

**Hepatitis C Virus Maintains Infectivity for Weeks after Drying on Inanimate Surfaces at Room Temperature: Implications for Risks of Transmission**

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## ABSTRACT

**Background:** Healthcare workers may come into contact with fomites containing infectious HCV during preparation of plasma, or following placement or removal of venous lines. Similarly, injection drugs users may come into contact with fomites. Hypothesizing that prolonged viability of HCV in fomites may contribute significantly to incidence; we determined the longevity of virus infectivity and the effectiveness of antiseptics.

**Methods:** We determined the volume of drops misplaced during transfer of serum or plasma. Aliquots equivalent to the maximum drop volume of plasma spiked with 2a HCV reporter virus were loaded into 24-well plates. Plates were stored uncovered at three temperatures: 4°, 22°, and 37°C for up to 6 weeks before viral infectivity was determined in a microculture assay.

**Results:** The mean volume of an accidental drop was 29  $\mu$ l (min - max of 20 - 33  $\mu$ l). At storage temperatures 4° and 22°C, we recovered viable HCV from the low titer spots for up to 6 weeks of storage. The rank order of HCV virucidal activity of commonly used antiseptics was bleach (1:10) > cavicide (1:10) > ethanol (70%).

### Conclusions:

The hypothesis of potential transmission from fomites was supported by the experimental results. The anti-HCV activity of commercial antiseptics varied.

## INTRODUCTION

The global burden of morbidity and mortality from hepatitis C virus (HCV) infection is truly pandemic with more than 170 million people currently infected <sup>1</sup><sub>\_ENREF\_1</sub>. Since there is currently no vaccine for HCV and available treatment regimens are limited by efficacy, cost, and side effects, prevention of HCV transmission remains the primary strategy for curbing the HCV epidemic. HCV is transmitted primarily through parenteral exposure to blood or body fluids contaminated with HCV. Injecting drug use (IDU), mother-to-child transmission, multiple heterosexual partners, accidental needle injuries, and transfusion of blood or blood products are among the most relevant risk factors for HCV acquisition<sup>2-4</sup>.

The epidemiology of HCV has changed in the last decade. Transmission from blood transfusions and surgical procedures have all but disappeared in developed countries<sup>5</sup>. There have been modest but insufficient declines in incidence among IDUs in locations with broad implementation of syringe exchange programs<sup>6-12</sup>. Nosocomial transmissions of HCV increasingly account for a large proportion of new HCV infections (i.e., 20% to 50%) in developed countries<sup>13-17</sup>. Thus, the relative impact and burden of nosocomial HCV transmission might be greater now than a decade ago. In an Italian study of 214 patients with acute HCV infections<sup>18</sup>, the most relevant associated risk factors were: history of medical procedures (32%) (e.g., hospitalization, surgery, endoscopy, dialysis, blood transfusion, dental treatment, or other invasive procedures) and intravenous drug use (30%). Interestingly, among the patients classified under medical procedures almost half of them did not have surgery or any invasive procedures while on admission. This has been corroborated by a study from Spain where the

investigators found that the only documented risk factor among patients with acute HCV infection was hospital admission<sup>19</sup>. One can speculate that these patients might have been exposed to HCV-contaminated surfaces during hospitalization. We hypothesized that occupational and iatrogenic HCV infections may be due in part to the ability of the virus to remain viable on fomites and other hospital equipment for prolonged periods.

We recently established a microculture assay for propagation of cell culture derived HCV (HCVcc) in small volumes by using a genetically engineered reporter virus derived from the HCVcc clone<sup>20,21</sup>. Using our microculture assay system, we performed a set of experiments to replicate the circumstances in which healthcare workers or patients may come into contact with HCV dried upon surfaces that include preparation of plasma, handling of hemodialysis equipment, and following placement or removal of venous lines. To our knowledge this constitutes the first study to closely simulate conditions leading to nosocomial transmission of HCV.

## **MATERIALS AND METHODS**

### **Plasmids and Viruses**

The construction of the Jc1/GLuc2A reporter virus, a derivative of the chimeric genotype 2a FL-J6/JFH with a luciferase gene from *Gaussia princeps* inserted between the p7 and NS2 genes, has been reported previously<sup>21,22</sup>. Viral stocks of Jc1/GLuc2A reporter virus were prepared by RNA transfection of Huh-7.5 cells. The titer of HCVcc was quantified by infecting cells with serial dilutions of the stock virus and determining the dilution that will infect 50% (TCID<sub>50</sub>) of the wells by using the method of Reed and Muench<sup>23</sup>.

## Cell Culture

Human hepatoma cells highly permissive for HCVcc (Huh-7.5 subline)<sup>24</sup> were maintained as subconfluent, adherent monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 1 mM non-essential amino acids (Invitrogen, Carlsbad, CA) at 37°C and 5% CO<sub>2</sub>.

## Determination of the volume of accidentally misplaced HCVcc-contaminated plasma on surfaces

The most likely circumstances in which healthcare workers or patients may come into contact with HCV dried upon surfaces are following spillage of HCV-contaminated blood, serum, or plasma during the course of preparing a blood sample for analysis or removing a venous line. To simulate such accidents, we obtained ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood from HIV and HCV seronegative donors. The tube was centrifuged at 2000 rpm for 15 minutes and a rubber dropper was used to aspirate and transfer the plasma into several vials for storage as per practice and recommendation of the clinical microbiology laboratory at Yale-New Haven Hospital. The procedure was done in a biosafety cabinet with a foil mat to collect accidental drops of plasma. The experiment was done on two occasions and at each occasion 10 accidental drops were weighed. The volume of the drop was calculated based on the formula that 1 ml weighs 1 gram. The mean, with standard deviation of the mean, and maximum volumes were calculated.

### **Desiccation of displaced HCVcc-contaminated plasma drops on work surfaces**

To determine how quickly the plasma dried on the surface we seeded the wells in uncovered 24-well tissue culture plates with the maximum accidentally dropped volume (33  $\mu$ l). The 24-well plates were stored in a refrigerator at 4°C, on a benchtop at 22°C, or in an incubator at 37°C, and observed every 60 minutes until all replicates (20 drops) had dried. The time to dryness in these storage conditions was recorded. To determine the effect of humidity on time to dryness, we, in a separate experiment, recorded the temperature and humidity using an analog thermo-hygrometer instrument (General Tools, New York, NY, USA) three times a day (7 - 9 am; 12 noon - 1 pm; and 3 - 5 pm) for a week. The mean humidity, with standard deviation of the mean was calculated.

### **Viability of dried HCVcc on surfaces**

We spotted 33  $\mu$ l of plasma spiked with HCVcc on the 24-well plates. They were either immediately tested for viable virus or stored at 4°C, 22°C, and 37°C for up to 6 weeks before testing. Twenty replicates were tested per condition and the experiment was repeated on two separate occasions. Negative controls comprised of plasma without virus. The day before each time point, 96-well plates were seeded with  $6.4 \times 10^3$  Huh-7.5 cells/well in 100  $\mu$ l of medium and incubated at 37°C in 5% CO<sub>2</sub>. To test for infectivity, the dried spots were rehydrated and reconstituted with 100  $\mu$ l of culture medium. The medium from the wells was gently aspirated from the cells and replaced with 100  $\mu$ l of the reconstituted virus mixture. After 5 h of incubation, the cells were washed with sterile PBS to remove the input virus; fresh medium was added and incubated for 3 days. After 3 days, culture supernatant was harvested and mixed with 20  $\mu$ l of lysis buffer

before luciferase activity was measured by using luciferase assay reagent kit (Promega, Madison, WI) and a luminometer (Synergy HT, BioTek, Winooski, VT). The relative luciferase activity (RLA) was determined to be linearly related to HCV infectivity<sup>16</sup>.

### **Virucidal effect of antiseptics on viability of contaminated HCVcc on surfaces**

We used three antiseptics – bleach (Clorox), ethanol, and caviocide (Metrex) - to determine the effect of antiseptics on infectivity of HCVcc contaminated spots by using a culture media without virus as negative control. Positive controls consisted of cell culture media with virus. These antiseptics are readily available in hospitals and research laboratories. Bleach is available as 6% sodium hypochlorite and is diluted 1:10 in tap water before use, while ethanol is available for use as 70% ethanol<sup>25-27</sup>. Caviocide is ready-to-use without dilution as per product insert. Prior to testing virucidal activity, it was necessary to determine the cytotoxic effects of the antiseptics on the Huh-7.5 cells. Briefly, 33  $\mu$ l of test antiseptic was pipetted onto a 24-well plate. The antiseptic was combined with 297  $\mu$ l of culture media (i.e., 1:10 dilution) and the mixture was passed through MicroSpin S-400 HR columns (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions. 300  $\mu$ l of column eluate or mixture not passed through the columns was added to Huh-7.5 cells seeded the previous day in a 48-well plate at  $3.0 \times 10^4$  cells/well in 300  $\mu$ l of medium, to make a final volume of 600  $\mu$ l and then incubated overnight at 37°C. After a day of further incubation, cell growth was determined with the alamarBlue® assay (Invitrogen) as manufacturer's instructions. Cell growth was determined as a function of relative fluorescence measured at 530 nm excitation and 590

nm emission (Synergy HT Plate Reader, BioTek, Winooski, VT). Five replicates were tested per condition and the experiment was repeated twice.

We modified a previously described protocol to test for the infectivity of HCVcc after exposure to test antiseptic<sup>28</sup>. In brief, an equal volume of test antiseptic was pipetted onto 33  $\mu$ l HCVcc contaminated spots for an exposure period of one minute, whereafter, 264  $\mu$ l of culture media was added to the virus-antiseptic mixture (i.e., 1:10 dilution) and reconstituted. To reduce the cytotoxicity of antiseptics, each mixture was passed through a MicroSpin S-400 HR column according to the manufacturer's instructions. Then 300  $\mu$ l of eluate passed through the column or mixture without column purification was added to Huh-7.5 cells in a 48-well plate at  $3 \times 10^4$  cells/well in 300  $\mu$ l of medium to make a final volume of 600  $\mu$ l. The cells were washed with sterile PBS after 4 h to remove input virus and incubated in 200  $\mu$ l fresh media for 3 days. The infectivity of HCVcc was determined by luciferase assay as described above. Ten replicates were tested per condition and the experiment conducted on three occasions.

## RESULTS

### Volume of accidentally misplaced HCVcc contaminated plasma

The experiment was done on three occasions, and on each occasion 10 drops were weighed. The mean volume of the drops, calculated on the basis that 1 ml weighs 1 gram, was  $29 \pm 5$   $\mu$ l and the range was 18 to 33  $\mu$ l. Since the maximum drop volume of 33  $\mu$ l presents the most risk of transmission, we used 33  $\mu$ l throughout our study.

### **Time to drying of HCVcc contaminated drops at different temperatures**

Dried droplets of serum contaminated with HCV may be inconspicuous and, therefore, more likely than a liquid droplet to cause accidental exposures to HCV. We determined how long it took a drop of HCVcc contaminated plasma to dry at 4°, 22° and 37°C. We determined the mean temperature and relative humidity in the refrigerator, the benchtop, and the incubator over a week. The temperature was  $4 \pm 1^\circ$ ,  $22 \pm 0^\circ$ , and  $37 \pm 0^\circ$ C in the refrigerator, the benchtop, and the incubator, respectively. The humidity was  $53 \pm 10\%$ ,  $44 \pm 5\%$ , and  $82 \pm 1\%$  at 4°, 22° and 37°C, respectively. The order of time to dryness was 4, 24, and 28 hrs at 22° (benchtop), 4° (refrigerator), and 37°C (incubator), respectively. Thus time to dryness correlated positively with the humidity of the storage condition.

### **Infectivity of dried HCVcc on surfaces at different temperatures**

We investigated the infectivity of HCVcc after drying on surfaces at different temperatures. Aliquots of 33  $\mu$ l of HCVcc contaminated serum were pipetted into 24-well plates and stored for up to six weeks. Twenty spots of dried HCVcc for each combination of storage time and temperature were reconstituted with culture media after storage and introduced into our assay system<sup>20</sup>. The proportion of HCVcc positive dried spots and the infectivity per HCVcc dried spot were determined. The results presented here came from at least three independent experiments.

First, we used a low titer stock of HCVcc, (i.e., equivalent to  $10^4$  infectious units/mL) to determine the infectivity of HCVcc after drying and storage for up to 6 weeks. We observed a negative correlation between storage temperature and HCVcc

infectivity (Figure 1A). With an assay detection limit of 1000 RLA (2-3 times over the background luciferase activity), we recovered viable HCVcc from dried spots stored at 37°C until day 7 of storage. In contrast, at storage temperatures 4° and 22°C, we recovered replicating HCVcc from all the spots for up to 6 weeks of storage. The infectivity, measured by RLA of the reconstituted spots, declined rapidly over time inversely to the storage temperature (Figure 1B). At storage temperatures of 4° and 22°C, we observed a sharp decline in infectivity over the first two weeks followed by persistent but lower infectivity through week six (Figure 1B). This is consistent with our previous report of biphasic decay rate of HCVcc<sup>20</sup>.

By using a high titer stock of HCVcc (equivalent to  $10^6$  infectious units/mL), we observed a prolonged infectivity of HCVcc at all storage temperatures. Almost 100% of the contaminated spots stored at 4° and 22°C remained positive for HCVcc through three weeks of storage (Figure 2A). At 37°C, 100% of the spots were positive till 10 days of storage and then declined to 40% and 0% at days 14 and 21, respectively (Figure 2A). The infectivity of the HCVcc recovered from the high titer HCVcc contaminated spots was in general 2 to 3-fold higher than the RLA of the low titer HCVcc at each time point. Infectivity was inversely proportional to the storage temperature. We observed a 50% reduction in infectivity at day 3, 14 and 21 for storage temperatures 37°, 22°, and 4°, respectively (Figure 2B).

### **Effect of antiseptics on infectivity of HCVcc on surfaces**

To investigate the virucidal effect of bleach, ethanol, and caviocide, we first determined the effects of these antiseptics on the growth of Huh-7.5 cells by using the alamarBlue® assay. When we tried undiluted bleach and caviocide, which were diluted

1:10 before addition to the tissue culture system, we found they were uniformly cytotoxic to Huh-7.5 cells whereas 70% ethanol had no significant effect on cell growth (Figure 3A). Cell growth was almost restored to control levels with a 1:10 dilution of bleach and a 1:20 dilution of cavicide following passage of the solution through MicroSpin S-400 HR columns (Figure 3A). Cavicide at a 1:10 dilution reduced growth by 70% relative to the control.

Based on the cytotoxicity results, experiments using bleach diluted 1:10 and 1:100, cavicide diluted 1:10 and 1:20, and ethanol at 70% and 7% were conducted by using MicroSpin S-400 HR columns<sup>29</sup> prior to adding eluate to the microculture system. After 1 min exposure to bleach (1:10 dilution), cavicide (1:10), and ethanol (70%), the percentage of positive contaminated HCVcc spots were 0%,  $3 \pm 6\%$ , and  $13 \pm 6\%$ , respectively (Figure 3B). Further dilutions of bleach (1:100), cavicide (1:20), and ethanol (7%) resulted in  $17 \pm 6\%$ ,  $43 \pm 6\%$ , and  $90 \pm 17\%$  positive spots, respectively. For certain viruses, passage through a MicroSpin column could reduce viral infectivity<sup>30</sup>, therefore, we performed a control experiment comprising HCVcc without exposure to any antiseptic and with or without passage through a MicroSpin column prior to infection of Huh 7.5 cells. The infectivity was  $80 \pm 10\%$  and 100% for HCVcc with and without passage through MicroSpin column, respectively (Figure 3B). We next tested the infectivity of HCVcc without MicroSpin column after exposure to antiseptic at concentrations that are least cytotoxic. After 1 min exposure to bleach (1:100 dilution), cavicide (1:20), 70% ethanol, and 7% ethanol, the percentage of positive contaminated HCVcc spots were  $30 \pm 10\%$ ,  $60 \pm 36\%$ ,  $30 \pm 35\%$ , and  $93 \pm 12\%$ , respectively (Figure 3B). The infectivity of residual HCVcc after passage through MicroSpin column (Figure

3C) was correlated with the likelihood of recovery of viable HCVcc. RLA was highest for 7% ethanol (with 27 of 30 spots yielding viable HCVcc) and lowest for 1:10 cavicide (1 of 30 spots yielding viable HCV).

## DISCUSSION

In our simulation of real world risks of HCV transmission in settings conducive to exposure to HCV-contaminated fomites, we observed that HCVcc could maintain infectivity for up to 6 weeks at 4° and 22°C. This finding supports our hypothesis that the increasing incidence of nosocomial HCV infections may be due to accidental contact with HCV-contaminated fomites and other hospital equipment even after prolonged periods following their deposition. Moreover, we found that HCVcc infectivity was influenced by HCVcc viral titer and the temperature and humidity of the storage environment. Furthermore, the commercially available antiseptics reduced the infectivity of HCVcc on surfaces only when used at the recommended concentrations<sup>25,27</sup>, but not when further diluted.

Although there have been two previous studies on infectivity and stability of HCV on surfaces<sup>28,31</sup>, to our knowledge, this is the first study that closely simulates the natural events likely to cause transmission of HCV. First, Kamili et al. reported that 100 µl aliquots of chimpanzee plasma contaminated with HCV was still infectious when dried and stored at room temperature for up to 16 hours<sup>31</sup>. Transmission of infection did not occur after 16 hours to up 7 days of storage. More recently, Doerrbecker et al. demonstrated that 50 µl of cell culture-derived HCV dried on steel discs remains infectious for up to 5 days at room temperature<sup>28</sup>. The limitations of these previous

studies include simulation of HCV transmission under artificial drying conditions. Furthermore, Doerrbecker et al. found that the infectivity of the virus recovered from the carrier system was 10-fold lower than that stored in liquid media. Therefore, one can speculate that the duration of infectivity observed in their study could be an underestimation. Moreover, differences in the three assay systems (e.g., *in vivo* versus *in vitro* assay; artificial versus passive desiccation) might account for the different durations of survival reported. Our study sought to overcome some of these limitations by determining the exact size of accidentally misplaced HCVcc-contaminated plasma and allowing the drops to dry under natural conditions. The fact that under these conditions we found HCVcc to be infectious for up to 6 weeks, consistent with our previous report that HCVcc survived in tuberculin syringes for up to 63 days<sup>20</sup>, is of public health concern. Taken together, these studies show that HCVcc remains potentially infectious for prolonged periods of time, ranging from 16 hours to 6 weeks depending on the assay system. We previously reported on the biphasic decay rate of our genotype 2a HCVcc at room temperature; a rapid decline of infectivity within the first 6 h followed by a second phase of a relatively slow exponential decay<sup>20</sup>. This is consistent with recent report on thermostability of 7 genotypes including 2a genotype<sup>32</sup>. Such prolonged infectivity could contribute to the increasing incidence of nosocomially acquired HCV infections.

Of infection control relevance is the fact that all the HCVcc-contaminated spots dried at room air within 4 hours, becoming inconspicuous and therefore more likely to cause accidentally exposures to HCV. HIV was also reported to dry at room temperature within 3 hours and retain infectivity for up to 7 days<sup>33,34</sup>. The infectivity of HCVcc and HIV when stored at room temperature for several days is consistent with that of other

envelope viruses<sup>35,36</sup>. The prolonged infectivity of these viruses has been attributed, in part, to their lipid envelope, which resists drying and protects the viral capsid from the deleterious effects of dehydration<sup>37</sup>. Hepatitis B virus, another lipid-enveloped hepatotropic virus, was reported to survive up to 7 days at room temperature; further time points were not available due to a laboratory mishap<sup>38</sup>. The resilience of these viruses at room temperature raises the possibility of their being transmitted through fomites. Our findings support the surveillance data on increasing incidence of nosocomial transmissions of HCV in developed countries<sup>13-17</sup>. Interestingly, most of the patients who acquired HCV in the hospital had no surgeries or invasive procedures; their only risk was hospital admission<sup>18,19</sup>. Fomites could, therefore, be an important vehicle for transmission of HCV in the hospital and household settings.

Finally, given the infection control implications of our findings, we decided to investigate if commonly used antiseptics are effective against HCV. We demonstrated that bleach, cavicide, and ethanol are effective at their recommended concentrations<sup>25-27</sup>. It is possible that the efficacy of cavicide at 1:10 is overestimated because the disinfectant itself reduced host cell viability by 70%. Further dilution of each antiseptic proved sub-optimal (Figures 3B and C). The finding for ethanol paralleled that of Ciesek et al., who found that HCV titers decreased at concentrations of 30% and 40% but complete inactivation did not occur at an exposure time of 5 min<sup>29</sup>. However, undiluted concentrations of several hand antiseptics (based on povidone-iodine, chlorhexidine digluconate, and triclosan) reduced HCV infectivity to undetectable levels<sup>29</sup>. Thus, there are several commercially available antiseptics that are effective against HCV.

Our study, which sought to improve upon prior studies, still has some limitations. First, the assay employs a genetically modified HCV laboratory clone derived from a genotype 2a virus that may not reflect survival characteristics of human isolates. However, the thermostability pattern of our virus is similar to that of other genotypes<sup>32</sup>. Second, the spiking of HCVcc-seronegative blood might not sufficiently replicate the biological factors present in the blood of HCV-infected individuals that could moderate HCV transmission and infectivity. However, the consistency of our results with previous *in vitro* studies and epidemiologic studies reporting transmission of HCV in healthcare setting and through sharing of injection paraphernalia<sup>39-43</sup> support our findings.

In conclusion, we have demonstrated that HCVcc can remain infectious at room temperature for up to 6 weeks. Our hypothesis of potential transmission from fomites was supported by the experimental results and provides the biological basis for recent observational studies reporting increasing incidence of nosocomial HCV infections and continued high incidence among people who inject drugs.

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Footnote:

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## Figure legend

Figure 1: Survival of low titer HCV after drying on surfaces. 33  $\mu$ l of HCV-spiked blood was spotted on 24-well plate and at 4°C, 22°C, and 37°C for up to 4 weeks before content was 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 20 drops at each time point. (A) The percentage of HCV positive dried spots after storage. (B) HCV infectivity per positive dried spot. Each value is mean + SD from at least 3 independent experiments.

Figure 2: Survival of high titer HCV after drying on surfaces. 33  $\mu$ l of HCV-spiked blood was spotted on 24-well plate and at 4°C, 22°C, and 37°C for up to 3 weeks before content was 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 20 drops at each time point. (A) The percentage of HCV positive dried spots after storage. (B) HCV infectivity per positive dried spot. Each value is mean + SD from at least two independent experiments.

Figure 3: Effect of commercially available antiseptic on Huh-7.5 cell growth and HCV infectivity. Three different antiseptics – bleach, cavicide and ethanol - were tested for their ability to inactivate HCV at different concentrations. (A) Effect of antiseptics on Huh-7.5 cell growth. 33  $\mu$ l of antiseptics were diluted 1:10 with cell culture medium and purified, or not, on S-400 HR columns and incubated on Huh-7.5 cells overnight. Cell

growth was determined by alamarBlue® assay. (B) The percentage of HCV positive dried spots after application of antiseptic. 33 µl of HCV-spiked blood was spotted on 24-well plate and exposed to 33 µl of antiseptics at different concentrations for 1 min. The reaction was stopped by diluting the antiseptic 1:10 with cell culture media and purified with or without MicroSpin S-400 HR columns. The eluents were used 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. Dark and grey bars represent experiments with and without passage through MicroSpin column, respectively. †, experiments without columns were not done because of the cytotoxicity of the antiseptic at these concentrations. (C) Residual infectivity of HCV contaminated surface after application of antiseptics. Each value is mean + SD from at least 3 independent experiments.

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