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Thermostability of seven hepatitis C virus genotypes in
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Thermo-stability of seven Hepatitis C virus genotypes *in vitro* and *in vivo*

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Abstract

Hepatitis C virus (HCV) is transmitted primarily through percutaneous exposure to contaminated blood especially in health care settings and among people who inject drugs. The environmental stability of HCV has been extrapolated from studies with the bovine viral diarrhea virus or was so far only addressed with HCV genotype 2a viruses. The aim of this study was to compare the environmental and thermo-stability of all so far known seven HCV genotypes *in vitro* and *in vivo*. Incubation experiments at room temperature revealed that all HCV genotypes showed similar environmental stabilities in suspension with viral infectivity detectable for up to 28 days. The risk of HCV infection may not accurately be reflected by determination of HCV RNA levels. However, viral stability and transmission risks assessed from *in vitro* experiments correlated with viral infectivity in transgenic mice containing human liver xenografts. A reduced viral stability for up to two days was observed at 37°C with comparable decays for all HCV genotypes confirmed by thermodynamic analysis. These results demonstrate that different HCV genotypes possess comparable stability in the environment and that non-infectious particles after incubation *in vitro* do not cause infection in an HCV *in vivo* model. These findings are important for estimation of HCV cross-transmission in the environment and indicate that different HCV genotypes do not display an altered stability or resistance at certain temperatures.

Keywords: Hepatitis C virus, thermo-stability, genotypes, thermodynamics

Introduction

Hepatitis C virus (HCV) is considered a major public health problem, infecting about 160 million people worldwide, representing 2.3% of the human population (1). These patients are at risk of potentially life-threatening hepatic complications including cirrhosis, liver failure and hepatocellular carcinoma. In fact, chronic HCV infection is associated with about 30 % of liver cancers worldwide and among the leading indications for orthotopic liver transplantation (2). HCV is a positive strand RNA virus belonging to the family *Flaviviridae* (3). Different patient isolates are grouped into 7 genotypes which differ on the nucleotide level by 31-33% and contain a variable number of subtypes with 20-25% nucleotide sequence variation. Current treatment, a combination of polyethylene glycol (PEG)-conjugated interferon alpha (IFN- α) and ribavirin, is only effective in a fraction of patients and associated with severe side effects (4). Several direct acting antiviral drugs are in clinical development and two specific protease inhibitors, Telaprevir and Boceprevir, were licensed in 2011.

The different HCV genotypes show a very distinct pattern in terms of geographic distribution, pathology and therapy response. While genotypes 1 and 4 are more resistant to interferon treatment, genotype 3 is associated with a high level of hepatic steatosis and also faster fibrosis progression. The most prevalent genotype in Europe and the USA is genotype 1 followed by genotype 2 and 3 which are especially common in people who inject drugs. Genotype 3 is very common in India and Thailand, whereas genotype 4 is the major cause for hepatitis C in Egypt and the Middle East. HCV genotype 5 is found mainly in South Africa and genotype 6 in Southeast Asia (5).

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 (6-8). Due to the lack of appropriate cell culture

and animal models susceptible to HCV infection, evidence-based guidelines on the prevention and management of HCV transmissions are incomplete or are based on studies with bovine viral diarrhoea virus (BVDV) as a surrogate virus. This hurdle could be overcome with the development of a HCV cell culture system that is based on the *Japanese Fulminant Hepatitis* (JFH) HCV isolate and reproduces the complete viral replication cycle *in vitro* (9-11). This system allowed for the first time to address environmental viability and inactivation of HCV (12-15). However, these studies were all based on genotype 2a isolates and thermo-stability of other HCV might be different which was not addressed so far.

In this study, we aimed to investigate the environmental and thermo-stability of all HCV genotypes at room temperature and 37°C. We further assessed if inactivation *in vitro* correlates with infectivity *in vivo* by infecting mice with a humanized liver (16). These results are important for assessment of HCV transmission risks and should help in defining stringent public health interventions to prevent HCV infections.

Material and Methods

Plasmids and viruses

The plasmid pFK-Jc1 has been described recently and encodes the intragenotypic 2a/2a chimeric virus Jc1 (17). Intergenotypic chimeras with core-NS2 of genotype 1 to 7 with UTRs and NS3-NS5B of JFH1 were described (18-22).

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).

In vitro transcription, electroporation and production of cell culture-derived HCV

Infectious HCV particles were produced as described previously (23). Briefly, Jc1 plasmid DNA was linearized and transcribed into RNA, which was then electroporated into Huh7.5 cells. Virus-containing culture fluids were harvested after 48 or 72 hours filtered through a 0.45 µm pore size filter. For determination of viral infectivity cell-free supernatants were used to infect Huh7.5 target cells.

Determination of HCV infectivity

Titers of infectious virus were determined by using a limiting dilution assay on Huh7.5 cells with a few minor modifications and tissue culture infectious dose 50 (TCID₅₀) was determined as described (9).

RNA quantification by reverse transcription PCR (qRT-PCR):

RNA from cell culture supernatants was isolated using viral RNA Kit (Roche). Two microliters of RNA sample was used for quantitative reverse transcription-PCR (qRT-PCR) analysis using a Light Cycler 480 device (Roche, Mannheim, Germany) as described previously (24).

Infection of Human liver-uPA^{+/+}-SCID mice

The mouse study was conducted at the Ghent University Hospital, with protocols approved by the Animal Ethics Committee of the Ghent University Faculty of Medicine. Transgenic SCID mice overexpressing the uPA gene under the control of an albumin promoter (uPA^{+/+}-SCID) were xenografted with primary human hepatocytes (BD Biosciences, Erembodegen, Belgium) as previously described (16). Chimeric mice were inoculated by intraperitoneal injection of 10-100 µl of the respective cell culture supernatants before or after incubation at room temperature for several weeks. EDTA plasma samples were collected at weekly intervals after inoculation and infection was monitored by a commercial qRT-PCR kit (Roche COBAS AmpliPrep/TaqMan48 assay, Roche Diagnostics).

Thermodynamic analysis

Viral structural stability was assessed based on the infectivity loss of viral particles using chemical kinetics formalism (25). TCID₅₀ measured over the time course was fitted to a linearized first-order kinetic equation (Eq. 1) in order to obtain the rate constant (k) of structural disintegration of viruses.

$$\ln C = \ln C_0 - kt \tag{1}$$

where C_0 and C are TCID₅₀ at time zero and t . Thermodynamic characteristics of the transition state in the viral structural disintegration was assessed by obtaining Gibbs

free energy (ΔG_a^0) enthalpy (ΔH_a^0) and entropic energy ($T\Delta S_a^0$) of activation of the process. The values of k at two different temperatures were analyzed using both Eq. 2 and Eq. 3 to obtain those thermodynamic values.

$$\Delta G_a^o = -RT \ln \frac{kh}{k_B T} \quad (2)$$

$$\Delta G_a^o = \Delta H_a^o - T\Delta S_a^o \quad (3)$$

where R is the gas constant ($= 8.314 \text{ J/(K mol)}$), h is Planck's constant ($= 6.63 \times 10^{-34} \text{ J s}$) (26), and k_B is the Boltzmann constant ($= 1.38 \times 10^{-23} \text{ J/K}$). Graph preparation and fitting was performed using SigmaPlot (version 11, Systat Software Inc, San Jose, CA).

Results

Stability of seven HCV genotypes at room temperature

Recently, it was demonstrated that cell culture derived HCV genotype 2a viruses retained infectiousness at room temperature for several weeks. However, infectivity of other HCV genotypes has not been analyzed (12, 14). To compare the environmental stability of all known seven HCV genotypes, we used HCV chimeras, in which core to NS2 proteins from each genotype was fused to the non-structural proteins of the JFH1 genome. Viral stocks of different genotypes were incubated in microfuge tubes at room temperature (21°C) for 35 days. Infectivity was determined by inoculation of Huh7.5 cells followed by a limiting dilution assay. As the various HCV genotypes propagate with different viral load depending on their viral fitness in tissue culture, the initial viral titers of the experiment ranged from 10^5 to 10^9 TCID₅₀/ml (Figure 1A). Infectivity at room temperature after 14 days of incubation was detectable for all viruses with half-life time of about 3-4 days and for genotype 2a viruses infectivity was still detectable after 28 days. For the genotypes with lower viral titers (1a, 1b, 4a, 3a) viral stability could be detected for up to 21 or 28 days, respectively. Assuming the infectivity loss was due to structural disintegration of the viral particles, rate constants of viral structural disintegration were obtained by fitting a linearized form of first-order kinetic equation. Based on that regression analysis, we found that the structural decay of HCV can be considered as a first-order kinetic process (Figure 1B). The decay in infectivity displayed as rate constants determined by structural disintegration is comparable for HCV genotypes 1-7 indicating similar stability at room temperature for all HCV strains (Figure 1B). As several studies addressing the risk of HCV transmission are based on the detection and quantification of HCV RNA by qRT-PCR, we quantified the RNA copy numbers of all

HCV genotypes over time (Figure 1C). In contrast to the viral titers, RNA copy numbers were only slightly reduced after 35 days demonstrating no clear correlation between the detection of genomic RNA and viral infectivity. In summary, the environmental stability of HCV genotypes is comparable at room temperature and the risk of HCV infection may not accurately be reflected by determination of HCV RNA levels.

In vivo infectivity of HCV genotypes after storage at room temperature

HCV displays a distinct and narrow tissue and host species tropism and naturally infects only humans and chimpanzees. The latter therefore are the only reliable immunocompetent animal model. However, their use is limited due to ethical reasons, high costs and restricted availability (27). Thus, transgenic mice containing human liver xenografts have been described and are permissive for HCV infection of all genotypes described so far (16, 28, 29). By using this model system, viruses from all genotypes that were incubated at room temperature were evaluated for their infectivity and risk of transmission *in vivo*. Therefore, we inoculated transgenic SCID mice overexpressing the uPA gene under the control of an albumin promoter (uPA^{+/+}-SCID) that harbor human primary hepatocytes in their liver with the viral samples of day 0 and a pool of all HCV genotypes that were non-infectious *in vitro* after several weeks (see Figure 1A). As depicted in Figure 2, all HCV genotypes from the initial inoculum of the incubation at room temperature experiment (day 0) were infectious *in vivo* with detectable HCV RNA copy numbers exceeding 10³ IU/ml three weeks after inoculation (Figure 2). The virus pool from all genotypes that were non-infectious in tissue culture caused no infection in the chimeric mice (Figure 2) and also after 8 weeks post-inoculation no HCV RNA could be detected (data not

shown). These results demonstrate that viral stability and transmission risks assessed from *in vitro* experiments correlate with viral infectivity *in vivo*.

Stability of all HCV genotypes at 37°C

Next, we evaluated HCV stability of all viral genotypes at 37°C. Different HCV genotypes were incubated in microfuge tubes at 37°C for 48 h and infectivity was determined by inoculation of Huh7.5 cells followed by a limiting dilution assay. Reduction of HCV titers to background level was detected for most genotypes at 48 h (Figure 3A). Genotype 1a with the lowest initial viral titer was undetectable after 32 h. Genotype 2a and 4a viruses that had starting titers over 10^6 TCID₅₀/ml were still infectious 48 h after incubation at 37°C. Considering the input titers no significant detectable differences in viral decay of all HCV genotypes could be observed. This was confirmed by calculating the rate constant of structural disintegration for all HCV strains. As seen in Figure 3B, the decay over time as rate constants determined by structural disintegration was comparable for the genotypes 1-7. Determination of HCV RNA copy numbers of the respective samples demonstrated no reduction in viral genome numbers after incubation for 24 h at 37°C and only a slight reduction after 48 h (Figure 3C). In conclusion, all HCV genotypes showed similar stability of around 2 days at 37°C.

Thermodynamic analysis of HCV genotypes

HCV was found to be more stable at 21°C than at 37°C as the rate constant in Eq. (1) has a higher value at 37°C (Table 1). To identify a thermodynamic explanation for this phenomenon we analyzed the kinetics on the basis of the transition-state theory, from which thermodynamic variables values, such as ΔG_a° , ΔH_a° , and ΔS_a° can be derived (26). Regardless of the genotype ΔH_a° of the process has a much larger

value than entropic contribution, $T\Delta S_a^\circ$ to the transition state (Figure 4A). This thermodynamic feature explains the temperature dependence on the viral structural stability. Loss of infectivity or disintegration of viral particles is an entropy-driven process and very unfavorable in terms of enthalpy. This suggests when the virus reaches the transition state a significant number of noncovalent bondings are broken (a positive value of ΔH_a°) and the overall system (virus and surrounding solvent) will be more disordered possibly due to the release of water molecules or ions associated with viral particles.

In our thermodynamic analysis we recognized that there is a significant variation in ΔH_a° among viral types suggesting the viral structures are quite different. However the overall stability (ΔG_a°) shows a limited variation (Figure 4B) because of a compensatory behaviour between enthalpy and entropy (Figure 4A). This indicates that the viral structure is a weakly coupled system (30-33).

Discussion

Experimental model systems simulating practical conditions are important for a better understanding and prevention of HCV transmission. Until now, HCV survival and stability in the environment were only described with a single HCV genotype or performed with surrogate markers for the presence and absence of infectious particles. In this study, we performed a stability analysis of all HCV genotypes at different temperatures and correlated these findings with an *in vivo* infectivity evaluation.

When comparing virus infectivity at room temperature, we found that the different HCV genotypes were still detectable for 21 to 28 days depending on the viral input. These data are in line with previous results demonstrating a stability of HCV genotype 2a viruses in serum for 3 to 4 weeks (12). In syringes, HCV survival was dependent on syringe type, time, and temperature and infectivity could even be demonstrated for up to 63 days in high void volume tuberculin syringes with a genotype 2a virus (14). When dried HCV particles were studied on inanimate surfaces, infectivity for up to 5 days could be observed (13). Kamili and colleagues dried and stored HCV plasma for 16 h, 4 days and 7 days at room temperature before inoculation of a chimpanzee with the samples (34). No infection occurred after inoculation with the material stored for 7 and 4 days, however, with the 16 h exposure sample HCV infection developed in the animal. The chimpanzee is the only reliable immunocompetent animal model for HCV, however their use is limited due to ethical reasons, high costs and restricted availability (27). Assessment of HCV infectivity with tissue culture assay described in this and previous studies should be a valuable way to assess the risk of HCV transmission (12-14). To confirm that non-infectious particles after incubation *in vitro* do not cause infection in an HCV *in vivo* model, we inoculated uPA-SCID mice that harbour human primary

hepatocytes in their livers with the viral samples of day 0 and a pool of non-infectious HCV genotypes from tissue culture. In contrast to the input particles from all genotypes that led to an infection *in vivo*, the virus pool caused no infection in the chimeric mice demonstrating that viral stability and transmission risks determined from *in vitro* experiments correlate with viral infectivity *in vivo*.

In line with the results of thermo-stability of HCV at room temperature, we observed no difference between viral genotypes at 37°C with virus infectivity detectable for 2 days. At both temperatures tested, HCV RNA copy numbers did not necessarily correlate with viral infectivity which was previously observed in *in vitro* and *in vivo* experiments (12, 34). This lack of correlation should be taken into account when interpreting HCV qRT-PCR-positive results in relation to the risk to human health.

According to our analysis HCV of all seven genotypes showed a similar structural stability although there was a significant variation in the values of enthalpy and entropy of activation. We found that this limited variation in the structural stability could be attributed to the phenomenon, enthalpy-entropy compensation. This suggests HCV particles are a weakly coupled system. This extra-thermodynamic phenomenon was also identified with respect to different environmental conditions. In our previous analysis of structural stability of genotype 2a reported by Painstil et al. (14) we found ΔH_a° and ΔS_a° were 25.6 kJ/mol and -254.3 J/(mol K), respectively (25). In the current study, we found that ΔH_a° and ΔS_a° of the same HCV genotype were 111.6 kJ/mol and 34.8 J/(mol K), respectively. Therefore, the values of $\Delta\Delta H_a^\circ$ and $T\Delta\Delta S_a^\circ$ at 37 °C due to the different environmental conditions are 86 kJ/mol and 89.6 kJ/mol. This suggests that environmental conditions can greatly affect both ΔH_a° and ΔS_a° in HCV structural decay. However, due to enthalpy-entropy compensation the overall effect can be negligible as the value of $\Delta\Delta G_a^\circ$ is just 3.6 kJ/mol.

In summary, we demonstrated that assessment of viral infectivity from *in vitro* experiments correlates with viral infectivity *in vivo*. All HCV genotypes displayed similar stability at different temperatures which is important for prevention strategies and estimating HCV transmission risks in the environment. Therefore, we recommend following strict hygienic guidelines to avoid HCV cross-transmission even if material has been contaminated several weeks ago.

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Figure legends

Figure 1: Stability of all HCV genotypes at room temperature. (A) Different HCV genotypes were incubated at room temperature for several weeks in suspension. At indicated time points, virus aliquots were tested for infectivity by a limiting dilution assay. (B) Kinetics of infectivity loss of different HCV genotypes at 21°C. Rate constants of viral structural disintegration were obtained by fitting a linearized form of first-order kinetic equation, $\ln C = \ln C_0 - kt$. R^2 values of the fitting are included in the figure. (C) HCV RNA of the respective viral supernatant was isolated and quantified by real-time polymerase chain reaction. A representative experiment of three independent repetitions is shown with standard deviations of the means.

Figure 2: *In vivo* infectivity of HCV genotypes stored at room temperature. Chimeric mice were inoculated by intraperitoneal injection with respective cell culture supernatants. EDTA plasma samples were collected at week 3 post injection and infection was monitored by a commercial qRT-PCR kit.

Figure 3: Stability of HCV genotypes at 37°C. (A) Different HCV genotypes were incubated at 37°C for several hours as a test virus suspension. At indicated time points, virus aliquots were tested for infectivity by a limiting dilution assay. (B) Kinetics of infectivity loss of different HCV genotypes at 37°C. Rate constants of viral structural disintegration were obtained by fitting a linearized form of first-order kinetic equation, $\ln C = \ln C_0 - kt$. R^2 values of the fitting are included in the figure. (C) HCV RNA of the respective viral supernatant was isolated and quantified by reverse-transcription polymerase chain reaction. A representative experiment of three independent repetitions is shown with standard deviations of the means.

Figure 4: Thermodynamic analysis of HCV genotypes. Enthalpy (ΔH_a^0) and entropic energy ($T\Delta S_a^0$) of activation of structural disintegration for eight different HCV genotypes at 37 °C (= 310 Kelvin). A simple linear equation ($y = ax + b$) was fit into the data. The fitting parameters a and b and their standard errors are 1.01 ± 0.01 and -101.8 ± 1.5 , respectively. R^2 of the fitting is 0.999.

Table 1: Rate constant for the structural decay of seven genotypes of HCV at two different temperatures