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Inactivation and survival of hepatitis C virus on inanimate surfaces

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Running title: Inactivation of dried hepatitis C virus by chemical biocides

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Abstract

Background: Hepatitis C virus (HCV) cross-contamination from inanimate surfaces or objects has been implicated in transmission of HCV in healthcare settings and among injection drug users. We established HCV-based carrier and drug transmission assays that simulate practical conditions to study inactivation and survival of HCV on inanimate surfaces.

Methods: Studies were performed with authentic cell culture derived viruses. HCV was dried on steel discs and biocides were tested for their virucidal efficacy against HCV. Infectivity was determined by a limiting dilution assay. HCV stability was analyzed in a carrier assay for several days or in a drug transmission assay using a spoon as cooker.

Results: HCV can be dried and recovered efficiently in the carrier assay. The most effective alcohol to inactivate the virus was 1-propanol and commercially available disinfectants reduced infectivity of HCV to undetectable levels. Viral infectivity on inanimate surfaces was detectable in the presence of serum for up to 5 days and temperatures of about 65-70°C were required to eliminate infectivity in the drug transmission assay.

Conclusions: These findings are important for assessment of HCV transmission risks and should facilitate the definition of stringent public health interventions to prevent HCV infections.

Key words: hepatitis C virus, inanimate surfaces, chemical biocides, injection drug users, nosocomial transmission

Introduction

Hepatitis C virus (HCV) is an enveloped virus that at present chronically infects about 130 million people worldwide [1]. One hallmark of HCV is its high degree of sequence variability which likely contributes to its ability to establish chronic infections. Different patient isolates are grouped into 7 genotypes and more than 100 subtypes within the genus *Hepacivirinae* of the family *Flaviviridae* [2]. Persistent infection is associated with a variable degree of liver damage often progressing in severity over the course of decades. Accordingly, a large number of patients are at risk of severe sequelae including life threatening conditions like cirrhosis and hepatocellular carcinoma [3]. The best available treatment, a combination of polyethylene glycol (PEG)-conjugated interferon alpha (IFN-α) and ribavirin, is not effective in every patient and can be associated with severe side effects [4]. A prophylactic or therapeutic vaccine is so far not available.

Hepatitis C is a blood-borne viral infection transmitted mainly through intravenous drug use, blood transfusions, accidental needle sticks, and other parental exposures, including nosocomial transmissions [5-9]. With the implementation of routine testing of blood products for HCV, transfusion-transmitted infections became rare [10]. However, outbreaks in healthcare settings have been consistently reported primarily attributed to contaminated medications or equipment and breaches in aseptic techniques in the United States, Europe and Japan [11-15]. Furthermore, cross-contamination continues to occur among injection drug users (IDU's) by the sharing of drug preparation equipment [16-18]. The seroprevalence of HCV among IDU's in the United States is high, ranging between 30% to 85%, with current estimates suggesting more than over 60 percent of newly acquired infections occur in individuals who have injected drugs [19, 20]. The adequate assessment of

transmission risks and the evaluation of the mechanisms of transmission have been difficult due to the lack of cell culture systems and animal models permissive to HCV infection. This obstacle has been overcome with the development of an HCV cell culture system based on the Japanese fulminant hepatitis (JFH1) HCV isolate, which reproduces the complete viral replication cycle *in vitro* [21-23]. This infection system was recently applied to evaluate the environmental stability of HCV and its susceptibility to chemical biocides in liquid suspensions [24]. Furthermore, Paintsil et al. analyzed in 2010 the survival of HCV in contaminated syringes and the duration of potential infectiousness [25], however, both studies did not analyze viability and infectivity of dried HCV.

Therefore, simulating realistic practical conditions, we established an HCV-based carrier and drug transmission assay to test inactivation and stability of HCV on inanimate surfaces. These results allow the further exploration of viral transmission from contaminated surfaces, objects, or devices and the potential for recommendations for effective measures interrupting this transmission.

Material and Methods

Plasmids and viruses

The plasmid pFK-Jc1 has been described recently [26]. Construct Luc-Jc1 encodes a chimeric HCV polyprotein which consist of codons 1-846 derived from J6/CF [27] combined with codons 847-3033 of JFH1. In this genome the HCV polyprotein-coding region is located in the second cistron and is expressed via an internal IRES element derived from the encephalomyocarditis virus. The first cistron contains the firefly luciferase reporter gene fused to the JFH1-derived 5´NTR and coding region of the N-terminal 16 amino acids of JFH1 core [28].

Chemical biocides

The alcohol substances 1-propanol, 2-propanol and ethanol were purchased from Carl Roth, Karlsruhe, Germany. Six commercially available biocides for surface disinfection were chosen to study the efficacy against dried HCV: Product A (based on ethanol, 2-propanol), product B (based on ethanol, 1-propanol), product C (based on glutaraldehyde), product D and E (based on quaternary ammonium compounds), and product F (based on peroxide compounds).

Cell Culture

Huh7.5 cells were cultured in Dulbecco`s modified Eagle medium (DMEM, Invitrogen) with 10% fetal bovine serum, 1x non-essential amino acids (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 100 IU/ml penicillin (Invitrogen).

1 ***In vitro transcription, electroporation and production of cell culture-derived***
2 ***HCV***
3 Infectious HCV particles were produced as described previously [28]. Briefly, Jc1 or
4 Luc-Jc1 plasmid DNA was linearized and transcribed into RNA, which was then
5 electroporated into Huh7.5 cells. Virus-containing culture fluids were harvested after
6 48 or 72 hours filtered through a 0.45 µm pore size filter. For determination of viral
7 infectivity cell-free supernatants were used to infect naive Huh7.5 target cells.
8
9 ***Determination of HCV infectivity***
10 Titers of infectious virus were determined by using a limiting dilution assay on
11 Huh7.5 cells with a few minor modifications and tissue culture infectious dose 50
12 ($TCID_{50}$) was determined as described [23]. For determination of Luc-Jc1 reporter
13 activity, infected cells were washed with PBS and lysed in luciferase lysis buffer (1 %
14 Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT, pH
15 7.8). Firefly luciferase activity was measured as described previously [28].
16
17 ***Preparation of the carrier***
18 Stainless steel discs with grade 2B finish on both sides (20 mm diameter, GK
19 Formblech GmbH, Berlin, Germany) were incubated in a 5% (v/v) Decon®90-solution
20 (Decon Laboratories Ltd., Hove, England) for 1 hour. Afterwards the discs were
21 rinsed off twice with freshly distilled water for 10 seconds, ensuring that the carriers
22 did not dry to any extent, and were then placed in 70% ethanol (v/v) for 15 minutes.
23 Finally, the carriers were dried by evaporation in sterile petri dishes under a
24 biological safety cabinet.
25
26

1

2 ***Experimental procedure of HCV carrier assay***

3 50 µl of the virus inoculum were pipetted in the center of each pretreated carrier and 4 dried in a desiccator or under a laminar flow for about 1 to 3 hours at room 5 temperature. After drying, the virus contaminated discs were transferred with forceps 6 into 25 ml plastic vial holders (Sarstedt AG & Co. KG, Nümbrecht, Germany), which 7 were previously filled with 0.5 g of sterile glass beads (0.25-0.50 mm diameter, Carl 8 Roth GmbH) to increase virus recovery by mechanical abrasion. Then, 100 µl of the 9 test substance were pipetted on the dried virus inoculum and incubated for 1 or 5 10 minutes. Control carriers received 100 µl of water instead of the chemical biocide. In 11 order to neutralize the test substance, 900 µl of culture medium were immediately 12 added at the end of the chosen exposure time. The vials were directly vortexed for 1 13 min to recover the residual virus, before the eluate was diluted to measure viral 14 infectivity. To determine cytotoxicity of the biocides, 1 part of PBS was mixed with 9 15 parts of the biocide and used to inoculate, permissive Huh7.5 cells. Cytotoxicity was 16 determined by examining permissive cells by microscopy for any significant changes 17 in the cell monolayer and calculated analogously to virus titer ($TCID_{50}$/mL).

18 For testing HCV stability and inactivation in the presence of serum whole blood 19 sample of healthy donors were centrifuged for 5 min at 5000 rpm to obtain serum. 20 The effect of serum on HCV stability was tested by mixing serum and virus 21 suspension in a ratio 1:1 in a total volume of 0.1 ml before the drying procedure.

22

23 ***Experimental procedure for HCV drug transmission assay***

24 To test the effect of different temperatures on HCV infectivity in a drug preparation 25 simulation, viral suspensions of 800 µl were used as inoculum of a standard 26 household spoon (stainless steel). A heating procedure was started with a tea

candle with a distance of about 4 cm between the spoon and the top of the flame. Temperatures of the suspensions were measured at specific time intervals using a thermometer for small liquids (YEW pocket thermometer 2542). At given temperatures 70 µl of the viral suspension was sampled. To judge the influence of human serum on virus stability in the drug transmission assay, virus suspension was diluted in a ratio of 1:8 with serum or water. Viral infectivity was determined by a luciferase reporter assay as described [28].

Results

Development of a HCV-based carrier test

In general, the carrier test method is designed to evaluate the ability of chemical biocides to inactivate vegetative bacteria, viruses, fungi, mycobacteria and bacterial spores on inanimate surfaces [29]. Here, the experimental procedure of the carrier assay was used for the first time to test the virucidal activity of biocides against dried HCV. First, stainless steel discs were inoculated with a virus preparation of the HCV genotype 2a chimera Jc1 [26] and dried under a laminar flow (Figure 1*A*). After drying, the virus-contaminated discs were transferred into plastic vial holders, which were previously filled with glass beads to increase virus recovery by mechanical abrasion. Next, the tested biocides were distributed onto the dried virus and incubated for 1 or 5 minutes. In order to neutralize the test substance, culture medium was immediately added at the end of the exposure time. The vials were directly vortexed to recover residual infectivity, before the eluate was diluted to determine viral infectivity using a limiting dilution assay.

It has been described that depending on which virus type is dried on the carrier the amount of infectivity recovered might vary [29]. Therefore, to determine the recovery efficiency for HCV, we titrated Jc1 incubated 1 hour in suspension and a virus inoculum that was dried for the same time on a carrier disc. As depicted in Figure 1*B*, the infectivity of HCV recovered from the carrier surface by our procedure was about 10-fold lower compared to the HCV stored in a liquid environment. Thus, approximately 10% of the viral infectivity was recovered in the carrier assay.

1 ***Virucidal efficacy of 1-propanol, 2-propanol and ethanol against dried HCV***

2 Surface disinfectants used in healthcare and other medical settings often contain 1-
3 propanol, 2-propanol or ethanol as active ingredients for decontamination of
4 surfaces. To assess the virucidal efficacy of these alcohols at concentrations ranging
5 from 10-60% on contaminated surfaces, we incubated each alcohol for 1 minute
6 (Figure 2*A*) and 5 minutes (Figure 2*B*) on dried HCV. The most effective alcohol to
7 inactivate HCV was 1-propanol, reducing viral titers to background levels at a
8 concentration of 30% with both incubation times (Figure 2). For 2-propanol, a
9 concentration of 30% decreased infectivity about 10-fold and complete inactivation
10 was observed at an alcohol content of 50% with a 1 minute exposure time and 40%
11 with 5 minutes incubation, respectively. Ethanol showed the lowest virucidal efficacy
12 with a required concentration of 50% to reduce viral titers to undetectable levels in
13 the 5 minute exposure (Figure 2*B*).

14

15 ***Effect of commercially available surface disinfectants against dried HCV***

16 To directly determine the efficacy of commercially available surface disinfectants, we
17 chose 6 different chemical biocides with different virucidal substances as
18 ingredients. Products A and B were both based on ethanol and 2-propanol or 1-
19 propanol, respectively. Product C contained glutaraldehyde as active ingredient.
20 Product D and E were on the basis of quaternary ammonium compounds whereas
21 for product F peroxide compounds were used as virucidal substance. The alcohol-
22 based biocides were tested as recommended with an incubation time of 5 minutes in
23 the concentrations of 10%, 50% and 100%. As depicted in Figure 3*A*, a
24 concentration of 50% for product A reduced viral titers about 50-fold. In an undiluted
25 preparation no infectivity could be detected, however, at a 100% concentration also
26 cytotoxicity was visible. Product B containing ethanol and 1-propanol demonstrated

a higher virucidal efficacy than product A reducing viral titers to background levels already at a concentration of 50% thus confirming the previous results that 1-propanol is superior over 2-propanol as biocide for HCV. The other commercially available disinfectants were tested at concentrations of 0.025%, 0.25% and 0.5% in the carrier test (Figure 3B). A complete inactivation could be achieved by all products at the highest concentration with only slight cytotoxicity for products C, D and E. These results show that ingredients like glutaraldehyde, quaternary ammonium and peroxide compounds have a high virucidal efficacy against HCV.

Survival of dried HCV on inanimate surfaces

Recently, it could be shown that HCV can be stable for several weeks in a liquid environment or in syringes [24, 25]. To evaluate the stability of non-liquid HCV, Jc1 virus was dried on carrier discs and incubated for several days at room temperature. As HCV infection is typically transmitted via blood, the effect of healthy serum on the stability of dried HCV was analyzed in parallel. Infectivity of dried virus in the presence of serum was reduced 10-fold after 2 days and reached undetectable levels after 6 days. Furthermore, the addition of serum resulted in reduced viral titers compared to the virus without serum (Figure 4*A*). In the latter case, we still could measure infectious HCV with a titer of about 30 $TCID_{50}$/ml after 7 days of incubation demonstrating a stability of dried HCV for more than a week on the carrier surface. In the next set of experiments, we analyzed if the addition of serum before the drying procedure influences the ability of the different biocides to inactivate HCV as reported for other viruses. The different alcohols or commercial disinfectants that were used in a concentration completely inactivated HCV as shown before (compare Figure 2 and 3). All tested biocides were able to inactivate HCV infectivity to

undetectable levels in the presence or absence of serum (Figure 4*B*), indicating that serum cannot confer viral resistance to the tested biocides.

Heat stability of HCV in a drug transmission assay

Epidemiologic studies indicate that the sharing of the drug preparation equipment among IDU's is an important risk factor for HCV transmission [18, 30]. Spoons and/or cookers are used to heat diluted heroin into solution. Cookers are mostly used in the US while spoons are mostly used in Europe. During the drug preparation spoons are often reused and shared between users. The drug dilution from the spoon is drawn into a syringe and blood contaminated with HCV can be exposed to the drug dilution by insertion of an HCV contaminated syringe into spoons that are shared. Therefore blood on spoons/cookers could be source for contamination with infectious HCV and the ability of the virus to survive on such surfaces can have a strong impact on cross-transmissions. To evaluate the transmission risk via this route, we contaminated a spoon with Jc1 reporter virus (Figure 5*A*). With the use of a tea candle increasing temperatures were simulated with the cooker device. At indicated time intervals, aliquots were taken and used to determine infectivity by luciferase reporter assay. Viral infectivity started to decrease at a temperatures of around 50°C and was below the detection limit at about 65-70°C in 9 independent measurement series (Figure 5*B*). The time required to reach certain temperatures depends highly on the experimental setup, but in our case about 80-95 seconds were necessary when small bubbles start to appear on the spoon. The half-life of HCV at different temperatures did not differ significantly between reporter virus and authentic wild-type HCV Jc1 (data not shown). Next, we tested the impact of water and serum in this drug transmission assay. As depicted in Figure 5C, the addition of

water or serum to the virus solution did not influence HCV stability. Again, about 65°C was the temperature required to inactivate viral infectivity to background levels.

Discussion

For better understanding and prevention of HCV transmission in medical settings and in the environment, experimental system simulating practical conditions are highly relevant. In this study, we addressed HCV inactivation and stability profiles on inanimate surfaces to mimic viral cross-transmissions among IDU's and in healthcare settings where HCV infections continue to occur. We demonstrated that HCV could be dried and recovered efficiently in a carrier assay that can therefore be used to validate chemical biocides in their virucidal efficacy against HCV. Importantly, it also confirms that reusing HCV contaminated cookers could lead to infection even if using sterile syringes. Furthermore, by simulating the procedure for heating drugs into solution, we showed that HCV could be eliminated at temperatures of 65-70°C. These data can be used for the design of public health recommendations and prevention of viral spread among IDU's. Until recently, experimental data about the environmental stability of HCV were not reported or performed with surrogate markers (antigens, RNA, enzyme activity) for the presence or absence of infectious particles. The HCV infection system used here is based on human hepatoma cells and viruses generated *in vitro* [21-23] and substantial progress has been made in HCV basic and translational research with this model [31]. However, limitations are that *in vivo* hepatocytes and patient-derived particles might be slightly different or that not all genotypes can be grown in cell culture.

In the environment, viruses are normally found on surfaces and/or embedded in body fluids like excrements, serum, blood or other excretions and the risk of viral transmission depends on the contact number, time, body parts and how readily the virus is released from such surfaces. The carrier test method for HCV developed here allows predicting the activity of chemical biocides simulating practical conditions. Dried HCV was exposed to a test product for a defined contact time. At

the end of the contact time, the virus-biocide mixture was recovered from the surface of the carrier and titrated to determine the degree of loss in virus infectivity. We could previously show in a quantitative suspension assay that 1-propanol is the most effective alcohol in activating HCV [24]. However, whereas in a suspension test a concentration of 20% 1-propanol was sufficient to eliminate Jc1 with a viral titer of 10^6 $TCID_{50}$/ml, higher concentration of the alcohol are needed to inactivate dried HCV due to a stronger challenge for the disinfectant [29]. Importantly, we could demonstrate that commercially available surface disinfectants have a high virucidal efficacy at concentrations recommended by the manufacturers as previously shown for hand antiseptics [24]. While dried virus in the presence of serum could survive for up to 5 days at room temperature, we could show that HCV in suspension could survive for even 3 weeks [24] and in syringes infectivity was detected for up to 63 days [25]. Kamili and colleagues demonstrated in a chimpanzee animal model that dried HCV derived from patient sera could survive for at least 16 hours, but was not detectable after storage of 4 or 7 days [32]. Differences in the viral dose, storage conditions or determination of infectivity *in vitro* or *in vivo* [33, 34] might account for the different survival times between these studies. We used here a highly sensitive detection assay and were able to determine precise survival times of the virus on dried surfaces in the presence or absence of serum. The transmission patterns for hepatitis B virus (HBV) are very similar to HCV and high stability in the environment has been reported for this hepatotropic virus as well [35]. In line with our results, infectivity after drying of HBV-positive human plasma could be detected for at least one week while no longer incubation times were analyzed [35]. In summary, these reports showed that HCV could remain viable for a prolonged time in the environment indicating that blood-contaminated surfaces can serve as HCV

reservoirs. Consequently, effective disinfection of surfaces is crucial in the prevention of HCV transmission.

Transmission of HCV remains high among IDU's in recent years, with incidence rates ranging from 16% to 42% per year [36]. Furthermore, the risk of HCV transmission estimated per exposure to a contaminated syringe is 5-fold to 20-fold higher than that of HIV [37-39]. Recently, Paintsil et al. contributed to the understanding of biological mechanisms of HCV transmission by studying contaminated syringes with HCV cell culture derived virus [25, 40]. They found that HCV survival was dependent on syringes type, time and temperature. Infectivity could be detected for up to 63 days in high void volume tuberculin syringes. These results suggest that this long survival contributes to the high prevalence of HCV in comparison to HIV among IDU's in spite of successful syringe exchange programs. Besides syringes, the sharing of drug cookers and cotton for filtration was also significantly associated with HCV infection independent of sharing needles and syringes [18, 30]. We could show here that HCV on a spoon as cooker can survive temperatures up to 65°C which corresponds to a heating time of 80-95 sec in this assay setup indicating that virus survival on cookers could be also a potential source of infectious HCV aside from syringes.

In summary, we could show that infectious HCV can persist as a dried sample for up to one week. The most effective alcohol to inactivate the virus was 1-propanol and commercially available disinfectants reduced HCV infectivity to undetectable levels emphasizing strict hygiene measurements. These experimental developments should facilitate testing the virucidal activity against HCV of chemical biocides used for surface disinfection. In addition, these results will further improve the understanding of HCV cross-contaminations and its prevention in healthcare settings and among injection drug users.

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Figure legends

Figure 1: Experimental setup for hepatitis C virus (HCV) carrier assay. A, Stainless steel discs were inoculated with 50 µl of Jc1 and dried under a laminar flow for about 1 hour. After drying, discs were transferred into 25 ml plastic vial holders, which were previously filled with glass beads. Biocides for testing were distributed onto the dried virus and incubated for 1 or 5 minutes. 900 µl of culture medium were immediately added at the end of the exposure time. The vials were directly vortexed for 1 minute, before the eluate was diluted to determine viral infectivity by immunohistochemistry. B, Jc1 virus was either incubated in suspension or dried on a carrier disc for 1 h before viral titers were determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infective dose 50 ($TCID_{50}$) values. The mean values of 8 independent experiments with standard errors are shown.

Figure 2: Effect of different kinds of alcohol against hepatitis C virus (HCV). A, 1-propanol, 2-propanol and ethanol were tested in a carrier assay for their efficacy in inactivating HCV. The alcohol concentrations ranged from 10% to 60% with an exposure time of 1 minute. Residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infective dose 50 ($TCID_{50}$)

values. The mean values of 2 independent experiments with standard errors are shown. B, different alcohols were tested in a carrier assay for their efficacy in inactivating HCV as described in A with an exposure time of 5 minutes. The mean values of 3 independent experiments with standard errors are shown.

Figure 3: Effect of commercial surface disinfectants against hepatitis C virus (HCV). A, Two alcohol-based commercial surface disinfectants (products A and B) were tested in a carrier assay for their virucidal efficacy against HCV. Concentrations of 10%, 50% and 100% were used with an exposure time of 5 minutes. Residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infective dose 50 ($TCID_{50}$) values. The mean values of 3 independent experiments with standard errors are shown. B, Four commercial surface disinfectants (products C-F) were tested in a carrier assay for their virucidal efficacy against HCV as described in *A* with concentrations of 0.025%, 0.25% and 0.5%. The mean values of 3 independent experiments with standard errors are shown.

Figure 4: Stability of dried hepatitis C virus. A, Jc1 in the presence or absence of human serum was dried on a carrier and incubated for several days at room temperature at indicated time points. Infectivity was determined by a limiting dilution assay. A representative experiment with 3 independent repetitions is shown. B, Different biocides at indicated concentrations and exposure time of 5 minutes were tested in a carrier assay for their efficacy in inactivating HCV in the presence or absence of human serum. Residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infective dose 50 ($TCID_{50}$)

values. The mean values of 3 independent experiments with standard errors are shown.

Figure 5: Hepatitis C virus stability on equipment for heating drugs into solution. A, For the drug transmission assay viral suspensions of 800 µl were used as inoculum of a spoon as cooker. A heating procedure was started with a tea candle. Temperatures of the suspensions were measured at specific time intervals and at given temperatures 70 µl of the viral suspension was sampled. Infectivity was determined by infection of naive Huh7.5 cells following luciferase reporter assay. B, Luc-Jc1 reporter virus was incubated at the indicated temperatures as a viral suspension with a volume of 800 µl on a spoon. Temperatures increased by the use of a tea candle. Infectivity of 70 µl aliquots was determined by infection of naive Huh7.5 cells following luciferase reporter assay. The values of nine independent measurement series are shown. C, Luc-Jc1 reporter virus was incubated on a spoon as described in A. Water and serum were added in a dilution of 1:8 with the virus suspension before temperatures were increased by the use of a tea candle. Infectivity of 70 µl aliquots was determined by infection of naive Huh7.5 cells following luciferase reporter assay. The values of at least two independent measurement series are shown.