serial dilutions of HCV into a culture system that included virus and target cells in 96-well plates. The day before the experiment, 96-well plates were seeded with 6.4×10^3 Huh-7 cells/well in 100 μL of medium and incubated at 37°C in 5% CO_2. On the day of the experiment, HCV stocks used for the experiment were mixed with ethylenediaminetetraacetic acid (EDTA)-anticoagualted blood from an HIV and HCV seronegative donor at a ratio of 1:10. Serial 1:2 dilutions of the HCV were made in triplicate. The medium from the wells was gently aspirated from the cells and replaced with 100 μL of the HCV-blood mixture. After 4 h of incubation, the cells were washed with sterile phosphate-buffered saline to remove the input virus, and fresh medium was added and incubated for 3 days. After 3 days, culture supernatant was harvested and mixed with 20 μL of lysis buffer before luciferase activity was measured using luciferase assay reagent kit (Promega) and a luminometer (FARCyte; Amersham Biosciences). The relative luciferase activity was determined as a function of HCV infectivity and plotted against concentration of HCV ($TCID_{50}$/mL) on a logarithmic scale. The set of experiments was performed on 2 separate occasions, and the data were combined for analysis.

HCV decay assay. To investigate the rate of decay of the infectivity of the stock Jc1/Glu2 virus, several 100 μL aliquots of the virus were stored for 0–96 h at room temperature. Aliquots were removed every 6 h or less and stored at −80°C. The stored aliquots were thawed and used to infect the Huh-7.5 cells. The relative infectivity was determined by measuring the luciferase activity after 3 days of infection as described above.

Syringes. The types of syringes used by IDUs vary. The residual volume that remains in a syringe after injection depends on the size and design of the syringe [34]. For syringes with fixed needles, the void volume (ie, dead-space fluid) remains in the tip of the syringe, at the base of the plunger, and in the needle itself when the plunger is fully depressed; these syringes generally have a low void volume. Syringes with detachable needles retain fluid in the hub of the syringe, as well as at the base of the plunger and in the needle; these syringes generally have a high void volume. We used 2 different kinds of syringes in our experiments: the low void volume U-100 1-mL insulin syringe with an attached 27-gauge, 0.5-inch needle and the high void volume 1-mL tuberculin syringe, with a detachable 26-gauge, 0.5-inch needle. The average void volumes after complete depression of the plunger for insulin and tuberculin syringes have been reported elsewhere as 2 and 32 μL, respectively [35, 36]

Viability of HCV in stored syringes. Syringes were loaded with HCV-spiked blood to replicate the practice of "booting" by IDUs [37, 38]. The usual intravenous injection sequence includes properly registering the syringe in the vein, in which visible blood is drawn into the syringe, then injecting the drug, which leaves a void volume of drug solution mixed with some blood. However, many injectors pull up on the plunger a second time, introducing blood into the barrel of the syringe that mixes with the remaining drug solution. This "booted" material is reinjected, leaving within the syringe a void volume that is predominantly blood. In our experiments, we chose to simulate the practice of booting because it constituted the worst-case scenario that maximizes the amount of HCV-contaminated blood.

After preparing syringes to replicate booting, they were either

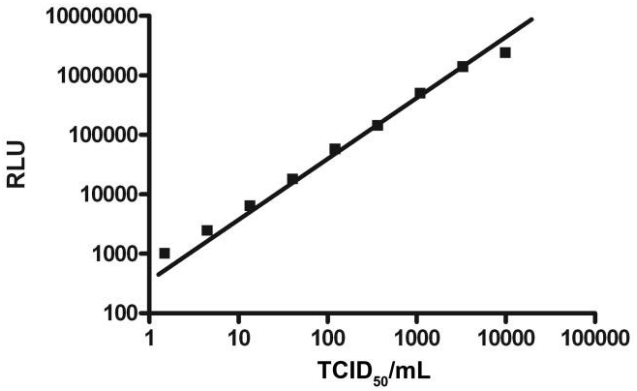

Figure 1. Linear dynamic range of microculture assay. Aliquots (100 μL) of serial 1:2 dilutions of stock virus were used to infect Huh 7.5 cells. After 3 days of incubation, the culture supernatant was harvested and the concentration of virus determined as a function of relative luciferase activity. The relative luciferase units (RLU) were plotted as a function of the concentration of input virus (median tissue culture infective dose [$TCID_{50}$]/mL) on a logarithmic scale. The experiment was performed on 2 separate occasions in triplicate, and the data were combined for analysis.

immediately tested for viable virus or stored at 4°C, 22°C, and 37°C for up to 63 days before testing. To test syringes, they were flushed with 100 μL of culture medium, which was introduced into Huh-7.5 cells in a 96-well plate and incubated for 3 days. After 3 days, culture supernatant was analyzed for relative luciferase activity as described above.

The range of different storage temperatures has been used in previous HIV studies, and they reflect different ambient temperatures in which injection practices may occur [36]. The storage duration captured the time used syringes remain in circulation in the absence of a syringe exchange program as was measured in New Haven, Connecticut (mean duration, 23.5 days) [26, 39].

Data analysis. The regression analysis for the standard curve comparing infectivity and relative luciferase activity and the exponential decay analysis were determined using GraphPad Prism program (version 4.0; GraphPad Software Inc). The relative luciferase activity and $TCID_{50}$ were plotted on a logarithmic scale; time of storage was plotted on a linear scale. For determining the decay rate, the slope was used to calculate $t_{1/2}$ values.

RESULTS

HCV microculture assay. We developed a microculture assay for investigating the viability of HCV recovered from contaminated syringes. The HCV used (Jc1/Gluc2A) had a luciferase gene from *G. princeps* inserted between the p7 and NS2 gene [29]. After infection of Huh-7.5 cells with Jc1/GLuc2A, the GLuc2A enzyme and infectious reporter virus are secreted into the culture medium. HCV replication could be determined over time by measuring secreted GLuc2A activity [29]. This system allowed us to use relative luciferase activity as a function of infectivity or viability of HCV recovered from syringes. Huh-7.5 cells were infected with HCV reporter virus and incubated for 3 days before culture supernatants were analyzed for luciferase activity.

Assay sensitivity. As a first step, we determined the linear dynamic range of the microculture system. Starting with a viral stock of known $TCID_{50}$, we prepared serial 1:2 dilutions that were introduced into our system. Negative controls (using HCV-contaminated blood without cells, uncontaminated blood, or Huh-7.5 cell alone) yielded uniformly negative results. When 100 μL aliquots of dilution series were introduced, supernatants had relative luciferase activity that showed a strict correlation to expected $TCID_{50}$ over a linear range of 4 logs, between 1 and 10^4 $TCID_{50}$ (Figure 1). This range of sensitivity allowed us to detect changes in infectivity comparable to a >100-fold reduction in viral load. The limit of detection in the microculture assay was 250 relative luciferase units, equivalent to ~0.1 $TCID_{50}$.

Decay of infectivity of HCV at room temperature. We next investigated the rate of decay of the infectivity of the stock virus at room temperature. Aliquots of the virus were left in room temperature for up to 96 h. Samples were collected at intervals of ≤6 h and stored at −80°C until the determination of infectivity. We observed a biphasic decay of HCV viability (Figure 2). There was a rapid decline of infectivity within the first 6 h, with a $t_{1/2}\alpha$ of 0.4 h, followed by a second phase of a relatively slow exponential decay with a $t_{1/2}\beta$ of 28 h.

Survival of HCV in syringes. We last investigated the survival of HCV recovered from syringes stored at different temperatures. We simulated 2 scenarios of residual volumes after complete depression of the plunger: low void volume (2 μL)

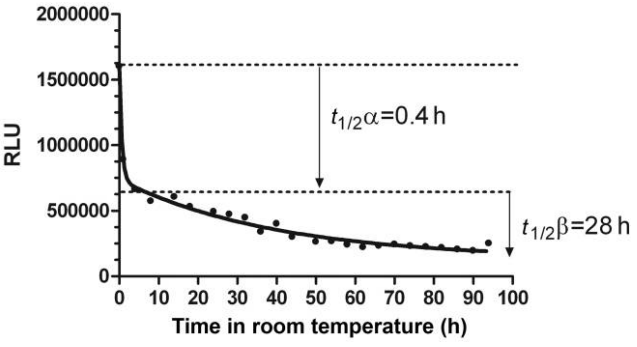

Figure 2. Hepatitis C virus decay rate at room temperature. Aliquots (100 μL) of the virus were stored 0–96 h at room temperature. Aliquots were removed from room temperature every 6 h or less and stored at −80°C. The stored aliquots were thawed and used to infect Huh-7.5 cells. The relative infectivity was determined by measuring the relative luciferase units (RLU) after 3 days of infection. Each value is the mean of 2 independent experiments.

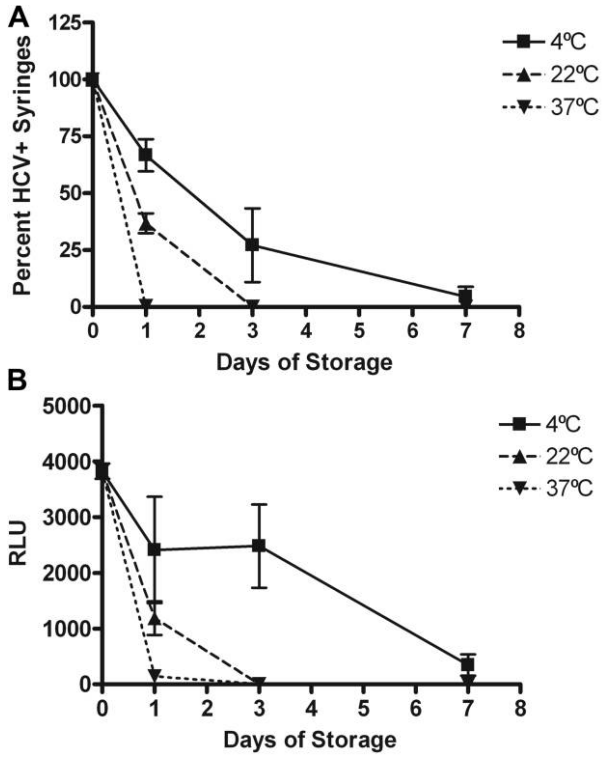

Figure 3. Survival of hepatitis C virus (HCV) in low void volume insulin syringes. Syringes were loaded with HCV-spiked blood to simulate "booting." Syringes were stored at 4ºC, 22ºC, and 37ºC for up to 14 days before contents were flushed to infect Huh-7.5 cells. HCV survival, a function of infectivity, was determined by the relative luciferase units (RLU) after 3 days of culture. There were 15 syringes at each time point in each experiment. *A*, The percentage of HCV positive syringes. *B*, HCV infectivity per positive low void volume syringe. Each value is the mean ± standard error of mean from at least 3 independent experiments.

with 1-mL insulin syringe (with permanently attached needle) and high void volume (32 μL) with 1-mL tuberculin syringe (with detachable needle). The syringes were loaded with HCV-contaminated blood and stored at different temperatures for up to 63 days. For each experiment to test for HCV survival, the contents of at least 15 stored syringes for each combination of storage time and temperature were introduced into our assay system. The proportion of HCV-positive syringes and the infectivity per HCV-positive syringe were determined. The results presented here came from at least 3 independent experiments.

The low void volume insulin syringes were stored for up to 14 days. We observed an inverse relationship between temperature and HCV survival (Figure 3*A*). Both the number of HCV-positive syringes and infectivity of HCV in the positive syringes declined rapidly over time. We recovered viable HCV from syringes stored at 4° for up to 7 days (5% HCV syringes), whereas syringes stored at 22° and 37° yielded no HCV-positive syringes beyond day 1 of storage. The loss in infectivity of HCV per positive syringe recovered after 1 day of storage at 22° and 37° was 30% and 96%, respectively. After storage at 4°, HCV-positive syringes showed 38% and 92% reductions in infectivity after 3 and 7 days of storage, respectively (Figure 3*B*).

The high void volume tuberculin syringes were stored for up to 63 days. In contrast to the results for the low void volume syringes, we observed a prolonged survival of HCV in the high void volume syringes at all storage temperatures; however, as with the low void volume syringes, storage at 4° was more favorable to survival of HCV than was storage at either 22° or 37°. The proportion of syringes with viable HCV declined sharply to 50% over the first 14 days of storage at 22° and 37° (Figure 4*A*). However, with storage at 4°, we observed a 50% decrease in viable HCV recovery only after 35 days of storage. There was a monotonic decline in the proportion of HCV-positive syringes stored at 22° (Figure 4*A*). However, for syringes stored at 4° and 37°, the monotonic decline ceased after day 35. The proportion of HCV-positive syringes recovered at

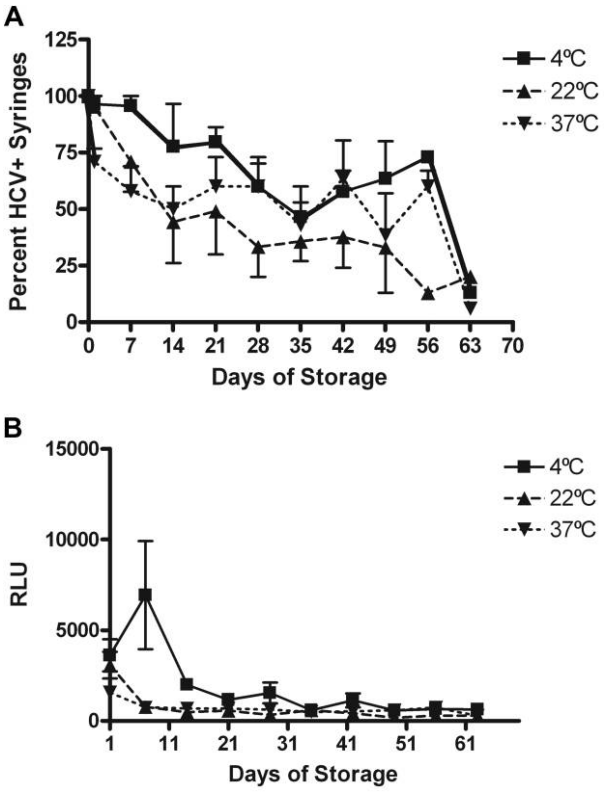

Figure 4. Survival of hepatitis C virus (HCV) in high void volume tuberculin syringes. Syringes were loaded with HCV-spiked blood to simulate "booting." Syringes were stored at 4ºC, 22ºC, and 37ºC for up to 63 days before contents were flushed to infect Huh-7.5 cells. HCV survival, a function of infectivity, was determined by the relative luciferase units (RLU) after 3 days of culture. There were 15 syringes at each time point in each experiment. *A*, The percentage of HCV-positive syringes at each time point. *B*, HCV infectivity per positive high void volume syringe after 1–63 days of storage. Each value is the mean ± standard error of mean from at least 3 independent experiments.

the final storage duration, 63 days, was 13% at 4°, 20% at 22°, and 6% at 37°.

The infectivity of the HCV recovered from the high void volume syringes was determined as a function of luciferase activity. We observed at least a 90% reduction in infectivity per positive syringe after 1 day of storage at all the temperatures (Figure 4*B*). The infectivity of recovered virus stored at 4° tended to be higher than at other temperatures over the first 21 days of storage. At the final storage duration, 63 days, the mean infectivity per positive syringe was 655 ± 30, 291 ± 10, and 275 ± 21 relative luciferase units for syringes stored at 4°, 22°, and 37°, respectively. Infectivity was therefore significantly higher for syringes harboring viable virus at 4° than at 22° and 37°.

DISCUSSION

In our experimental simulation of IDU injection practices, we observed that HCV survived in HCV-contaminated syringes for up to 63 days in high void volume syringes. Our finding supports our hypothesis that the efficient transmission of HCV among IDUs may be partly due to the ability of the virus to remain viable in contaminated syringes for prolonged periods. Moreover, we found that HCV survival was dependent on syringe type, time, and temperature. These parameters can be manipulated in the design of public health recommendations and interventions for preventing the spread of HCV among IDUs.

To our knowledge, this is the first study to establish the survival of HCV in syringes. Until recently, the absence of a sensitive tissue culture assay had made it impossible to develop suitable models to estimate HCV infectivity during the drug injection process. Lindenbach and colleagues [30, 31] recently developed a full-length HCV genotype 2a infectious clone (HCVcc) that replicates, producing infectious virus in cell culture. To conduct the experiments, we used a genetically modified HCVcc (Jc1/GLuc2A reporter virus) virus that expresses luciferase after viral replication. After infection of Huh-7.5 cells, the GLuc2A enzyme and infectious reporter virus are released into the culture medium. This allowed us to use the relative luciferase activity to determine HCV infectivity and HCV survival in syringes. Phan et al [29] previously demonstrated that GLuc2A expression was dependent on HCV replication, and Gluc2A expression correlated positively over time with the level of intracellular HCV RNA using quantitative RT-PCR.

We observed that HCV survival is dependent on the type of syringe; syringes with detachable needles (high void volume) appear far more likely to transmit HCV. This observation is consistent with experimental studies in HIV [36] and epidemiologic studies in HIV and HCV, providing evidence that the probability of transmission is associated with viral burden (ie, a function of viral load and volume of inoculum) [22, 40–42]. In a recent study, Zule et al [41] found an independent association between a history of sharing high void volume syringes and the prevalence of HIV and HCV among IDUs in North Carolina. Interestingly, the investigators likened the protective role played by the use of low void volume syringes to that of male circumcision and antiretroviral therapy in reducing HIV transmission [43, 44]. The type of syringe used by IDUs depends on locality and individual preference. IDUs in the United States predominantly use fixed-needle insulin syringes (low void syringes); pharmacies no longer sell detachable insulin syringes [40]. In areas where injection practices require volumes of water >1 mL, IDUs frequently resort to the use of syringe volumes >1 mL [41]. Interestingly, syringe exchange programs often stress the importance of providing IDUs with syringes that they prefer and meet their needs [45]. Our finding suggests the use of low void volume syringes should be stressed by syringe exchange programs to reduce HCV transmission.

The infectivity of HCV, in both low and high void volume syringes, declined sharply over the first few days. This was consistent with the observed biphasic decay rate of HCV at room temperature. Our finding of decay in infectivity of extracellular HCV is consistent with previous reports [46]. The survival of HCV in the low void volume syringes had an inverse relation to the storage temperature in many but not all conditions tested. Lower temperatures preserved the viability of HCV in the low void volume syringes to a greater extent than it did in the high void volume syringes. With the high void volume syringes, the infectivity was comparable at all temperatures after the first 14 days of storage. The time course of HCV survival in low void volume syringes is consistent with previous studies with HIV, although HCV appears to survive longer than HIV in high void volume syringes [36]. This raises the question whether the disproportionally high prevalence of HCV in comparison to HIV among IDUs could be partly explained by the differences in survival of these viruses in syringes. HCV has been shown by blood bank services to be stable for at least 7 days in plasma and serum stored at 4°C [47]. Although consistent with our finding of HCV survival in small void volume syringes stored at 4°C, these studies used PCR detection of HCV RNA, which is not a direct demonstration of viable virus. Is it possible that the duration of survival of our laboratory clone differs substantially from the duration of survival of common strains of HCV? Yes, but there is no currently available method to determine this, because there is no tissue culture system that can assess the replication or infectivity of HCV isolates from HCV-infected individuals. Furthermore, the fact that the prevalence of HCV consistently surpasses that of HIV in IDU populations may be attributed to higher viral titer and a different range of cells susceptible to HCV infection. HIV-1 requires entry into activated $CD4^+$ cells for productive infection, whereas HCV needs only to enter hepatocytes, which

the virus is likely to encounter because injection of HCV-contaminated drugs brings virus to the liver on its first pass through the circulatory system. Clearly, additional research is needed to elucidate those factors that result in the higher transmissibility of HCV because virus viability alone does not seem to explain this difference.

Interestingly, harm reduction programs have effectively reduced the incidence of HIV but not HCV among IDUs [8, 24–26]. Our findings have implications in the design of public health recommendations for preventing the spread of HCV among IDUs. Successful syringe exchange programs have reduced circulation time of used syringes from 23.5 days to <3 days [26, 39]. Thus, HCV in contaminated syringes may still be viable and hence transmissible throughout the circulation time of syringes found, once syringe exchange programs are established. The types of syringes used by IDUs vary from place to place and usually depend on availability, local preferences, and cultural practices [41]. Although most IDUs in developed countries use low void volume syringes, there are still individuals who prefer high void volume syringes, and syringe exchange programs provide syringes according to an individual's preference. In some places where homemade drugs are more common (eg, the former Soviet Union), the weaker drug solutions made in this way encourage the use of larger volume syringes that almost invariably come with detachable needles [48]. Because replacing the larger volume syringes is not practical, control of HCV transmission will require substantially expanded access to sterile syringes for IDUs in these regions.

Our study has some limitations. First, the simulation of the survival of HCV in syringes under laboratory-controlled conditions may not accurately reflect the natural transmission dynamics among IDUs. Second, the data come from the use of a genetically modified HCV laboratory clone derived from a genotype 2a virus. Third, the spiking of HCV-seronegative blood might not have sufficiently replicated the biological factors (eg, the presence of anti-HCV antibodies, immune complexes, or cytokines) present in the blood of HCV-infected individuals that could moderate HCV transmission and infectivity. However, the consistency of our results with previous epidemiologic studies that reported high HCV prevalence among IDUs supports our findings [22, 40–42].

This is the first study to our knowledge to establish the survival of HCV in contaminated syringes and the duration of potential infectiousness. The finding of prolonged duration of survival of HCV in syringes is a public health concern and adds additional evidence of the need for effective syringe exchange programs and other mechanisms to expand syringe access for IDUs.

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