Chlorine inactivation of hepatitis E virus and human adenovirus 2 in water

Rosina Girones, Anna Carratalà, Byron Calgua, Miquel Calvo, Jesús Rodriguez-Manzano and Suzanne Emerson

ABSTRACT

Hepatitis E virus (HEV) is transmitted via the fecal–oral route and has been recognized as a common source of large waterborne outbreaks involving contaminated water in developing countries. Thus, there is the need to produce experimental data on the disinfection kinetics of HEV by chlorine in water samples with diverse levels of fecal contamination. Here, the inactivation of HEV and human adenovirus C serotype 2 (HAdV2), used as a reference virus, was monitored using immunofluorescence and quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays. HEV has been shown to be susceptible to chlorine disinfection and presented equivalent kinetics to human adenoviruses. The $C(t)$ values observed for a 2-log reduction of HEV were 0.41 in buffered demand-free water and 11.21 mg/L × min in the presence of 1% sewage. The results indicate that the inactivation kinetics of HEV and HAdV2 are equivalent and support the use of chlorine disinfection as an effective strategy to control HEV waterborne transmission.

Key words | chlorine, disinfection, HAdV 2, HEV, sewage, water

Rosina Girones (corresponding author)
Anna Carratalà
Byron Calgua
Jesús Rodriguez-Manzano
Department of Microbiology,
Faculty of Biology, University of Barcelona,
Avd. Diagonal 643,
Barcelona, 08028, Catalonia,
Spain
E-mail: *rgirones@ub.edu*

Anna Carratalà
Present address: Laboratory of Environmental Chemistry, School of Architecture,
Civil and Environmental Engineering (ENAC),
École Polytechnique Fédérale de Lausanne,
Lausanne 1015,
Switzerland

Miquel Calvo
Department of Biostatistics,
University of Barcelona, Barcelona, 08028,
Spain

Rosina Girones
Suzanne Emerson
Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases,
National Institutes of Health,
Bethesda, Maryland 20892-8009,
USA

INTRODUCTION

Hepatitis E virus (HEV) is a non-enveloped virus with positive sense single-stranded RNA and is responsible for epidemic and sporadic cases of acute hepatitis worldwide, but especially in Asia, Africa and the Middle East. HEV outbreaks have been described among civilians, the military, and refugees (Boccia *et al.* 2006; Guerrero-Latorre *et al.* 2011). HEV is transmitted via the fecal–oral route and has been recognized as a common cause of large waterborne outbreaks through fecally contaminated water (Labrique *et al.* 2013). Every year an estimated 20 million hepatitis E infections occur, over three million acute cases of hepatitis E, and 70,000 hepatitis E-related deaths (WHO 2012). Chlorination is commonly applied to eliminate pathogens from water (Sobsey *et al.* 1991; Page *et al.* 2009). Some studies have suggested that drinking chlorinated surface water did not appear to reduce the risk of infection (Boccia *et al.* 2006). This suggests that there is a need to evaluate the efficacy of current water treatment methods and to produce experimental information on the disinfection kinetics of HEV by chlorine. Unfortunately, traditionally HEV cell culture has been very inefficient (Emerson *et al.* 2006) and no experimental data are yet available on HEV susceptibility to chlorine inactivation.

In this study we evaluated the disinfection kinetics of HEV using 1–2 mg/L residual-free chlorine in order to evaluate (a) the inactivation of HEV (obtained from both cell culture and pooled feces) and HAdV2 suspensions (used as a reference), (b) the effect of sewage (1%) on the

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inactivation of HEV, and (c) the inactivation of HAdV2 suspensions at higher concentrations. For HAdV2, both the level of reduction of viral infectivity and viral genome decay were measured.

It is well established that organic compounds may interfere with disinfection treatments (Sobsey *et al.* 1991; Thurston-Enriquez *et al.* 2003). Thus, in addition to HEV in buffered demand-free water (BDF) inactivation was monitored in the presence of 1% of raw sewage to evaluate the interfering effect that a heavy contamination event with sewage may represent in the chlorine disinfection of water. Disinfection kinetics of HEV from both cell culture and fecal suspensions is analyzed in order to produce realistic data applicable to water disinfection since it has been described that cell culture-generated HEV and also blood HEV are associated with lipids (Takahashi *et al.* 2010) and this may not be the case for HEV excreted in feces.

Among enteric viruses prevalent in the environment, adenovirus is considered as an indicator of human fecal contamination (Pina *et al.* 1998; Albinana-Gimenez *et al.* 2009). In previous studies, free chlorine has been demonstrated to be efficient against adenovirus (Clarke *et al.* 1956; Thurston-Enriquez *et al.* 2003; Baxter *et al.* 2007; Page *et al.* 2009). Adenovirus type 2 (HAdV2), a member of species C in the *Mastadenovirus* genus, is primarily associated with respiratory infections and is often also shed in the feces of infected people regardless of infection site; thus, transmission of HAdV2 and other non-gastrointestinal adenoviruses can occur via water (Yates *et al.* 2006). HAdV2 was used as a representative adenovirus in the current study because this type is frequently detected in fecally contaminated water and its viability assay in the laboratory is relatively robust.

MATERIALS AND METHODS

The disinfection dynamics of HEV strains obtained both from cell culture and from fecal samples were evaluated under diverse conditions, including in the presence of low levels of urban sewage, with HAdV2 serving as a comparative reference viral strain. As an example of representative wastewater matrices, raw sewage samples from two different geographical areas were collected at the entry of urban wastewater treatment plants (WWTPs) in Spain (Barcelona) and in Egypt (Cairo), and were stored frozen at −80 °C until their use in these experiments. Both urban sewage samples presented common levels of human fecal contamination as measured by quantification of human adenoviruses 10^3–10^5 genome copies (GC)/mL.

A HEV suspension was prepared using an in-house subclone of Caco-2 cells (Emerson *et al.* 2010) transfected with Sar55 RNA genomes transcribed *in vitro*. Cell culture medium from day 14 to 21 post-transfection was collected, filtered through a 0.45-μm pore-size polyvinylidene difluoride (PVDF) Millex-HV filter (Millipore), aliquoted and frozen at −80 °C. Virus particles were prepared by diluting 1 mL aliquots of medium with phosphate buffered saline (PBS) in a total volume of 5 mL and ultracentrifuging for 2 hours at 237.020 *g*. The pelleted viruses were resuspended in 100 μL PBS.

A 10% fecal suspension of Sar55 HEV virus was prepared in PBS from feces collected from an experimentally infected rhesus macaque at Bioqual, Rockville, MD in compliance with the guidelines of Bioqual's and NIAID'S Institutional Animal Care and Use Committees. Human adenovirus suspensions were obtained from cell culture following the same protocol as detailed for HEV.

Infectious HEV and HAdV were quantified in triplicate by immunofluorescence assays (IFA) as previously described (Emerson *et al.* 2005; Calgua *et al.* 2011), and genomic decay was monitored using qPCR assays developed in earlier studies (Emerson *et al.* 2001; Hernroth *et al.* 2002).

Disinfection experiments were conducted as detailed in previous studies (Thurston-Enriquez *et al.* 2003; de Abreu Corrêa *et al.* 2012) with minor modifications. Experiments were conservatively designed and BDF water at a pH of 8 was used as a worst-case scenario, since it is known that the free chlorine species present at this pH (OCl^-) shows a lower disinfection efficacy as compared to HOCl (Thurston-Enriquez *et al.* 2003; Deborde & von Gunten 2008).

Briefly, a solution of sodium hypochlorite (Sigma-Aldrich, 425044) was prepared at approximately 1,000 mg/L. The experiments were performed at room temperature (25–26 °C) in 4 and 10 mL glass reactors for HEV and HAdV, respectively. Glassware used in the experiments was made chlorine-demand free, as previously described (Thurston-Enriquez *et al.* 2003). Preliminary assays (data not shown)

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were used to estimate the initial chlorine concentration required to achieve residual concentrations between 1 and 2 mg/L after adding viral concentrates in the reactors (Table 1). Reactors without chlorine were included as controls and monitored in parallel. HEV and HAdV2 numbers in these reactors were stable in all the experiments (Figures 1–3).

Viral suspensions were seeded to reactors containing BDF water (3.5 mL for HEV and 5.5 mL for HAdV2) and their initial concentrations (Table 1) were measured at the beginning of the experiments (time 0). Aliquots of 400 µL were collected from the reactors at 0.5, 10, 20, and 30 minutes and the chlorine was quenched with sodium thiosulfate (0.1%). Free chlorine residual concentration in the BDF water was quantified before the addition of the samples to batch reactors and after 0.5 and 20 minutes of contact time. In order to determine $C(t)$ values and the times needed to inactivate from 1 to 4-log of the viruses, (t_{90}, t_{99}, $t_{99.9}$ and $t_{99.99}$), the efficiency factor Hom (EFH) model was applied to the bench-scale results as previously described (Haas & Joffe 1994; Thurston-Enriquez *et al.* 2003; Black *et al.* 2009; de Abreu Corrêa *et al.* 2012). The EFH model is a widely used model which takes into account the decreasing chlorine concentrations caused by the virus itself and organics present in the solution. The chlorine concentration in each experiment was therefore modeled as a first-order kinetic equation:

$$C(t) = C_0 e^{-k't} \quad (1)$$

where $C(t)$ is the residual chlorine (mg/L) at time t (min), C_0 is the initial chlorine concentration, and k' is the first-order chlorine decay rate constant. C_0 and k' from Equation (1) were used in the following equation to model the disinfection kinetics:

$$\ln\left(\frac{N(t)}{N_0}\right) = -kC_0^n t^m \left(\frac{1 - e^{-nk't/m}}{nk't/m}\right)^m \quad (2)$$

where k is the inactivation rate constant, n is the coefficient of dilution, and m is the Hom's exponent, $\ln(N(t)/N_0)$ is the natural log of the survival ratio and $N(t)$ the number of organisms remaining at time t divided by N_0 the number at time zero. To determine the parameter values for both equations, Microsoft Excel Solver (Microsoft Excel 2010, Microsoft Corp.) was used to minimize the error sum of squares between the observed and predicted $C(t)$ and $\ln(N(t)/N_0)$ for each viral disinfection in each experiment.

Once the parameters of the EFH model equation were estimated for all the experiments, they were used to predict the time values for 2- and 3-log inactivation of the viruses. These times are noted respectively as t_{99} and $t_{99.9}$. Once the different parameters of Equation (2) are estimated, the time $t_{100\theta}$ necessary to reach any desired θ% percentage of reduction was obtained by reversing Equation (2), thus:

$$t_{100\,\theta} = -\frac{m}{nk'} \ln\left(1 - \frac{nk'}{m}\left(\frac{\ln(1-\theta)}{kC_0^n}\right)^{1/m}\right) \quad (3)$$

Table 1 | Times of inactivation (minutes) predicted to inactivate from 1 to 4-log of HEV and HAdV2, along with the calculated $C(t)$

Virus tested	Experimental condition	Viral initial concentration (FFU/mL)	Initial free chlorine (mg/L)	R^2	1-log		2-log		3-log		4-log	
					T90	$C(t)$	T99	$C(t)$	T99.9	$C(t)$	T99.99	$C(t)$
HEV	BDF + viruses from cell culture	1.97×10^3	7.3	0.61	0.02	0.15	NR		NR		NR	
	BDF + viruses from pooled feces	2.10×10^4	5.8	0.77	0.02	0.12	0.07	0.41	NR		NR	
	BDF + 1% sewage	1.86×10^3	23.1	0.98	0.22	5.09	0.49	11.21	NR		NR	
HAdV2	BDF + high viral load	7.20×10^5	2.7	0.96	0.27	0.73	0.35	0.95	0.44	1.19	0.61	1.65
	BDF	1.40×10^4	5	0.82	0.23	1.15	0.78	3.9	NR		NR	
	BDF	1.40×10^4	2.2	0.89	0.36	0.79	NR		NR		NR	

BDF, buffered demand-free water; NR, not reached; $C(t)$, disinfectant concentration (in mg/liter) × contact time (in minutes).

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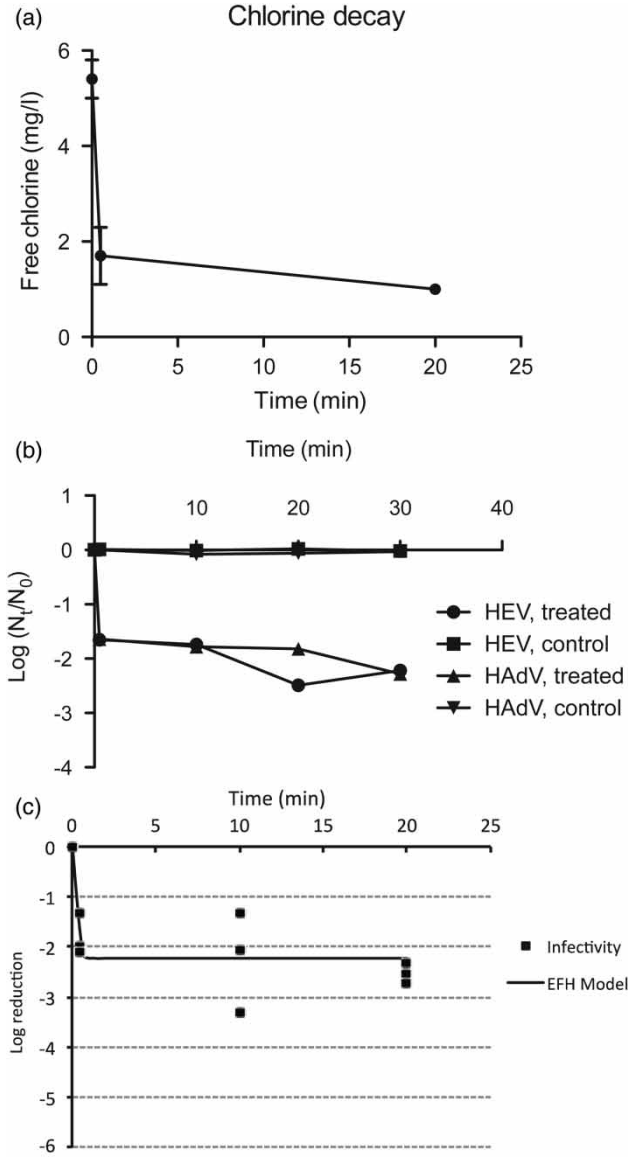

Figure 1 | Inactivation kinetics of HEV suspensions obtained from pooled feces in BDF water treated with free chlorine at 5 mg/L. (a) Free chlorine decay curve in the disinfection assays using BDF water. Dots represent an average of two replicates. (b) Log reduction curves for seeded HEV and HAdV2, as a comparative reference. Data points correspond to one experiment. (c) EFH model representing log reduction of HEV plotted as a continuous line. The observed data are plotted as squares.

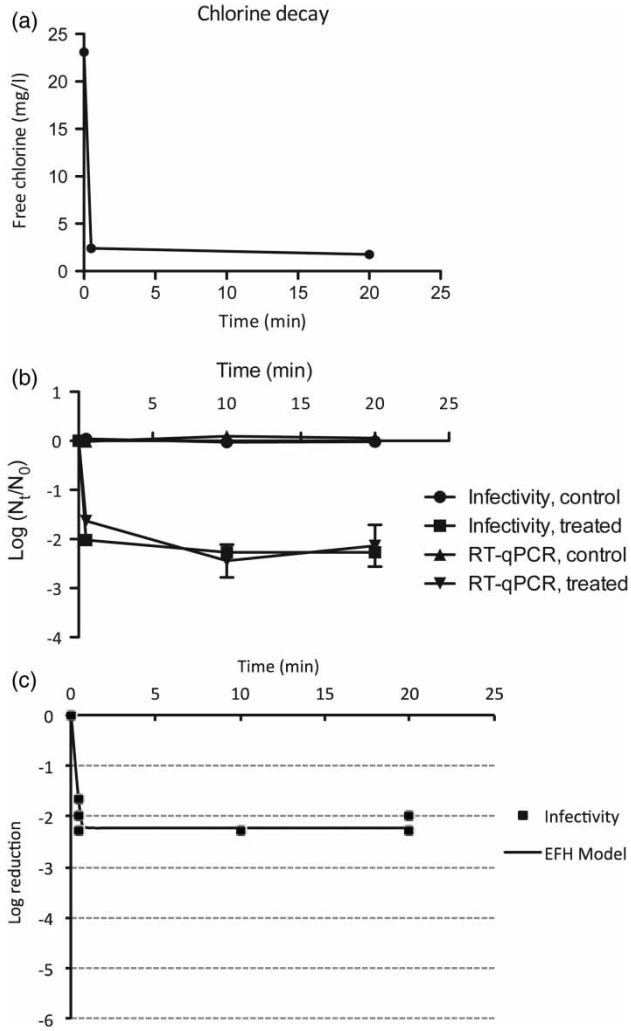

Figure 2 | Inactivation kinetics of HEV suspensions in BDF water with 1% of urban sewage treated with free chlorine at 23 mg/L. (a) Free chlorine decay curve in the disinfection assays using BDF water with 1% of urban sewage. Dots represent an average of two replicates. (b) Log reduction curves for seeded HEV. Each data point corresponds to an average of two independent experiments. (c) EFH model representing log reduction of HEV in the presence of 1% of sewage plotted as a continuous line. The observed data are plotted as squares.

Notice that the values of θ must lie in the interval:

$$0 < \theta < \min\left(1;\ 1 - \exp\left(-kC_0^n(m/nk')^m\right)\right) \quad (4)$$

Equation (3) provides the time when a reduction of θ% of the initial concentration is reached, for instance, if $\theta =$ 0.99, a 2-log inactivation (or t_{99}). However, if condition (4) is not satisfied for the desired θ, this percentage of reduction cannot be reached at any time if the kinetics are modeled as (2).

The $C(t)$ values were calculated by multiplying inactivation times obtained from the disinfection models (in minutes) by the chlorine concentration (mg/L). The reported $C(t)$ values represent the mean of the $C(t)$ values calculated for each experimental condition

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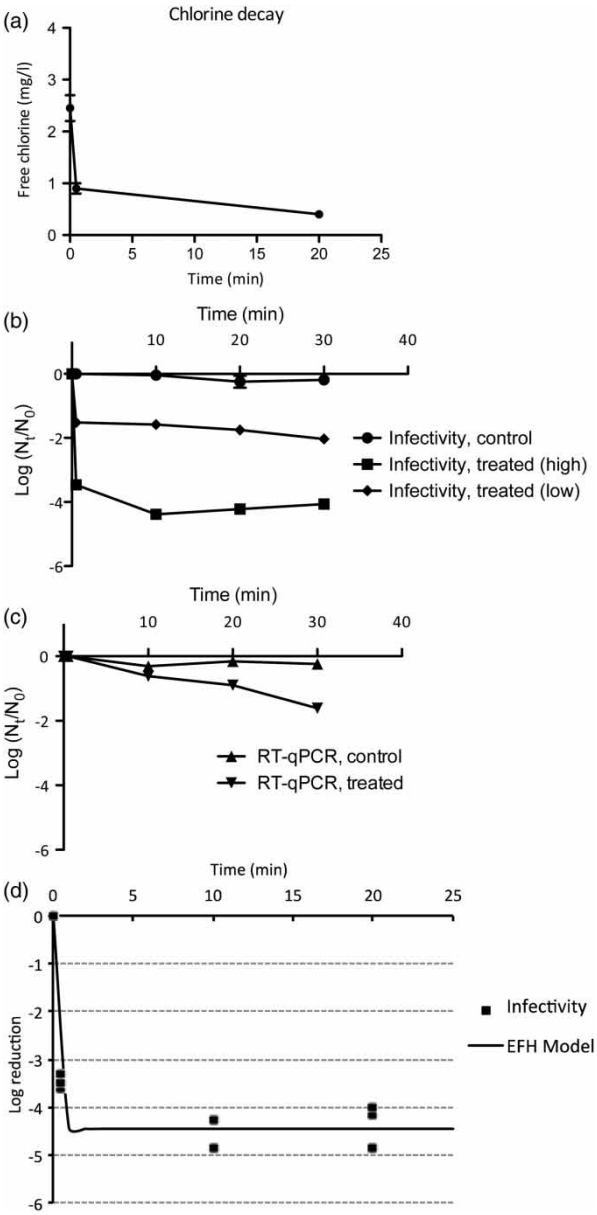

Figure 3 | Inactivation kinetics of HAdV2 suspensions at different initial concentrations in BDF water treated with free chlorine at 2.5 mg/L. (a) Free chlorine decay curve in the disinfection assays. Dots represent an average of two replicates. (b) Log reduction curves for HAdV2 infectivity seeded at different initial concentrations of 7.2×10^5 (high) and 1.4×10^4 FFU/mL (low). Data points correspond to one experiment. (c) Log reduction curves for HAdV2 as measured by qPCR. Data points correspond to one experiment. (d) EFH model representing log reduction of HAdV2 plotted as a continuous line. The observed data are plotted as squares.

analyzed. The disinfection kinetics for HEV in the different conditions and for HAdV were compared by using an *F*-approximate test, in order to assess the homogeneity of the overall pair.

RESULTS AND DISCUSSION

HEV and HAdV2 inactivation curves followed biphasic decays (Figures 1–3). Biphasic kinetics in chlorine treatments may be explained by the action of different chlorine species and the presence of potential viral aggregates (Thurston-Enriquez *et al*. 2003) as most viruses in surface water and wastewater are likely aggregated or associated with organic matter (Sobsey *et al*. 1991). Viral inactivation due to chlorine may initially be associated with reactions of capsid proteins with free chlorine forms such as HOCl and ClO^- at pH 8, and at a later stage, in the presence of organic compounds to reactions involving secondary chlorine species such as organic chloramines (Thurston-Enriquez *et al*. 2003; Kahler *et al*. 2010).

HEV and HAdV2 inactivation curves are presented in Figure 1. Comparison with an *F*-test showed that the studied viruses presented equivalent inactivation kinetics (p-value ≈ 1). Infectious HEV and HAdV2 were still detected after 30 minutes of contact time, and no significant inactivation was observed in the qPCR assays. In terms of inactivation parameters (Table 1), $C(t)$ values to achieve 1-log viral reduction were between 0.15–0.12 mg/L × min for HEV from both cell culture and fecal suspensions. Higher values were observed for HAdV2: 1.15 mg/L × min for 1-log reduction and 3.90 mg/L × min for 2-log.

The inactivation curves obtained for HEV in the presence of urban sewage (1%) are presented in Figure 2. $C(t)$ values increased from 0.41 to 11.12 mg/L × min for a 2-log inactivation. A similar reduction was observed with qPCR, showing that free chlorine at high initial concentrations (such as 23 mg/L) acts on both viral genome and proteins. The objective of these experiments is to analyze HEV disinfection in conditions as similar as possible to potential episodes of contamination of water by sewage. The concentration 1% of raw sewage was selected as representative of these potential events.

The analysis of HAdV2 allowed the use of viral suspensions at higher concentrations (7.2×10^5 FFU/mL). The results obtained (Figure 3) showed quantifiable values of HAdV2 after 60 minutes of contact time and a $C(t)$ value of 1.65 mg/L × min to achieve a decay of 4-log (Table 1).

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$C(t)$ values may increase with higher pH, lower temperatures and vary according to the experimental design. However, the $C(t)$ values obtained for HAdV2 were consistent with those described in previous studies on HAdV40 disinfection in groundwater (pH 8–8.2) at 15 °C, ranging from 0.72 to 2.4 mg/L × min for a 2-log reduction (Thurston-Enriquez *et al.* 2003).

To the best of our knowledge, this study is the first to present experimental data demonstrating the susceptibility of HEV to chlorine. The results indicate that the inactivation kinetics of HEV and HAdV2 are equivalent. However, more experimental data are needed to support evidence-based policy and action on water safety and the protection of vulnerable populations.

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