How Stable Is the Hepatitis C Virus (HCV)? Environmental Stability of HCV and Its Susceptibility to Chemical Biocides

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Background. In the absence of a cell culture system for propagation of the hepatitis C virus (HCV), the antiviral activity of disinfectants against HCV was extrapolated from studies with the bovine viral diarrhea virus. The recent development of an HCV infection system allowed the direct assessment of environmental stability and susceptibility to chemical disinfectants.

Methods. Studies were performed using cell-culture grown HCV. Infectivity was determined by limiting dilutions. HCV RNA levels were analyzed by quantitative real-time polymerase chain reaction. Genome stability was determined by transfection of recovered RNA into Huh7.5 cells and immunostaining.

Results. HCV infectivity in a liquid environment was detectable for up to 5 month at lower temperatures. The risk of HCV infections may not accurately be reflected by determination of HCV RNA levels, because viral infectivity and HCV RNA copy numbers did not directly correlate. Different alcohols and commercially available antiseptics reduced the infectivity of HCV to undetectable levels. However, diluting the hand disinfectants abrogated the virucidal activity.

Conclusions. This study assessed the environmental stability and susceptibility to chemical biocides of HCV. The results should be useful in defining rigorous disinfection protocols to prevent nosocomial transmission of HCV.

Hepatitis C virus (HCV) infection is considered a major public health problem, with >130 million chronic carriers worldwide. HCV infection causes acute and chronic liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. Presently, there is no vaccine against HCV available, and health care workers are at constant risk of acquiring HCV infection from occu-

pational exposure. Moreover, nosocomial transmissions of HCV account for a large proportion of new HCV infections in the Western world. For instance, hospital admission was the only risk factor in about two-thirds of acute hepatitis C cases in Spain [2, 3] and Italy [3], and 15% of patients registered in the large German Hep-Net acute HCV database became nosocomially infected [4, 5]. Importantly, HCV transmission in health care units is often associated with breaches in aseptic techniques [6, 7]. For instance, a patient-to-patient outbreak of HCV genotype 3a infection was caused by shared saline bags, which were contaminated when reused syringes were used to draw blood from venous catheters [8]. Furthermore, due to the lack of appropriate cell culture and animal models susceptible to HCV infection, evidence-based guidelines on the prevention and management of occupational exposures are incomplete, because until now no data have been available regarding HCV stability and sensitivity toward chemical disinfectants.

HCV is a positive strand RNA virus belonging to the family Flaviviridae. Its 9.6-kb genome is composed of the 5′-nontranslated region (NTR), an open reading frame encoding a large polyprotein, and the 3′-NTR [9]. The polyprotein is cleaved into 10 individual proteins building up the virus particle (core, E1, or E2) or required for RNA replication (nonstructural proteins). Recently, a cell culture system based on the Japanese fulminant hepatitis (JFH) HCV isolate was developed, which reproduces the complete viral replication cycle in vitro [10–12]. Cell culture–derived HCV particles are infectious in animal models, thus confirming their authenticity. This system allowed us to address for the first time the environmental stability of HCV and its susceptibility against routinely used hand antiseptics.

In the past, inactivation of HCV by chemical disinfectants has been studied mainly with bovine viral diarrhea virus (BVDV) as a surrogate virus, because of the similarity in genome structure and mode of replication to HCV [13]. In this study, we aimed to systematically investigate the long-term ex vivo stability and sensitivity of HCV to different disinfectants and antiseptics.

MATERIAL AND METHODS

Plasmids and viruses. Constructs JFH1 and Jc1 have been described recently [10, 14]. Construct Luc-Jc1 encodes a chimeric HCV polyprotein, which consist of codons 1–846 derived from J6/CF [15] 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 ribosome entry site 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 the JFH1 core [16].

BVDV strain NADL (VR-534) was obtained from Dr Stephanie Bendtfeld (Institute of Virology, School of Veterinary Medicine, Hannover). Before inactivation assays, the virus was passaged once in primary bovine kidney cells and 5 times in KOP-R cells (primary cells from bovine oropharyngeal tissue).

Hand antiseptics. Seven hand antiseptics were chosen to study the efficacy against HCV, Including 3 hand scrubs and 4 hand rubs. Hand scrubs included product A (based on povidone-iodine), product B (based on chlorhexidine digluconate), and product C (based on triclosan). Hand rubs included product D (55 g of ethanol and 10 g of 1-propanol), product E (>90 g of ethanol), product F (55 g of ethanol and 10 g of 1-propanol), and product G (45 g of ethanol and 18 g of 1-propanol).

Cell culture. Huh7.5 cells were cultured in Dulbeccòs modified Eagle medium (Invitrogen) with 10% fetal bovine serum, 1× nonessential amino acids (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 100 IU/mL penicillin (Invitrogen).

KOP-R cells for BVDV replication originated from the Bundesforschungsanstalt für Viruskrankheiten der Tiere (Dr R. Riebe).

In vitro transcription, electroporation, and production of cell culture–derived HCV. On the basis of a DNA plasmid encoding the JFH1 isolate or derivatives thereof [11, 14, 17], in vitro transcripts of the individual constructs were generated, as described elsewhere [16]. After 48 h, supernatants were collected and filtered through filters with 0.45-μm pores. To determine viral infectivity, cell-free supernatants were used to infect naive Huh7.5 target cells.

Preparation of BVDV suspension. For the preparation of the test virus suspension, KOP-R cells were cultivated with Eagle's minimal essential medium (Cambrex Bio Science Verviers) supplemented with l-glutamine, sodium pyruvate, and 10% or 2% fetal calf serum (Biochrom; antibodies against BVDV not detectable) and infected with BVDV (stock virus suspension). As soon as cells showed a constant cytopathic effect, they were subjected to a rapid freeze-thaw procedure. This was followed by low-speed centrifugation (10 min at 1000g) to sediment cell debris. Test virus suspension was stored at −80°C.

Determination of BVDV infectivity. Infectivity was determined by means of end-point dilution titration in a microprocedure. For this, samples were diluted with ice-cold Eagle's minimal essential medium with 2% fetal calf serum, and 100 μL of each dilution was placed in 8 wells of a microtiter plate. KOP-R suspension was adjusted to reach approximately 10–15 $\times$ 10^3 cells per well. Finally, cultures were observed for cytopathic effects for 10 days of inoculation.

Luciferase assay. Infected or transfected cells were washed twice with phosphate-buffered saline (PBS) and lysed in 1000 μL/well (6-well plate) or 350 μL/well (12-well plate) luciferase lysis buffer (1% Triton X-100, 25 mmol/L glycylglycine, 15 mmol/L magnesium sulfate, 4 mmol/L ethylene glycol tetraacetic acid, and 1 mmol/L dithiothreitol, pH 7.8). Firefly luciferase activity was measured as described elsewhere [16], using a luminometer (Lumat LB 9507; Berthold).

RNA quantification by reverse-transcription polymerase chain reaction (RT-PCR). RNA was isolated from infected cells using a NucleoSpin RNA II Kit (Macherey-Nagel). HCV RNA was quantified using a LightCycler 480 (Roche), as described elsewhere [18, 19].

Indirect immunofluorescence. Huh7.5 cells were seeded onto glass coverslips in 24-well plates at a density of 2 $\times$ 10^4 cells/cm^2 per well; they were fixed 48 h after infection using PBS supplemented with 3% paraformaldehyde for 10 min at room temperature. Staining of NS5A was performed as described elsewhere [18].

Testing HCV stability in the environment. To test the effect of different temperatures on HCV infectivity, viral suspensions
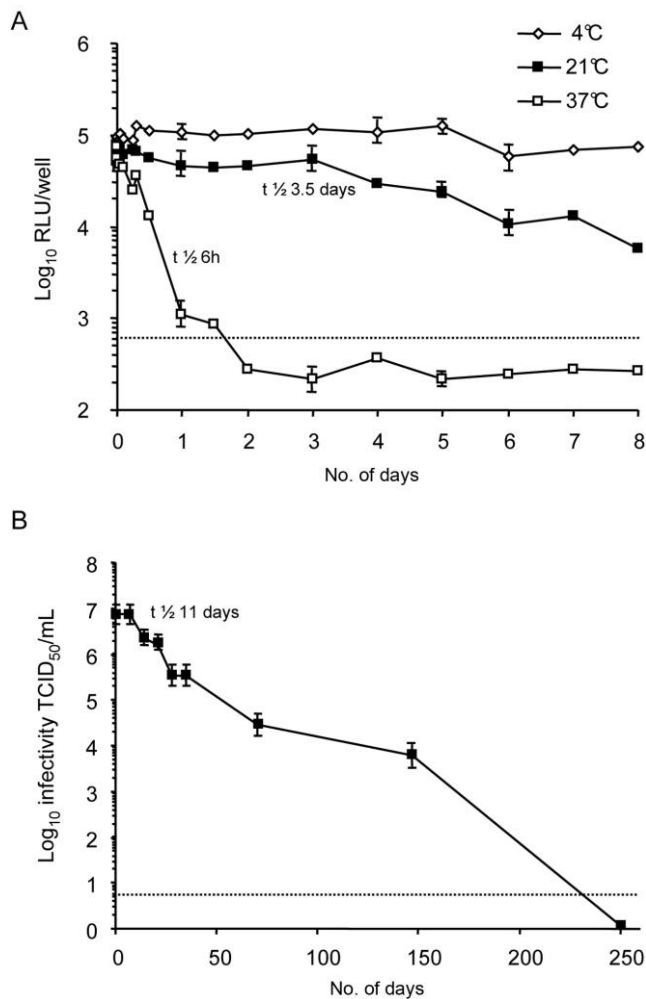
A

B

Figure 1. Stability of hepatitis C virus (HCV) at different temperatures. *A*, Luc-Jc1 virus was incubated at the indicated temperatures and time intervals as a test virus suspension. Infectivity was determined by inoculation of naive Huh7.5 cells for 4 h. HCV infection was quantified by measuring HCV reporter activity 48 h later. A representative experiment with 3 independent repetitions is shown. RLU, relative light units; $t_{1/2}$, half-life. *B*, Jc1 wild-type virus was stored for up to 252 days at 4°C. At the indicated time points, virus aliquots were tested for infectivity by a limiting dilution assay; $TCID_{50}$, 50% tissue culture infective dose. A single long-term experiment was performed.

were stored in microfuge tubes at 4°C, 21°C, or 37°C for the respective time period. Whole-blood samples from healthy donors were centrifuged for 5 min at 850*g* to obtain serum. The effect of serum on HCV stability was tested by mixing serum and virus suspension at a ratio of 1:1 in a total volume of 1 mL. To assess the influence of virus exposure to different surfaces, microfuge tubes (Sarstedt) were used as plastic material, screws (stainless steel, 1.4301 CrNi 18/8) as a steel surface, and Peha-soft vinyl powder-free gloves (Paul Hartmann) as gloves. Virus suspension (1 mL) was incubated on the surfaces without drying. Reporter activity after different incubation times was

measured, as described above. To address the importance of different pH values, viral suspensions were diluted in TN buffer (10 mmol/L Tris-HCl, pH 7.0; 50 mmol/L sodium chloride) and incubated at a ratio of 1:1 for 4 h during the infection process, as described elsewhere [20].

Quantitative suspension assay. For HCV and BVDV in vitro inactivation, experiments were carried out by mixing 1 part of test virus suspension with 9 parts of the different kinds of alcohol or hand antiseptics. After incubation with short exposure times, test mixtures were immediately serially diluted
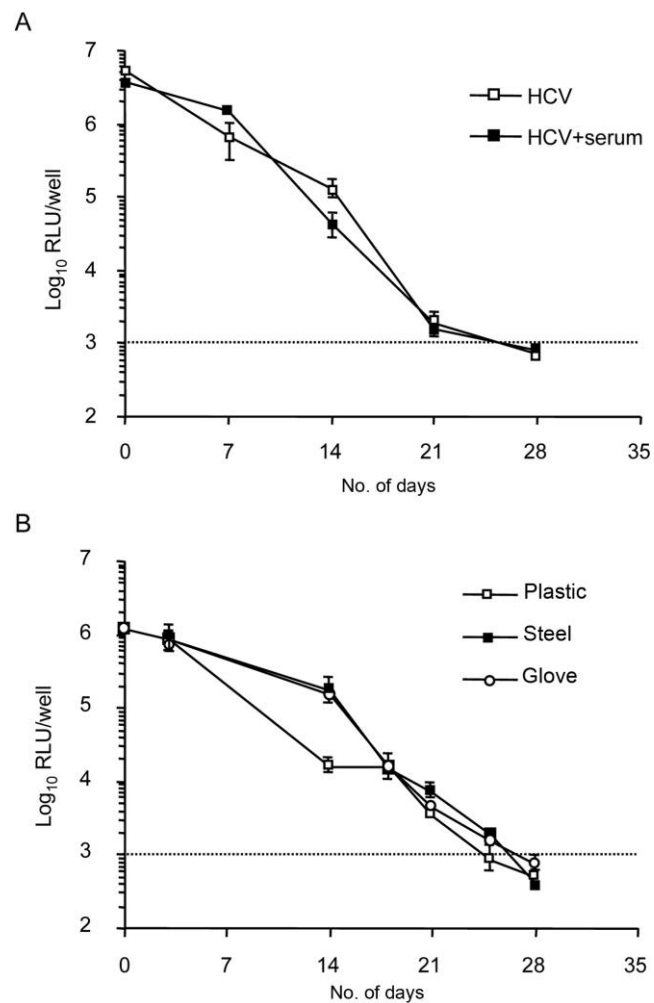
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Figure 2. Importance of serum and different surfaces for hepatitis C virus (HCV) stability. *A*, Luc-Jc1 virus was stored in the presence of human serum or medium at room temperature at the indicated time points. Infectivity was determined by inoculation of naive Huh7.5 for 4 h. HCV infection was quantified by measuring HCV reporter activity 48 h later. A representative experiment with 3 independent repetitions is shown. RLU, relative light units. *B*, HCV reporter virus Luc-Jc1 was incubated as a viral suspension on plastic, steel, or gloves at room temperature for the indicated time points. HCV reporter infectivity was determined by inoculation of naive Huh7.5 cells. A representative experiment with 3 independent repetitions is shown.
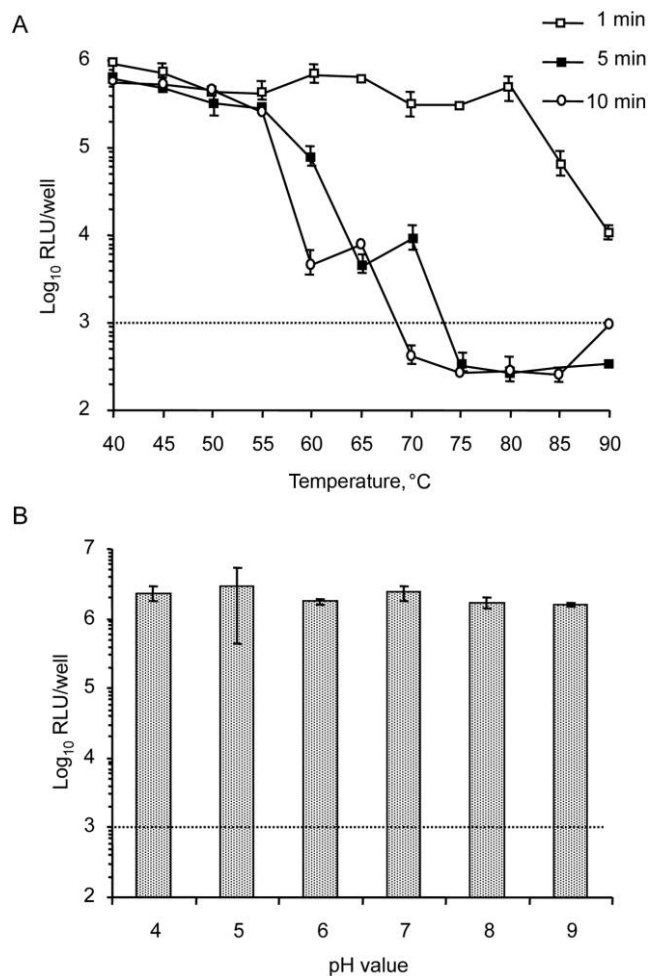
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Figure 3. Effect of heat and different pH values on hepatitis C virus (HCV) stability. *A,* Luc-Jc1 virus was incubated at the indicated temperatures and time intervals as a viral suspension with a volume of 300 μL in a heating block. Infectivity was determined by inoculation of naive Huh7.5 cells for 4 h. HCV infection was quantified by measuring HCV reporter activity 48 h later. RLU, relative light units. *B,* Luc-Jc1 reporter viruses were treated with different pH values after determination of HCV infectivity by inoculation of naive Huh7.5 cells and detection of luciferase activity. A representative experiment with 3 independent repetitions is shown (*A* and *B*).

in Dulbeccòs modified Eagle medium to stop activity of the biocides and then titrated in 96-well microplates. To reduce cytotoxicity of biocides, the test mixture was purified via gel filtration with MicroSpin S-400 HR columns (GE Healthcare), according to the manufacturer's instructions. Virus titers were measured using end-point dilution titration, as described elsewhere [11]. Virus titers (50% tissue culture infective dose [$TCID_{50}$/mL]) were calculated according to the method of Spearman and Kärber [19, 21]. To determine the cytotoxicity of the disinfectants, 1 part of PBS was mixed with 9 parts of the disinfectant and inoculated into permissive cells. Cytotox-

icity was determined by examining permissive cells by microscopy for any significant changes in the cell monolayer and calculated analogously to virus titers ($TCID_{50}$/mL).
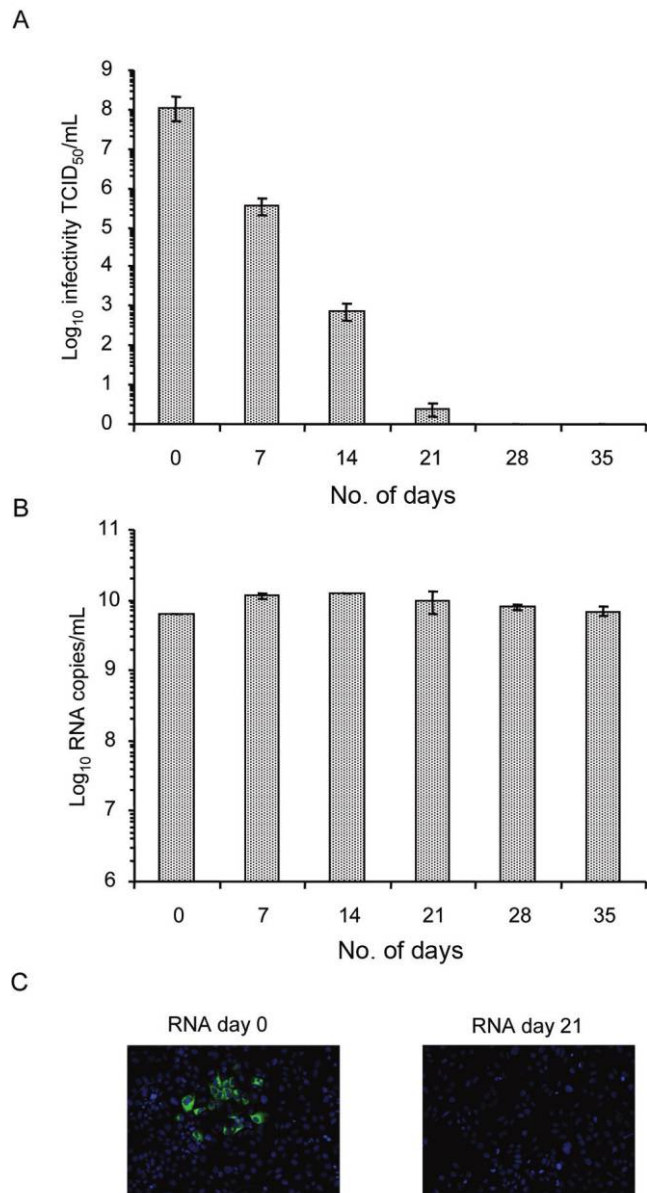
A

B

C

RNA day 0   RNA day 21

Figure 4. Correlation of viral infectivity with hepatitis C virus (HCV) RNA copy numbers. *A,* Wild-type Jc1 virus was stored for up 35 days at room temperature. Every 7 days viral titers were determined by a limiting dilution assay; $TCID_{50}$, 50% tissue culture infective dose. *B,* HCV RNA of the respective viral supernatant was isolated and quantified by reverse-transcription polymerase chain reaction. *C,* The HCV RNA isolated from days 0 and 21 was used for reelectroporation of Huh7.5 cells. After 48 h, cells were fixed and stained by immunofluorescence for the HCV protein NS5A. A representative experiment with 2 independent repetitions is shown (*A–C*).
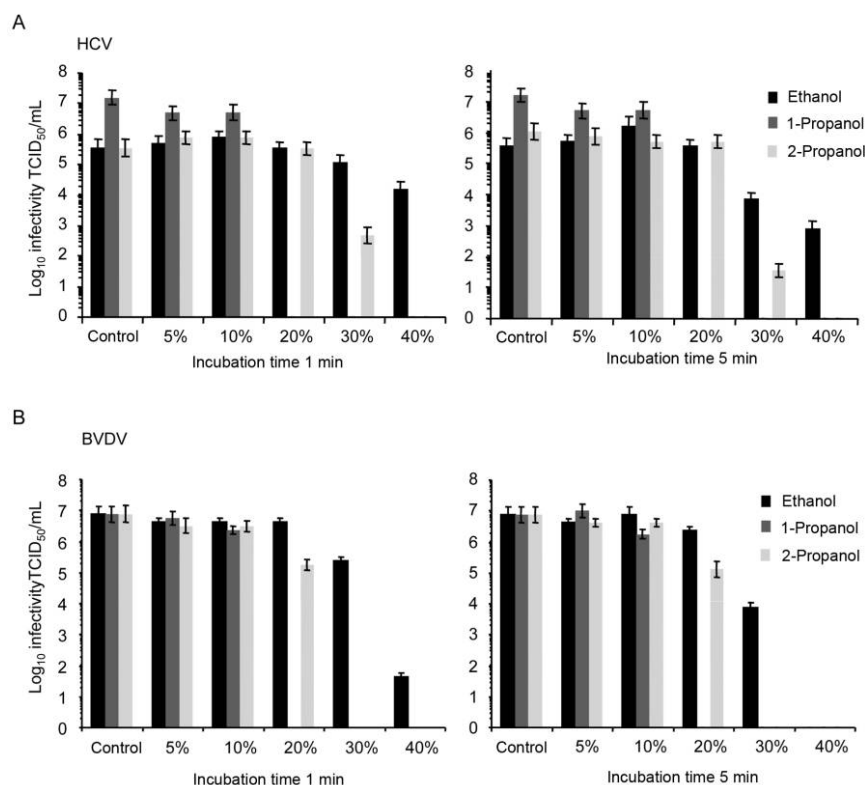
Figure 5. Comparison of the inactivation of hepatitis C virus (HCV) and bovine viral diarrhea virus (BVDV) by different kinds of alcohol. *A,* Ethanol, 1-propanol, and 2-propanol were tested for their efficacy in inactivating HCV. The biocide concentrations ranged from 5% to 40%, with exposure times of 1 or 5 min. For this inactivation assay, 1 part virus is mixed with 9 parts alcohol. Residual infectivity was determined by end-point dilution titration. Viral titers are displayed as 50% tissue culture infective dose ($TCID_{50}$) values. *B,* Efficacy of ethanol, 1-propanol and 2-propanol against BVDV was addressed by a quantitative suspension assay, as described for panel *A.* Viral titers are given as $TCID_{50}$ values. A representative experiment with 3 independent repetitions is shown (*A* and *B*).

RESULTS

Stability of HCV infectivity at different temperature. To evaluate HCV stability at different temperatures, HCV reporter viruses encoding for the firefly luciferase gene were incubated at 4°C, 21°C, and 37°C for up to 10 days, respectively. Infectivity was determined by inoculation of Huh7.5 cells and determination of HCV luciferase activity was performed for reporter viruses (Figure 1*A*) or by a limiting dilution assay for wild-type virus (Figure 1*B*). At 4°C, HCV infectivity was only slightly reduced after 8 days, whereas incubation at room temperature resulted in a 10-fold decrease of infectivity at this time point. Inactivation of HCV to background levels was detected at 37°C after just 2 days, indicating that HCV stability is temperature dependent (Figure 1*A*). Next, we performed long-term incubations of HCV at 4°C for up to 252 days. Interestingly, after 147 days of incubation at 4°C, infectivity was still detectable and decreased to background levels only after 252 days (Figure 1*B*). The half-life of HCV at different temperatures did not differ between reporter virus and authentic wild-type HCV (data not shown).

Influence of serum and different surfaces on HCV stability. Because HCV in vivo is typically associated with serum, the effect of a healthy individual's serum was analyzed by incubation of HCV at room temperature for ≥28 days in the presence and absence of serum. Infectivity was determined by measuring HCV reporter activity. The addition of serum did not change viral titers that decreased over time, reaching undetectable levels after 21 days of incubation at 21°C, indicating that human serum has no influence on the stability of HCV (Figure 2*A*).

Because health care workers or patients could be exposed to HCV by contaminated samples on different surfaces, we next determined whether incubation of the virus in a liquid suspension on plastic, steel, or gloves could change HCV stability. As depicted in Figure 2*B*, no significant difference between the tested surfaces was detected.

Influence of high temperature and different pH on HCV infectivity. Careful handling of HCV-positive materials in hospitals or medical practice can prevent nosocomial transmission of the virus. However, it is still unknown how efficiently
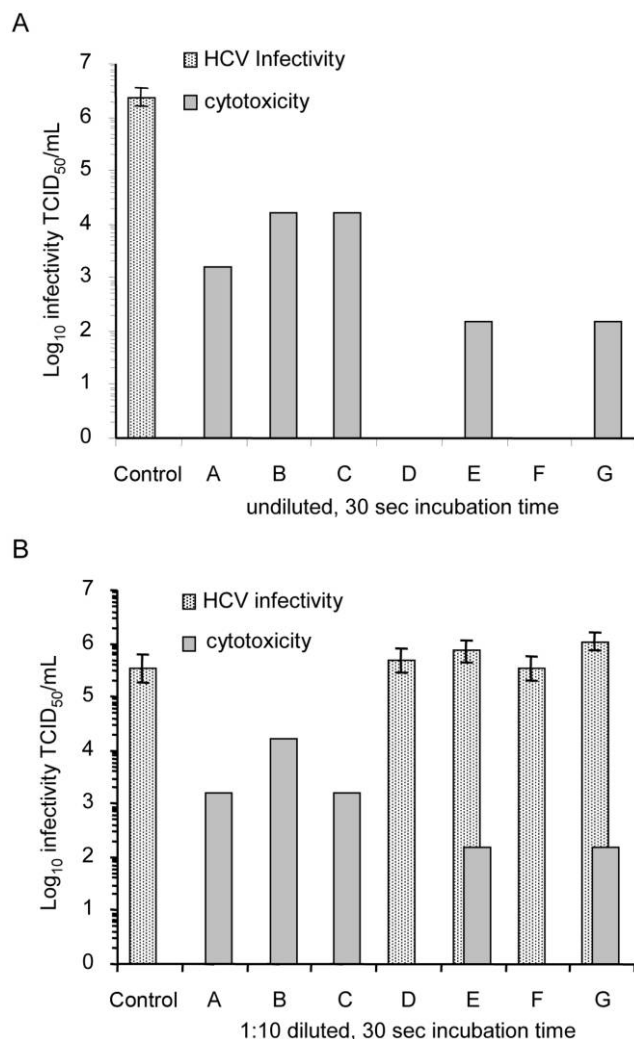
Figure 6. Effect of commercial hand antiseptics against hepatitis C virus (HCV). *A*, Seven commercial hand antiseptics (products A–G) were tested in a quantitative suspension assay for their efficacy in inactivating HCV. Incubation times of 30 s were used, with different products at 90% concentrations. $TCID_{50}$, 50% tissue culture infective dose. *B*, Commercial disinfectants (products A–G) were diluted 1:10 with water and evaluated in a quantitative suspension assay, as described for *A*. A representative experiment with 3 independent repetitions is shown (*A* and *B*).

HCV can be inactivated by heat. Viral suspensions were incubated for 1, 5 or 10 min at temperatures ranging from 40°C to 90°C. Residual HCV infectivity was still detectable at temperatures of 60°C–65°C and incubation times of 5 or 10 min. More than 100-fold reduction of infectivity to less than the detection limit of the assay was observed after 5-min exposure of the virus to 75°C or 1-min exposure at 70°C (Figure 3*A*). In addition, the importance of changes in the pH for viral stability was tested by incubating HCV at different pH values for 4 h after determination of HCV reporter activity. HCV infectivity was not affected at pH values ranging between 4 and 9 (Figure 3*B*).

HCV infectivity in comparison with HCV-RNA copy numbers. An unconventional route of HCV transmission can be household objects, such as tooth brushes, razors, or nail scissors. There are several reports demonstrating that HCV RNA is detectable on such devices, as well as on medical equipment [22–25]. Moreover, the presence of HCV genomes in biological fluids other than blood has been described. However, it is unclear to what extent presence of HCV genomic RNA on household devices, medical equipment, or different body fluids correlates with a risk of infection.

We therefore investigated viral infectivity and HCV RNA copy numbers during prolonged storage of the virus at room temperature. Whereas viral infectivity was significantly reduced after 21 days and below the detection limit of the assay at later time points (Figure 4*A*), RNA copies numbers of almost 10^{10} copies/mL were measured throughout the whole incubation time of 35 days (Figure 4*B*). To determine whether the loss of infectivity was due solely to the inability of the virus particle to penetrate into cells via the normal route of entry, or whether the viral genome itself was no longer infectious, we transfected the virus particle associated RNA from the preparation at days 0 and 21 into highly permissive Huh7.5 cells. Successful initiation of viral RNA replication was assessed by visualizing HCV antigen–positive cells with immunofluorescence. HCV RNA recovered from virions at day 0 was infectious with 180 focus-forming units and replicated efficiently in the human hepatoma cell line when introduced by transfection. In contrast, no HCV antigen positive cells were detected when HCV RNA from viruses at day 21 was transfected (Figure 4*C*).

Effect of ethanol, 1-propanol, and 2-propanol on HCV stability. Ethanol, 1-propanol, and 2-propanol are active ingredients of commercial alcohol-based antiseptics and disinfectants used in medical settings. In recent years, BVDV as surrogate virus has been used to assess virucidal efficacy of antiseptics because of the lack of appropriate cell culture models for HCV. To determine the efficacy of different kinds of alcohol on HCV (Figure 5*A*) and BVDV (Figure 5*B*) stability, we incubated the 2 viruses for 1 or 5 min with ethanol, 1-propanol, or 2-propanol at concentrations ranging from 5% to 40%. The most effective alcohol to inactivate HCV and BVDV was 1-propanol, reducing viral titers to background levels at a concentration of 20% after exposure times of 1 and 5 min, respectively. In the case of ethanol, HCV titers decreased at concentrations of 30% and 40%, but complete inactivation was observed only for BVDV, at a concentration of 40% and a 5-min incubation time. Compared with BVDV, HCV was slightly more resistant to ethanol and 2-propanol. Furthermore, we tested the HCV inactivation efficiency of glutaraldehyde and peracetic acid as 2 types of high-level disinfectants. Glutaraldehyde at 0.5% and peracteic acid at 0.05% were able to com-

pletely inactivate HCV within a 1-min incubation time (data not shown).

Virucidal efficacy of commercially available disinfectants against HCV. In addition, 7 commercial hand antiseptics were tested for their ability to inactivate HCV in a quantitative suspension test. An incubation time of 30 s was chosen, and different products (products A–G) were evaluated at concentrations of 90% (Figure 6A) or 1:10 dilutions (10%) (Figure 6B). Undiluted, all tested commercial antiseptics inactivated HCV infectivity to undetectable levels (Figure 6A). Next, we investigated whether a 1:10 dilution of the commercial antiseptics affected the virucidal activity of the products. As depicted in Figure 6B, additional dilution of the alcohol-based hand rubs D–G abrogated the inactivation properties of these hand antiseptics. In contrast, the hand-washing products A–C—on the basis of povidon-iodine, chlorhexidine digluconate, and triclosan, respectively—were effective against HCV at a concentration of 10%.

DISCUSSION

In this study, we performed a comprehensive analysis of the stability of the HCV using the recently developed cell culture system fully permissive for HCV infection and replication. This infection system is based on human hepatoma cells and viruses generated in vitro, which might differ from normal human hepatocytes and patient-derived viral particles in some aspects. However, given the recent progress that has been made in HCV research applying this system [26], cell culture–derived HCV particles are considered the best tool to address the questions described here.

Whereas HCV was surprisingly stable at room temperature and 4°C, addition of human serum or incubation on different surfaces did not change the stability profile of the virus [27]. Given that high-titer preparations of HCV retain well-detectable infectivity for several days even when kept at room temperature, it is conceivable that HCV contaminated materials and infusions represent a substantial risk for transmission. Therefore, these data have important implications for the prevention of nosocomial transmission and patient-to-patient outbreaks of viral hepatitis [28]. The transmission pattern of hepatitis B virus (HBV) is similar to those of HCV, and this hepadnavirus was also shown to be quite stable in the environment. After drying and storage of HBV-positive human plasma for 1 week, inoculation of a chimpanzee resulted in active HBV infection [29]. Longer incubation times were not addressed in this study.

An experimental chimpanzee model was also used by Kamili et al [30], who dried and stored HCV plasma for 16 h, 4 days and 7 days at room temperature after inoculation of the samples in a chimpanzee. No infection occurred after inoculation with the material stored for 7 or 4 days, however, but with the 16-h exposure sample, an HCV infection developed in the animal. It is important to keep in mind that so far no clear correlation between tissue culture infectious dose and chimpanzee infectious dose has been established for HCV. Therefore, and also because of different storage conditions and divergent viral doses of the starting material, it is not possible to directly compare the duration of HCV infectivity between these studies. Moreover, development of HCV-specific cellular immunity might also affect the results [31, 32]. Because chimpanzee experiments are highly limited owing to ethical considerations and high costs, assessment of HCV infectivity with the tissue culture assays described in this study should be a valuable way to assess the risk of viral transmission [10, 11].

Importantly, we demonstrate that the quantity of HCV RNA does not necessarily correlate with viral infectivity, which was also observed in the in vivo study by Kamili et al [30]. Several studies that addressed different risks of HCV transmission [22–24, 33] were based on the detection of HCV RNA by RT-PCR. The lack of correlation between the detection of genomic copies (determine by RT-PCR) and infectivity (determined by $TCID_{50}$), or even viral genome intactness (determined by retransfection assay), should be taken into account in interpreting HCV RT-PCR–positive results in relation to the risk to human health; similar findings have also been reported for other viruses [34–36]. One possible explanation for these findings is that only short fragments of HCV RNA genome are amplified by RT-PCR, and improvements in the diagnostic tools for amplifying the full-length RNA genome would substantially strengthen the reliability of test results. Additional clinical and experimental studies are required to better evaluate the infectivity and stability of HCV in breast milk or semen and the risk of transmission associated with HCV in these fluids.

Regarding the inactivation profiles of HCV for different kinds of alcohols, we demonstrated that 1-propanol was the most effective alcohol in quantitative suspension tests. Overall, the comparison of HCV and its often-used surrogate BVDV demonstrated only slight variations in susceptibility to the substances. Inactivation of HCV by commercial antiseptics showed an advantage of hand scrubs over alcohol-based disinfectants at a dilution of 1:10, which is due to the different mechanisms of inactivation for an enveloped virus. Because under practical conditions antiseptics are not diluted, no conclusion can be drawn favoring scrubs for hand hygiene.

In summary, this first systematic investigation of HCV stability and sensitivity to different disinfectant and antiseptics has important implications for the management of occupational exposures to HCV. We propose that very strict compliance with established hygienic guidelines should be mandatory to avoid nosocomial and occupational HCV infections, even if materials have been contaminated with HCV several weeks earlier.

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