Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Thermal stability of structurally different viruses with proven or potential relevance to food safety

E. Tuladhar[1,2], M. Bouwknegt[3], M.H. Zwietering[2], M. Koopmans[1,4] and E. Duizer[1]

1 Laboratory for Infectious Diseases and Screening, Center for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
2 Laboratory of Food Microbiology, Wageningen University and Research Center, Wageningen, the Netherlands
3 Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
4 Department of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands

Keywords
foodborne virus, influenza A virus, norovirus, parechovirus, suspension test, thermal inactivation.

Correspondence
Erwin Duizer, National Institute for Public Health and the Environment, Centre for Infectious Disease Control, PO Box 1 3720 BA Bilthoven, the Netherlands.
E-mail: erwin.duizer@rivm.nl

2012/0057: received 11 January 2012, revised 24 February 2012 and accepted 2 March 2012

doi:10.1111/j.1365-2672.2012.05282.x

Abstract

Aims: To collect comparative data on thermal stability of structurally different viruses with proven or potential relevance to food safety.
Methods and Results: Suspensions with poliovirus Sabin1, adenovirus type5, parechovirus1, human norovirus (NoV) GII.4, murine NoV (MNV1) and human influenza A (H1N1) viruses were heated at 56 and 73°C. Infectivity was tested by culture assay for all but human NoV GII.4 that cannot be cultivated *in vitro*. Time to first $\log_{10}$ reduction (*TFL*-value) was calculated based on best fit using the monophasic, biphasic or Weibull models. The Weibull model provided the best fit at 56°C for all viruses except influenza virus. The *TFL* at 56°C varied between a high of 27 min (parechovirus) to a low of 10 s (adenovirus) and ranked parechovirus > influenza > MNV1 > poliovirus > adenovirus. The monophasic model best described the behaviour of the viruses at 73°C, in which case the *TFL* was MNV1(62s) > influenza > adenovirus > parechovirus > poliovirus(14s).
Conclusions: Viruses do not follow log-linear thermal inactivation kinetics and the thermostability of parechovirus and influenza virus is similar to that of proven foodborne viruses.
Significance and Impact of the Study: Resistant fractions of viruses may remain infectious in thermal inactivation processes and inactivation of newly discovered or enveloped viruses in thermal food preparation processes should not be assumed without further testing.

Introduction

In recent years, viruses have been increasingly recognized as an important cause of foodborne diseases. The viruses most frequently reported as involved in foodborne outbreaks are the human noroviruses (NoVs) and hepatitis A virus (Friesema *et al.* 2009; Koopmans 2009; Westrell *et al.* 2010). Other viruses such as rotavirus, hepatitis E virus, astrovirus, Aichi virus, sapovirus, enterovirus, coronavirus, parvovirus and adenovirus can also be transmitted by food, and anecdotal evidence suggests that the list of foodborne viruses may be even longer (Greening 2006; Duizer and Koopmans 2008; FAO/WHO 2008). Most recognized viruses transmitted by foods are nonenveloped small spheres (around 30 nm in diameter) with single-stranded positive sense RNA genomes. The primary mode of transmission is person-to-person spread, but indirect transmission via faecally contaminated food is common.

The purpose of this study was to obtain data on the thermal stability of a variety of viruses, specifically human NoV [and the cultivable surrogate, murine NoV (MNV1) (Cannon *et al.* 2006)] poliovirus, parechovirus, adenovirus

and influenza A virus. These viruses represent different families that are shed via faeces or respiratory secretions and because of this, might ultimately contaminate foods via food handler related contamination. Only, human NoV and poliovirus have been confirmed to be transmitted by foods and/or water. Foodborne transmission of adenoviruses is considered likely (Duizer and Koopmans 2008) and the serotype 5 strain is interesting because it can be detected in respiratory excretions as well as faeces and has a different genome structure than the small RNA viruses (Rosario *et al.* 2006). Adenovirus is a nonenveloped virus, approximately 80 nm in diameter with a double-stranded DNA genome. We also evaluated the thermal stability of parechovirus, which causes a mild gastrointestinal illness in young children (Benschop *et al.* 2008).

The respiratory influenza A (H1N1) virus was included, because these viruses are shed in respiratory secretions and may thus contaminate foods. As the oropharynx is a common replication site for many respiratory viruses, including influenza virus (Lieberman *et al.* 2009) infection resulting from ingestion, while not considered likely, cannot be excluded. Furthermore, a relatively high thermostability for highly pathogenic avian influenza viruses has been shown (Thomas and Swayne 2007) and foodborne transmission has been suggested (Duizer and Koopmans 2008). However, influenza A viruses are much larger (around 300 nm), do have an envelope and a different genome (negative strands RNA) than the common foodborne viruses. Temperatures of 56 and 73°C for different time intervals were evaluated because these are commonly used in cooking and pasteurization processes (Cannon *et al.* 2006; Isbarn *et al.* 2007).

As the observed reduction in virus number under progressing treatment (e.g. time-temperature) can follow different patterns depending on the different inactivation mechanisms or changing experimental circumstances (Cerf 1977), we analysed our data using different models. The simplest reduction pattern involves a constant reduction rate, with a log-linear decrease in virus numbers (i.e. monophasic reduction) (Chick 1908). Alternatively, the initial population of viruses in the sample can consist of several viral fractions and/or several different experimental circumstances, with each fraction displaying a particular reduction rate. For example, when two such rates are observed, then the reduction is biphasic (de Roda Husman *et al.* 2009). Alternatively, the reduction rate(s) need not be constant over time, but may continuously change under progressing treatment. This situation is modelled with the Weibull model (van Boekel 2002). By applying statistical modelling, hypothesis can be tested regarding constant or variable inactivation rates or the presence of mixture virus populations to support data interpretation in terms of intervention measures.

Comparable data on thermal stability from different viruses and characterization of the kinetics of inactivation will help assessing the likelihood of viral survival through food production processes and it points to the need for considering foodborne transmission of viruses for which this route is unexpected, such as the enveloped influenza viruses.

Materials and methods

Viruses and cells

Viruses used for the study were poliovirus Sabin 1 (vaccine strain), adenovirus type 5 (Hu/adenovirus/type 5/6270/1988/Ethiopia), influenza A (H1N1) virus (Hu/influenza A/266/2008/Netherlands (H1N1) virus), parechovirus 1 (Hu/parechovirus/type 1/147/2008/Netherlands), MNV1 (Mu/NoV/GV/MNV1/2002/USA) and human NoV GII.4 (stool sample, Hu/NoV/GII.4/1803/2008/Netherlands).

For all but human NoV, viral stocks were prepared by infecting monolayers of respective host cells (Tuladhar *et al.* 2012). Poliovirus and adenovirus were cultivated on human epidermoid cancer (Hep-2) cells, MNV1 on raw mouse macrophage (Raw −264·7) cells, parechovirus 1 on human colon adenocarcinoma (HT-29) cells (Abed and Boivin 2006) and influenza A virus was cultivated on Madin-Darby canine kidney (MDCK-1) cells. Human NoV suspension was prepared as 10% w/v stool homogenates in Dulbecco's Modified Eagle Medium (DMEM) as described before (Svraka *et al.* 2009) that was filtered through a 0·2-μm pore size filter. The suspension was free of all other enteric viruses tested (rotaviruses, enteric adenoviruses, astroviruses and sapoviruses) as determined before by PCR assays (Svraka *et al.* 2009). All the virus stocks were stored at −70°C until used in experiments.

Preparation of sterile stool suspension

A 20% (w/v, wet weight) stool suspension from a healthy volunteer was prepared in phosphate buffer (0·01 mol l^{-1}, pH 7·2) and sterilized by autoclaving at 121°C for 15 min. The suspension was vortexed, centrifuged at 1500 ***g*** for 20 min, and the supernatant recovered, aliquoted and stored at −20°C. As described previously (Svraka *et al.* 2009), the stool suspension was tested and found to be free of evidence of viral RNA or DNA corresponding to human NoVs GI and GII, rotaviruses, enteric adenoviruses, astroviruses and sapoviruses. The stool suspension was further diluted to 6% in sterile phosphate buffer to perform the heat inactivation experiments at the final concentration of 1% stool in the sample.

Thermal inactivation of viruses

Thermal inactivation experiments were carried out using a suspension assay design in which viruses were suspended with and without stool. The viral stocks titres used were as follows: poliovirus Sabin 1: $6{\cdot}3 \times 10^8$ 50% Tissue Culture Infective Dose ($TCID_{50}$) ml^{-1} ($1{\cdot}6 \times 10^{10}$ PCR unit (PCRU) ml^{-1}), adenovirus type 5: $6{\cdot}3 \times 10^7$ $TCID_{50}$ ml^{-1} ($3{\cdot}2 \times 10^9$ PCRU ml^{-1}), parechovirus 1: $1{\cdot}3 \times 10^8$ $TCID_{50}$ ml^{-1} ($2{\cdot}0 \times 10^9$ PCRU ml^{-1}), influenza A (H1N1) virus: $1{\cdot}3 \times 10^6$ $TCID_{50}$ ml^{-1} ($1{\cdot}5 \times 10^8$ PCRU ml^{-1}), MNV1: $1{\cdot}7 \times 10^7$ PFU ml^{-1} ($5{\cdot}0 \times 10^8$ PCRU ml^{-1}) and human NoV GII.4 was at a concentration of 1×10^8 PCRU ml^{-1}. The viral stocks were dispensed in 100 μl fractions in reaction tubes and 100 μl stool suspension (6% w/v) or DMEM (control) was inoculated separately. As human stool is not the natural matrix for influenza A virus, this virus was suspended in DMEM only. The viral suspensions were preheated to 30°C, followed by the addition of 400 μl of DMEM preheated to 69 or 94·5°C to instantaneously achieve temperatures of 56 or 73°C, respectively. The final temperatures obtained were recorded using a digital thermometer (Sling, China). The suspensions were maintained at the desired temperatures in a digitally controlled water bath at 56°C for 0, 5, 10 and 30 min and at 73°C for 0, 30 s, 1 and 3 min. At each time point, a suspension was removed and cooled immediately by ice immersion. The samples were stored at −70°C prior to analysis by infectivity assay or quantitative PCR, as appropriate. The infectivity reduction was determined by cell culture assays. As human NoV cannot be cultured (Duizer *et al.* 2004), only the viral nucleic acid reduction was determined. Each experiment was performed in triplicate.

Enumeration of viruses

Infectivity (Plaque) assay.

Plaque assays for MNV1 enumeration were performed in 6-well culture plates as described by Wobus *et al.* (2004) except for the addition of 2 ml of warm DMEM complete medium on top of the agarose after solidification. After removing the medium, plaques were visualized by adding 2 ml of freshly prepared 0·015% w/v neutral red solution in DMEM at room temperature. After 2 h, plates with 5–50 plaques were counted. The results were expressed as plaque forming unit (PFU) per ml of sample.

Infectivity ($TCID_{50}$) determination.

The other viruses were enumerated by titration in 96-well plates using the $TCID_{50}$ approach. For poliovirus Sabin 1, parechovirus 1, adenovirus type 5 and influenza A (H1N1) virus, 10-fold serial dilutions were prepared followed by inoculation in 96-well plate using cell monolayers as previously described (Tuladhar *et al.* 2012). For Hep-2, MDCK-1 and HT-29 cells, seeding density was 2×10^5 cell ml^{-1}. Poliovirus Sabin 1 and adenovirus type 5 virus suspensions were added on freshly trypsinized Hep-2 cells. Parechovirus 1 was titrated on 1-day-old HT-29 cells. Influenza A (H1N1) virus was titrated on 3-day-old MDCK-1 cell monolayers after washing twice with phosphate buffer solution prior to infection. The influenza A (H1N1) virus suspensions were prepared in DMEM with 2·5 g ml^{-1} TPCK (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone) treated trypsin (Sigma porcine pancreatic type IX). Cytopathic effect was observed after 5 or 6 days of incubation at 37°C in 5% CO_2. Viral titres were calculated by the Spearman–Karber method (Karber 1931).

Viral Nucleic acid extraction and Real time (RT) PCR

In addition to infectivity assay, semi quantitative PCR or RT-PCR was performed on all viral suspensions before and after heat treatment. Viral nucleic acid extraction was performed using the MagNA Pure Light Cycler total nucleic acid isolation kit as described previously (Svraka *et al.* 2009). Amplifications for MNV1 (Bae and Schwab 2008), poliovirus Sabin 1 (Donaldson *et al.* 2002), adenovirus type 5 (Svraka *et al.* 2009), human NoV GII.4 and influenza A (H1N1) virus have been previously described (Tuladhar *et al.* 2012). For parechovirus 1, forward and reverse primers were designed by our laboratory and are as follows: 5′-GCCATCCTCTAGTAAGTTTG-3′ and 5′-TACCTTCTGGGCATCCTTC-3′ (location 326-582), respectively. The probe sequence, which was labelled with 6-carboxyfluorescein (FAM) at the 5′ end and conjugated with black hole quencher at the 3′ ends, was TAACAGGTGCCTCTGGGGCCAA. The amplifications consisted of 95°C for 10 s for denaturation and annealing temperature of 50°C for 20 s. PCR or RT-PCR amplifiable units (PCRU or RT-PCRU) were determined by the slopes of standard curves made for each virus. The standard curve was made by plotting cyclic threshold (C_t) value verse log PCRU or RT-PCRU of 10-fold dilutions of the viral stock. The highest dilution giving a positive result was assigned a value of 1 amplifiable unit. The $\log_{10}$ reduction in PCRU or RT-PCRU was calculated by subtracting the 30 min time point value from 0 min value for 56°C treatment and subtracting the 3 min value from the 0 min value for the 73°C treatment.

Data analyses

For the infectivity data, viral inactivation was expressed as reduction in the infectious units ($TCID_{50}$ or PFU). The

infectious units were $\log_{10}$-transformed and assumed to be distributed normally after transformation. The data were fitted to three potential mathematical models: a monophasic using model (1) as described by Chick (1908):

$$\log_{10}(C(t)) = \log_{10}(C_0) - \lambda t \quad (1)$$

a biphasic model (2) as described by de Roda Husman *et al.* (2009):

$$\log_{10}(C(t)) = \log_{10}\left[C_0(we^{-\lambda_1 t} + (1-w)e^{-\lambda_2 t})\right] \quad (2)$$

and a Weibull model (3) as described by van Boekel (2002):

$$\log_{10}(C(t)) = \log_{10}(C_0) - (\lambda t)^p \quad (3)$$

where C_0 is the $\log_{10}$ number of infectious viruses at time zero, the λ's and P are inactivation parameters (with λ in model (3) being $1/\delta$ as described by van Boekel (2002), and w is a parameter that directs the biphasic shape in eqn 2. Models (1) and (2) and models (1) and (3) are nested, justifying the likelihood ratio test for assessing statistically superior fits (α set at 0·05). Models (2) and (3) are non-nested, and therefore, model selection was based on the corrected Akaike Information Criterion (Burnham and Anderson 2002). Parameter values were estimated by maximizing the likelihood of the respective models. Zero-counts were included as censored observations, with contributions to the total likelihood based on the cumulative density function using a detection limit of a single infectious virus. Parameter uncertainty was assessed by Markov Chain Monte Carlo sampling using the Metropolis and Hastings algorithm (Gilks *et al.* 1996). The time to the first $\log_{10}$ reduction (*TFL*-value) was estimated by solving t from Eqns. (1–3) using the maximum likelihood estimates for the parameters. The 95% confidence intervals were generated likewise using parameter values from the Markov Chain Monte Carlo posterior and taking the 2·5 and 97·5 percentiles.

The best fitting model was chosen to describe the inactivation for each virus-temperature-matrix combination. MATHEMATICA software (ver. 8; Wolfram Research, Champaign, IL, USA) was used for the analysis.

Results

Infectivity reduction

No decrease in virus titre was detected after heating to 30°C for 20 min in a heat block (data not shown). The time-dependent infectivity reductions observed at 56 and

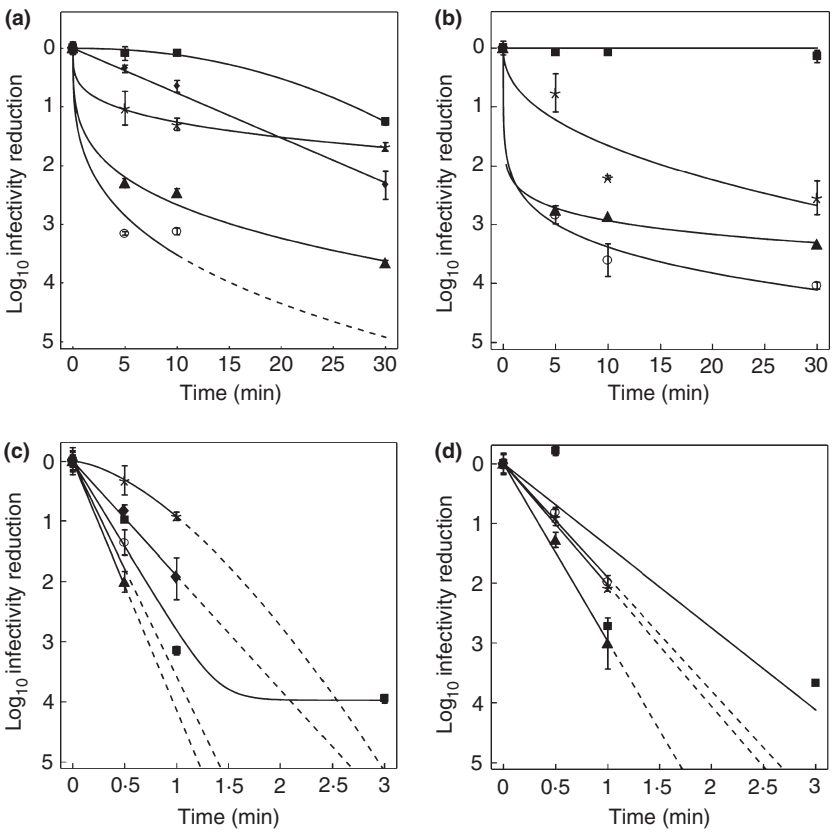

Figure 1 Infectivity reduction of viruses suspended in Dulbecco's Modified Eagle Medium and treated at 56°C (a) or 73°C (c), or suspended in 1% stool and treated at 56°C (b) or 73°C (d). Markers indicate the mean (n = 3) (○) adenovirus type 5, (▲) poliovirus Sabin 1, (*) MNV1, (◆) influenza A (H1N1) virus, (■) parechovirus 1. Dotted lines indicate complete inactivation and error bars show standard deviation.

73°C are presented in Fig. 1. The inactivation data were fitted to the monophasic, biphasic and Weibull models and the model providing the best fit was chosen to calculate *TFL*-values. *TFL*-values for the viruses both in DMEM and 1% stool at 56°C are presented in Table 1. In most cases, the Weibull model provided the best fit for the 56°C treatment, except for influenza A (H1N1) virus (monophasic reduction). When the viruses were suspended in DMEM, the *TFL*-values at 56°C ranked (from highest or most resistant, to lowest or least resistant) as follows: parechovirus 1 > influenza A (H1N1) > MNV1 > poliovirus Sabin 1 > adenovirus type 5 (Table 1).

After the maximum treatment time of 30 min at 56°C, $\log_{10}$ reductions of viruses suspended in DMEM ranked as follows: parechovirus 1 > MNV1 > influenza A (H1N1) virus > poliovirus Sabin 1 > adenovirus type 5 with <1 $\log_{10}$ reduction for parechovirus 1 to over 4 $\log_{10}$ reduction for adenovirus type 5 (Fig. 1a). After 30 min at 56°C, there is a moderate stabilizing effect of stool on the thermal stability of parechovirus 1 and adenovirus type 5, but no clear effect on thermal stability of MNV1 and poliovirus Sabin 1 (Fig. 1a,b). Parechovirus 1 showed no significant reduction in infectivity at 56°C in stool.

The *TFL*-values at 73°C calculated from the best fitting model are presented in Table 2. The monophasic model provided the best fit for poliovirus Sabin 1, adenovirus type 5 and influenza A (H1N1) virus at 73°C in DMEM. The biphasic model was most appropriate for parechovirus 1, while the Weibull model was best for MNV1 (Fig. 1c). At 73°C, all the viruses tested, except parechovirus 1, were inactivated completely (defined as >4 $\log_{10}$ infectivity reduction) within 3 min. *TFL*-values at 73°C ranked (from highest to lowest) as MNV1 > influenza A (H1N1) virus > adenovirus type 5 > parechovirus 1 > poliovirus Sabin 1 and varied between 14 s for poliovirus Sabin 1 to 64 s for MNV1. Based on infectivity reduction after 3 min at 73°C in DMEM, thermal stability of the tested viruses rank as parechovirus 1 > MNV1 > influenza A (H1N1) virus > adenovirus type 5 > poliovirus Sabin 1 with nearly 4 $\log_{10}$ reduction for parechovirus 1 to complete inactivation (>4 $\log_{10}$ reduction) for all the other tested viruses (Fig. 1c).

Table 1 Best fitting inactivation model per virus-matrix combination at 56°C, and estimated required time (min) and 95% confidence interval for the first $\log_{10}$ reduction (*TFL*) and the estimated values for the parameter *P*

Virus	Matrix	Best Fitting model	*TFL*		*P*	
			Mean	95% interval	Mean	95% interval
Adenovirus type 5	Dulbecco's	Weibull	0·16	0·05–1·61	0·30	0·21–0·47
Poliovirus Sabin 1	Modified Eagle	Weibull	0·30	0·16–0·5	0·28	0·27–0·37
MNV1	Medium	Weibull	4·21	2·7–13·4	0·27	0·06–0·36
Influenza A virus		Monophasic	13·1	11·6–15·4	1	–
Parechovirus 1		Weibull	27·0	23·0–29·9	2·18	1·24–3·06
Poliovirus Sabin 1	1% stool	Weibull	<0·01	10^{-8}–<0·01	0·11	0·05–0·12
Adenovirus type 5		Weibull	0·01	0·006–0·17	0·18	0·16–0·33
MNV1		Weibull	3·20	2·03–11·4	0·44	0·23–0·86
Parechovirus 1		No decay	–	–	–	–

Table 2 Best fitting inactivation model per virus, matrix, estimated time (min) to first $\log_{10}$ reduction (*TFL*) at 73°C, 95% confidence interval (a value of 0·5 means 30 s) and the estimated values for the parameter *P*

Virus	Matrix	Best fitting model	TFL		*P*	
			Mean	95% interval	Mean	95% interval
Poliovirus Sabin 1	Dulbecco's	Monophasic	0·24	0·17–0·36	1	–
Parechovirus 1	Modified Eagle	Biphasic	0·35	0·27–0·61	–*	–
Adenovirus type 5	Medium	Monophasic	0·40	0·25–0·57	1	–
Influenza A virus		Monophasic	0·53	0·40–0·77	1	–
MNV1		Weibull	1·06	0·78–1·77	1·57	1·32–12·6
Poliovirus Sabin 1	1% stool	Monophasic	0·34	0·29–0·53	1	–
MNV1		Monophasic	0·49	0·41–0·62	1	–
Adenovirus type 5		Monophasic	0·53	0·43–0·74	1	–
Parechovirus 1		Monophasic	0·73	0·51–6·26	1	–

*Parameter values were λ_1: 6·5 (4·3–8·7), λ_2: approximately 0 (97·5% upper limit: 10^{-14}), *w*: 0·9999 (0·99–0·9999).

Table 3 Log_{10} PCR unit reduction of genetic material on heating at 56°C between 0 and 30 min and between 0 and 3 min at 73°C in Dulbecco's Modified Eagle Medium (DMEM) and 1% stool

	56°C		73°C	
Virus	DMEM	1% Stool	DMEM	1% Stool
Poliovirus Sabin 1	0·1 ± 0·2	0·1 ± 0·1	0·1 ± 0·2	0·0 ± 0·1
Adenovirus type 5	0·1 ± 0·2	0·1 ± 0·1	0·1 ± 0·1	0·0 ± 0·0
Parechovirus 1	0·1 ± 0·1	**0·6 ± 0·2**	**1·1 ± 0·3**	**1·4 ± 0·1**
Influenza A (H1N1) virus	0·4 ± 0·3	ND	0·0 ± 0·0	ND
MNV1	0·3 ± 0·3	0·1 ± 0·2	**0·8 ± 0·2**	0·4 ± 0·1
Human NoroGII.4	0·2 ± 0·0	0·1 ± 0·0	0·1 ± 0·1	0·0 ± 0·0

Bold font type indicate a significant reduction of RNA, $n = 3$, ±SD, ND = not done.

Viral nucleic acid reduction

The PCRU or RT-PCR reduction after heating at 56°C for 30 min and 73°C for 3 min is shown in Table 3. Overall, the PCRU reduction was <1 log_{10} for all the viruses tested except for parechovirus 1 at 73°C. The results show that virus infectivity loss occurs much more rapidly than does loss of amplifiable viral RNA or DNA, for all viruses and all conditions tested, with viral RNA persisting even after completed loss of infectivity.

Discussion

The aim of the study was to estimate time and temperature-dependent inactivation of structurally different groups of viruses at 56 and 73°C. In general, it is assumed that small nonenveloped viruses are among the most stable viruses and that their sensitivity to heat (and other environmental stresses) increases with size and because of the presence of a viral envelope. However, few studies have compared the thermostability of structural different viruses under identical conditions.

In this study, we did not find a relation between viral structure and thermal stability. We did find a wide range in thermo-stability when comparing the small nonenveloped positive stranded RNA viruses to one another. In our comparisons, we assumed that *TFL* reduction values represent the inactivation of the most sensitive viral fraction in the suspension, that is, the monodispersed fraction. Based on *TFL* reduction values at 56°C, the two representatives of the *Picornaviridae* (poliovirus and parechovirus) showed very different thermostabilities, while the nonenveloped adenovirus was less thermostable than the enveloped and larger influenza virus. The relatively high thermal stability of highly pathogenic influenza virus has been previously reported for highly pathogenic avian influenza viruses (Thomas and Swayne 2007) and may thus be an important characteristic of influenza A viruses in general. Additionally, a recent study (Sauerbrei and Wutzler 2009) also showed that the reduction in poliovirus Sabin 1 by heat (dry) was faster than that of the enveloped bovine viral diarrhoea virus and vaccinia virus. These results suggest that the viral envelope may have less impact on thermal stability of viruses than previously thought (Richards 1999; Baert *et al.* 2008; Sauerbrei and Wutzler 2009). This suggests that some enveloped viruses may remain infectious throughout the food chain if only mild heat treatment is performed.

To the best of our knowledge, this is the first study reporting thermal stability of parechovirus. Parechovirus is a recently discovered virus of the family *Picronaviridae* and found to be associated with gastrointestinal and respiratory syndromes similar to human enteroviruses (Stanway and Hyypia 1999; van der Sanden *et al.* 2008). The monodispersed virus fraction showed the highest thermostability of the viruses tested at 56°C, a relatively rapid reduction in a large fraction of the virus at 73°C, but inactivation was still incomplete after 3 min at 73°C (Fig. 1a,c), indicating the potential presence of a highly stable fraction. For parechovirus, we found that limited reductions in infectious viruses were concomitant with relatively high reductions in RT-PCRU. This might suggest that the RNA of the large fraction of noninfectious viruses, as present in all viral stocks (Jaykus 1997), is more sensitive to breakdown than the RNA of infectious viruses. Overall, parechovirus was the most thermostable of all viruses tested. While its transmission by foodborne routes has not yet been reported, the fact that they are shed in faeces, replicate in the gastrointestinal tract and are relatively heat stable warrants further investigation of their role in foodborne illness.

Except for influenza A virus, we conducted our experiments on viral suspensions with and without stool components. When enteric viruses are found on foods, it is usually the result of faecal contamination. This low level of contamination was chosen because we expected that by adding 1% stool the levels of viral aggregation would already increase significantly, thereby decreasing the magnitude of the most sensitive (monodispersed) viral fraction. Additionally, very low levels of interfering substances have been shown to have a profound impact on chemical inactivation of viruses in suspensions (Young and Sharp 1977; Mattle *et al.* 2011). In most cases in our study, the *TFL*-values were not significantly affected by the presence of 1% stool, which is consistent with the assumption that *TFL*-values represent the inactivation of the most sensitive (monodispersed) virus fraction. Apparently, 1% stool in suspension is not enough to cause aggregation of such a large fraction (>90%) of the viruses that the *TFL* is affected. Overall, we conclude that 1%

stool is not a significant interfering substance for assessing the thermo stabilities of the viruses we tested.

We calculated the *TFL*-values from the inactivation data based on best fit statistics, considering the monophasic (log linear), Weibull and biphasic models as candidates. At 56°C, the time-dependent reduction in the nonenveloped viruses was best modelled using the Weibull model. Most of the data for the 73°C showed monophasic inactivation kinetics, although two of the nine virus condition combinations demonstrated non-monophasic kinetics. When inactivation occurs in two or more phases, as is suggested by the biphasic and Weibull models, viral infectivity loss is not constant over time, but rather changes under progressing treatment. At 56°C, the shape of the inactivation curves for adenovirus, poliovirus and MNV1 were characterized by a rapid initial drop in the infectivity, followed by tailing caused by diminishing reduction rates as time increased (Fig. 1a,b; $P < 1$ Table 1). On the other hand, a shoulder was observed for parechovirus at 56°C and MNV at 73°C ($P > 1$). The presence of tails and shoulders (and hence biphasic inactivation kinetics) tends to indicate the presence of viral fractions having different native thermal stability. The most likely cause of this phenomenon would be that some of the viral suspension presents itself as single or monodispersed virions, while other parts of the suspension consist of aggregated viruses. The monodispersed fraction would be considered more heat labile and should theoretically show monophasic (log linear) reduction. On the other hand, the aggregated virus fraction will display a higher thermostability, depending upon the degree of aggregation, among other factors. This phenomenon has been described for MS2 bacteriophage (Langlet *et al.* 2007). Interestingly, for the enveloped influenza A (H1N1) virus, a monophasic model provided the best fit at both 56 and 73°C, indicating the constant rate of reduction over time and apparent limited heterogeneity of viral fractions.

TFL-values are generally calculated under the assumption of log-linear inactivation kinetics (Thomas and Swayne 2007; Hewitt *et al.* 2009), even though the inactivation curves often do not follow this pattern. Values that are falsely calculated by linear extrapolation, thereby ignoring shoulders or tails, could lead to overestimation or underestimation of the time-temperature combinations needed to achieve a desired degree of log reduction.

Because of the large number of viruses tested, this study was performed with a limited number of time points at 56 and 73°C. For those viruses demonstrating rapid $\log_{10}$ reduction with a matter of seconds, or a wide 95% confidence interval, more frequent sampling and additional replicates would be warranted. Nonetheless even with these study limitations, the deviations from monophasic reduction are apparent in almost half the cases, especially at the lower temperature, thereby providing valuable insight for intervention by heat treatment.

In summary, heating at 73°C for 3 min was sufficient to inactivate (i.e. to <1 infectious virus ml^{-1}, >4 $\log_{10}$ reduction) all the tested viruses except parechovirus 1. Influenza A virus and parechovirus 1 showed thermal stability similar to, or greater than, other viruses that have been proven to be transmitted by foodborne routes, and hence, their inactivation by common food processing methods should not simply be assumed. Caution should be taken in using and extrapolating *TFL*-values when analysing thermal inactivation, for example, in food or blood products, because reductions in numbers of infective viruses often do not follow log-linear (monophasic) kinetics and resistant fractions may remain infectious.

Acknowledgements

We would like to thank Dr Lee-Ann Jaykus (North Carolina State University, USA) for critically reviewing the manuscript and Dr Christiane Wobus (University of Michigan Medical School, USA) for providing MNV1 and Raw-264.7 cell. This work was partly funded by research school VLAG.

References

Abed, Y. and Boivin, G. (2006) Human parechovirus types 1, 2 and 3 infections in Canada. *Emerg Infect Dis* **12**, 969–975.

Bae, J. and Schwab, K.J. (2008) Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Appl Environ Microbiol* **74**, 477–484.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J. and Uyttendaele, M. (2008) Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Appl Environ Microbiol* **74**, 543–546.

Benschop, K., Molenkamp, R., van der Ham, A., Wolthers, K. and Beld, M. (2008) Rapid detection of human parechoviruses in clinical samples by real-time PCR. *J Clin Virol* **41**, 69–74.

van Boekel, M.A.J.S. (2002) On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int J Food Microbiol* **74**, 139–159.

Burnham, K. and Anderson, D. (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. New York: Springer.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A. and Vinje, J. (2006) Surrogates for the study of norovirus stability and inactivation in the environment: a

comparison of murine norovirus and feline calicivirus. *J Food Prot* **69**, 2761–2765.

Cerf, O. (1977) Tailing of survival curves of bacterial spores. *J Appl Bacteriol* **42**, 1–19.

Chick, H. (1908) An investigation of the laws of disinfection. *J Hyg (Lond)* **8**, 92–158.

Donaldson, K.A., Griffin, D.W. and Paul, J.H. (2002) Detection, quantitation and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using real-time RT-PCR. *Water Res* **36**, 2505–2514.

Duizer, E. and Koopmans, M. (2008) Emerging food borne viral diseases. In *Food Borne Viruses Progress and Challenges* ed. Koopmans, M.P.G. and Cliver, D.O. pp. 117–145. Washington DC: ASM Press.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P.G. and Estes, M.K. (2004) Laboratory efforts to cultivate noroviruses. *J Gen Virol* **85**, 79–87.

FAO/WHO (2008) Viruses in Food. In *Microbiological Risk Assessment Series No 13*: Scientific advice to support risk management activities: meeting report.

Friesema, I.H., Vennema, H., Heijne, J.C.M., de Jager, C.M., Morroy, G., van den Kerkhof, J.H.T.C., de Coster, E.J.M., Wolters, B.A. *et al.* (2009) Norovirus outbreaks in nursing homes: the evaluation of infection control measures. *Epidemiol Infect* **137**, 1722–1733.

Gilks, W.R., Richardson, S. and Spiegelhalter, D.J. (1996) *Markov chain Mante Carlo in practise*. New York, NY: CRC Press.

Greening, G. (2006) Viruses in foods. In *Human and Animal Viruses in Food* ed. Goyal, S.M. pp. 5–42. New York, USA: Springer.

Hewitt, J., Rivera-Aban, M. and Greening, G.E. (2009) Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *J Appl Microbiol* **107**, 65–71.

Isbarn, S., Buckow, R., Himmelreich, A., Lehmacher, A. and Heinz, V. (2007) Inactivation of avian influenza virus by heat and high hydrostatic pressure. *J Food Prot* **70**, 667–673.

Jaykus, L.A. (1997) Epidemiology and detection as options for control of viral and parasitic foodborne disease. *Emerg Infect Dis* **3**, 529–539.

Karber, G. (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuce. *Arch Exp Pathol Pharmakol* **162**, 480–483.

Koopmans, M. (2009) Noroviruses in healthcare settings: a challenging problem. *J Hosp Infect* **73**, 331–337.

Langlet, J., Gaboriaud, F. and Gantzer, C. (2007) Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. *J Appl Microbiol* **103**, 1632–1638.

Lieberman, D., Shimoni, A., Keren-Naus, A., Steinberg, R. and Shemer-Avni, Y. (2009) Identification of respiratory viruses in adults: nasopharyngeal versus oropharyngeal sampling. *J Clin Microbiol* **47**, 3439–3443.

Mattle, M.J., Crouzy, B., Brennecke, M., Wigginton, K.R., Perona, P. and Kohn, T. (2011) Impact of virus aggregation on inactivation by peracetic acid and implications for other disinfectants. *Environ Sci Technol* **45**, 7710–7717.

Richards, G.P. (1999) Limitations of molecular biological techniques for assessing the virological safety of foods. *J Food Prot* **62**, 691–697.

de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F. and Teunis, P.F.M. (2009) Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Appl Environ Microbiol* **75**, 1050–1057.

Rosario, R.F., Kimbrough, R.C., Van Buren, D.H. and Laski, M.E. (2006) Fatal adenovirus serotype-5 in a deceased-donor renal transplant recipient. *Transpl Infect Dis* **8**, 54–57.

van der Sanden, S., de Bruin, E., Vennema, H., Swanink, C., Koopmans, M. and van der Avoort, H. (2008) Prevalence of human parechovirus in the Netherlands in 2000 to 2007. *J Clin Microbiol* **46**, 2884–2889.

Sauerbrei, A. and Wutzler, P. (2009) Testing thermal resistance of viruses. *Arch Virol* **154**, 115–119.

Stanway, G. and Hyypia, T. (1999) Parechoviruses. *J Virol* **73**, 5249–5254.

Svraka, S., van der Veer, B., Duizer, E., Dekkers, J., Koopmans, M. and Vennema, H. (2009) Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral gastroenteritis. *J Clin Microbiol* **47**, 1674–1679.

Thomas, C. and Swayne, D.E. (2007) Thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat. *J Food Prot* **70**, 674–680.

Tuladhar, E., Terpstra, P., Koopmans, M. and Duizer, E. (2012) Virucidal efficacy of vaporized hydrogen peroxide disinfection. *J Hosp Infect* **80**, 110–115.

Westrell, T., Dusch, V., Ethelberg, S., Harris, J., Hjertqvist, M., Jourdan-da Silva, N., Koller, A., Lenglet, A. *et al.* (2010) Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. *Euro Surveill* **15**, pii=19524.

Wobus, C.E., Karst, S.M., Thackray, L.B., Chang, K.O., Sosnovtsev, S.V., Belliot, G., Krug, A., Mackenzie, J.M. *et al.* (2004) Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol* **2**, e432.

Young, D.C. and Sharp, D.G. (1977) Poliovirus aggregates and their survival in water. *Appl Environ Microbiol* **33**, 168–177.