FSA Project FS101074:

A critical review of the effect of heat, pH and water activity on the survival of Hepatitis A and E viruses

A Report to the United Kingdom Food Standards Agency
July 2014

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Executive Summary

A critical review has been performed on published work on the survival and elimination of hepatitis A and E viruses. Information on hepatitis A virus survival in water, on surfaces, on hands, and other matrices found in food production environments has been included, as have data on survival in various foods.

Hepatitis A virus is capable of prolonged survival in the environment and on foods, and is highly resistant to freezing. On surfaces, including china, tile, aluminium, paper and cotton clothes, the virus can remain infectious for several months, depending on temperature and relative humidity. Hepatitis A virus can tolerate low water activity, and can survive desiccation. It can survive in an infectious state for at least several hours on hands, and can survive, i.e. persist in an infectious state, for several months in frozen berries. On contaminated vegetables, the virus will remain infectious from contamination to consumption. The type of matrix has a significant influence on hepatitis A virus survival, i.e. the temperature does not have the same effect according to whether the matrix is simple (e.g. water) or complex (e.g. berries). The virus is highly stable at low pH, and is capable of tolerating the acidity of berry fruits.

Some data is available on the effect of disinfection and elimination procedures. From studies on hepatitis A virus in suspension, it appears resistant to some heating regimes e.g. those encountered during mild pasteurization. Few studies have been conducted on the effect of heat on hepatitis A virus in foods; the available data indicates that some food components, e.g. fats and sugars, can increase the virus' resistance to higher temperatures. Hepatitis A virus is completely eliminated by boiling. A statistical analysis of the available data on the response of hepatitis A virus to heat is also included, describing the virus' greater resistance in complex matrixes.

Common household washing or laundering practices may not eliminate hepatitis A virus contaminating clothing or articles such as tablecloths. The use of bleach in such procedures would constitute an effective measure if items are suspected of being heavily contaminated. Domestic bleach and other sodium hypochlorite disinfectants can produce reductions in hepatitis A virus on surfaces, but only at high concentrations. Glutaraldehyde compounds and sodium hydroxide are highly efficient for HAV inactivation. Quaternary ammonium

compounds may be useful for eliminating HAV in liquids, but not on dry surfaces. Washing hands can reduce contaminating hepatitis A virus by 75-95%. Chlorination can be effective against infectious HAV in aqueous suspension, although it is less so when the virus is associated with foodstuffs.

There is very little information available on the survival of hepatitis E virus; it may be capable of robust survival, although inactivation by heating at 71°C for 20 minutes has been demonstrated.

It is concluded that hepatitis A virus appears to be a highly robust virus which is able to survive on surfaces and foods, and is resistant to mild heat. There is however only limited information on the effects of heating regimes used in the food industry on hepatitis A virus on at-risk foods such as berry fruits and their products, and it is recommended that further studies be performed, modeling industrial features as closely as possible, to acquire the necessary information.

Hepatitis E virus may be similarly robust, but the lack of a reliable infectivity assay has hampered extensive study. It is recommended that studies be undertaken to develop an efficient propagation system (based on in vitro cell culture), to facilitate the acquisition of extensive information on survival of hepatitis E virus, and its response to disinfection and elimination procedures.

Keywords: hepatitis A virus, hepatitis E virus, HAV, HEV, survival, elimination, berry fruit, vegetables, pork products, heat, disinfection.

Glossary

Antigenicity: the capacity to stimulate the production of antibodies or the capacity to react with an antibody.

Capsid: the protein shell of a virus.

Cryo-electron microscopy: a form of transmission electron microscopy (EM) where the sample is studied at cryogenic temperatures.

CT value: concentration of a disinfectant in mg l^{-1} multiplied by the contact time in min.

Electron beam irradiation: a process which involves using electrons, usually of high energy, to treat an object for a variety of purposes (e.g. sterilization).

Elimination: removal of infectious virus by a deliberately applied procedure.

Fomite: any object or substance capable of carrying infectious organisms, and hence transferring them from one individual to another.

High hydrostatic pressure (HHP): a food preservation technology based on the inactivation of microorganisms by applying extreme pressures.

Inactivation: the decline in virus infectivity effected by a deliberately applied procedure

kGy: Kilo Geigy, a measure of radioactivity.

MegaPascals (MPa): a measure of force per unit area, defined as 1,000,000 newton per square metre.

Open reading frames (ORFs): is the part of a gene that contains no stop codons.

Picornavirus: is a virus belonging to the family *Picornaviridae*. They are non-enveloped, positive-stranded RNA viruses with an icosahedral capsid.

Pfu: plaque-forming units; number of virus units which can be detected through formation of discrete zones of cytopathic effect in a lawn of cultured cells.

Positive-sense RNA virus: a virus whose genetic information consists on a single strand of RNA that is the positive (or sense) strand which encodes mRNA and protein.

RT-PCR-detectable units: Number of virus units which can be detected by reverse transcription polymerase chain reaction used in quantitative format (qRT-PCR).

Stability: persistence of the intact virus capsid.

Survival: the persistence of virus infectivity when no treatment is deliberately applied

T90: Time taken for the first 90% reduction in virus infectivity, where the decay rate is linear.

$TCID_{50}$: Tissue culture infectious $dose_{50}$: the dilution of a virus suspension which can infect 50% of cultured cell sheets exposed to it (analogous to a most probable number test in bacteriology). $TCID_{50}$ can be related to infectious virus units.

TFL: log_{10} reduction in virus infectivity, where the decay rate is non-linear.

Virion: a complete virus particle that consists of an RNA or DNA core with a protein coat sometimes with external envelopes and that is the extracellular infective form of a virus.

1. Introduction

Human enteric viruses are known to be a major cause of infectious intestinal disease in the UK, with foodborne transmission thought to be an important route of infection; the Food Standards Agency (FSA)-funded second study of infectious intestinal disease in the community (IID2 Study) provides extensive information on the enteric virus-associated burden of infection (http://www.foodbase.org.uk/results.php?f_report_id=711). Recently, foodborne transmission of hepatitis A virus (HAV) and hepatitis E virus (HEV) has become a concern (Carvalho *et al* 2012; Colson *et al* 2010), raising questions about the survival of these hepatic viruses in a range of different foods. There are significant gaps in our knowledge regarding the survival of these viruses in foods and the environment (including food contact surfaces), and also regarding the effect of elimination procedures used in food supply chain settings, particularly for HEV.

HEV can be considered as an emerging pathogen, and neither it nor HAV are specifically targeted by food safety management systems. As part of its Foodborne Disease Strategy 2010 – 2015, the FSA will evaluate the effect of interventions designed to reduce foodborne disease, including illness caused by emerging pathogens and agents not specifically addressed by individual risk management programmes. To assist the FSA with this evaluation, it is timely to review the available information on survival and elimination of HAV and HEV, to inform risk analysis and to assist in identifying effective control measures to reduce the occurrence of foodborne transmission of these pathogens.

HAV belongs to the genus *Hepatovirus*, in which it is the only species; the genus itself lies within the family *Picornaviridae*. HAV particles are approximately 28 nm in diameter, possessing an icosahedral capsid enclosing the single-stranded positive-sense RNA genome. The HAV genome is 7.5 kb in length, and contains four structural and seven non-structural genes. The infectious dose of HAV particles may be very low (http://www.seafoodhaccp.com/SeafoodData/BadBugBook/chap31.html), perhaps in some cases even single particles (Cliver, 1985). After ingestion, the virus passes through the gastrointestinal tract and is carried to the liver (O'Connor, 2000). Damage to the liver may result primarily from the immune response to the virus-infected cells (Hollinger and Emerson, 2001; Pintó *et al.*, 2014). Released virions enter the bile duct, pass into the gastrointestinal tract, and are then excreted in faeces. During active virus replication, infected

persons remain generally asymptomatic. Peak virus shedding occurs approximately 4-6 weeks after ingestion of the agent, just before the onset of acute symptoms (which include jaundice, fever, malaise, nausea, abdominal discomfort, and dark urine), and infected persons can have virus in their stools before becoming aware of any illness. Virus excretion can continue generally up to 8 days after onset of symptoms (Hollinger & Emerson, 2001; Pinto *et al.*, 2012). In infants and young children, symptoms are generally milder, but faecal excretion persists for longer compared to adults (Hollinger and Emerson, 2001). The fatality rate is approximately 0.3% (Issa & Mourad, 2001), with serious complications being more likely to occur in persons over 40 years of age. Infection with HAV confers lifelong immunity (Melnick, 1995; Ryder & Beckingham, 2001); Pinto *et al.*, 2014).

In developing countries, most HAV infections occur among children, and adults are generally immune; in North America and Western Europe, overall population infection-derived immunity to HAV is declining, most likely due to increased standards of public health in recent decades which have led to less circulating virus and consequently a lesser exposure to the virus than previously (Pinto *et al.*, 2010). This creates a risk of the occurrence of large outbreaks, with contaminated foods imported from countries of high endemicity being one of the potential hazards (Pebody, 1998).

Next to noroviruses, Hepatitis A virus is currently the most significant viral agent of foodborne disease (Rzeżutka & Cook, 2004). The types of foodstuff most often implicated in outbreaks of hepatitis A are those which are eaten raw or only slightly cooked, such as soft fruit, salad vegetables, and shellfish, or handled extensively prior to consumption, such as prepared salad (Fiore, 2004). Foods may acquire viral contamination by contact with infected persons during harvesting or preparation, when viruses are transferred or shed on to foodstuffs *via* faecally contaminated hands. One milligram of faeces may contain up 10^7 genome copies in an immunocompetent HAV-infected patient (faeces from immunocompromised patients, such as people who are HIV-positive, may contain up to 10^{11} genome copies per mg), so microscopic quantities of faeces could harbour sufficient virus particles to constitute a hazard (Pinto *et al.*, 2012). Fresh produce such as leafy green vegetables and berry fruit can be extensively handled during harvesting, especially strawberries and raspberries which are often picked by hand. Extensive handling post-harvest may also occur; for instance with green onions at least three workers can be required to peel, trim and bundle them (Dentinger *et al.*, 2001). Such practices increase the chances of

contamination by infected persons. Contamination can also occur through contact with sewage-polluted waters, used for irrigation or washing. The effectiveness against foodborne viruses of treatments used in the preparation of minimally processed foods is in many cases doubtful (Cliver, 1997; Hirneisen *et al.*, 2010); this Report details the information to date on the effectiveness of such treatments, as applied on virus suspensions or in experimental systems on foodstuffs.

HEV is a small, non-enveloped, single-stranded, positive-sense RNA virus, 35 nm in diameter. The genome of approximately 7.2 Kb in length (Tam *et al.*, 1991) is capped at the 5′ end and polyadenylated at the 3′ end. It contains short stretches of untranslated regions (UTR) at both ends. The HEV genome has three open reading frames (ORFs). ORF1 encodes the non-structural polyprotein (nsp) that contains various functional units: methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp) (Chandra *et al.*, 2008). ORF2 encodes the viral capsid protein, the N-terminal signal sequence and glycosylation loci. ORF3 encodes a small regulatory phosphoprotein. HEV was designated in 2004 as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.*, 2005). The mammalian HEV genome was first cloned from cDNA libraries prepared from the bile of macaques experimentally inoculated with stool suspensions from human patients (Reyes *et al.*, 1991). Later PCR amplification products were obtained of the genomes of multiple geographically distinct isolates of HEV (Purcell & Emerson, 2001).

Ingestion of HEV particles is assumed to be the most important infection route. The virus may replicate in the intestinal tract but this has not been demonstrated to date. Via the portal vein, HEV can reach the liver (Panda *et al.*, 2007), which is believed to be the main HEV target organ. Hepatocytes most likely are the main replication cell type (Williams *et al.*, 2001). HEV attaches to host cells via specific high affinity receptors and enters the cytoplasm by clathrin-mediated endocytosis (Kapur *et al.*, 2012). Virtually nothing is known about the process by which HEV RNA enters target cells. It has been estimated that the infectivity titre of HEV for macaques is 1000-fold higher when inoculated intravenously compared to when it is ingested (Meng, 2003); however there is no information on the infectious dose for humans.

HEV is a main cause of epidemic hepatitis in developing countries and single cases of hepatitis in higher income countries. According to the World Health Organisation (http://www.who.int/mediacentre/factsheets/fs280/en/) worldwide there are 20 million hepatitis E infections, over three million acute cases of hepatitis E and 57,000 hepatitis E-related deaths every year.

HEV variants most closely related to those infecting humans can be divided into at least four genotypes (Panda *et al.*, 2007). Genotypes 1 and 2 only seem to affect humans. Genotype 1 viruses are predominantly isolated from outbreaks and sporadic cases in Asia and Africa, whereas genotype 2 strains mainly have been observed in outbreaks in Mexico and Africa. Genotypes 3 and 4 are zoonotic and are observed in different animal species and sporadic human cases, worldwide for HEV genotype 3 and mainly in Asia for HEV genotype 4.

Foodborne transmission of HEV was first demonstrated in clusters of Japanese patients after eating raw or undercooked meat from swine, wild boar or Sika deer (Tei *et al.*, 2003; Takahashi *et al.*, 2004). The genomic sequences of HEVs identified from the infected patients were identical to those recovered from the frozen leftover meat (Tei *et al.,* 2003; Yazaki *et al.*, 2003).

Through either detection of HEV sequences and/or epidemiological study, more hepatitis E cases have been linked to the consumption of food products contaminated with the virus. This includes infection via locally produced meat products (Colson *et al.*, 2010) but also from game meat, processed pork (Legrand-Abravanel *et al.*, 2010) mussels, shellfish and other bivalves (Donia *et al.*, 2012). Eating raw or undercooked meat products has been identified as a higher risk factor (Colson *et al.*, 2010). Bivalves are known transmitters of enteric viruses and especially oysters are eaten worldwide as raw seafood. More recently HEV sequences have been detected on soft fruits and vegetables, with irrigation water as the suspected contamination origin (Brassard *et al.*, 2012; Kokkinos *et al.*, 2012).

Transmission of a food- or environmentally-transmitted virus is dependent not only on its interaction with a host, but also on its interaction with the environment outside of the host. Viruses outside a host may be regarded as inert particles, and, possessing no intrinsic metabolism, they do not require any nutrients to persist. Nonetheless, they possess a degree of robustness which allows them to remain infectious during the various conditions they may

encounter between one host and another. This is illustrated by the number of outbreaks of enteric viral disease attributable to water or foodborne transmission.

The longer a virus can survive outside of a host the greater is its potential for transmission. This potential will be affected by various environmental conditions and factors as heat, moisture and pH. These and other factors will vary in presence and extent among various environments. In general, studies to determine the potential for survival of viruses, or the effect of procedures intended to eliminate them, have been conducted using basic principles in common (Nuanualsuwan & Cliver, 2003a). Infectious virus in defined numbers is artificially introduced into a sample of water, soil, food, etc., and the sample stored under conditions (e.g. temperature, moisture) relevant to those experienced naturally, for selected time periods. Viruses are then extracted from the sample and enumerated. There are various methods which can be used to extract viruses from food and environmental samples (Croci *et al.*, 2008); basically, these methods involve separating virus particles from solid material in the sample, and then concentrating them so that they may be delivered to a detection system. For enumeration of infectious viruses it is necessary to use cell culture in quantal format, e.g. plaque assay. The number of infectious virus remaining in the sample is compared with the number which was introduced, and statistical procedures can be performed to calculate any degree of decline. The use of cell culture is the only way (excluding human or animal experiment) to evaluate infectivity of viruses. It is quantitative (e.g. plaque assay), specific and sensitive. Nevertheless it has some drawbacks. It cannot be used for the most important pathogenic viruses (Norovirus, hepatitis A and E viruses) as it is time consuming and may be linked to some bias because of viral aggregation. Aggregation will provoke a decrease of viral titre because one aggregate will be counted as one plaque-forming unit even if it is formed by several infectious viruses. Such phenomena occur especially for high viral loads which is usually the case in inactivation studies. Alternatively, viral aggregates may express as lower an inactivation rate, compared to their respective isolated viral particle. Even if it is usually omitted, it is interesting to have information about the aggregation state of the viral suspension used for the inactivation studies.

Previous reviews (Rzezutka & Cook, 2004; Vasickova & Kovarcik, 2013) have highlighted generic features of enteric virus survival e.g. their ability to persist in an infectious state for prolonged periods in aqueous environments, and their resistance to freezing. Published reviews of the effects of procedures used within the food industry, such as thermal processing

(Baert, 2013), have discussed their potential for virus elimination, and indicated issues such as the protective effect of food components. In this current review, these generic features of virus survival and elimination will be reviewed with a focus on HAV and HEV.

In this report, a distinction is made between virus "survival" and virus "inactivation" and virus "elimination". Survival signifies the persistence of infectivity when no treatment is deliberately applied; and conversely, inactivation signifies decline in infectivity effected by a deliberately applied procedure. "Stability" in this report generally refers to stability of the intact virus capsid, but is occasionally used in other contexts for clarity of expression. "Elimination" has been used instead of "removal", except in the context of pharmaceutical products treatment, where it is a standard term.

Having in mind the drawbacks of cell culture, it is convenient to express virus survival or inactivation as a decrease of the initial viral load as a function of time. The kinetic may follow a log linear function. In such cases the T90 (the time to reduce 90 percent of initial load) or inactivation coefficient is a convenient way to compare decay between different viruses for different situations. If the function expresses a non-linear pathway, T90 cannot be used considering its initial definition. Another denomination should then be used to express a reduction for a given time. Time to reduce first log_{10} (TFL) of infectivity has therefore been introduced to compare decay rate of different viruses expressing a non-linear decay. In this report, where the reviewed data are clearly linear, T90 has been used to describe the initial decrease in infectious viral load; where the data is not explicitly linear, TFL has been used.

In the case of the use of disinfectants such as those based on chlorine, time and concentration should be taken into account, giving CT (concentration of disinfectant in mg l^{-1} multiplied by the contact time in min) values which must then be given in order to compare virus inactivation in different conditions.

The best way to estimate the survival or inactivation of one particular pathogenic virus is to use the virus itself. However, as most of them are not able to be cultivated, a surrogate is needed. Using a cultivatable virus from the same family as the pathogenic target is a convenient way to estimate viral inactivation. Such approach is not a complete guarantee of a good estimation of virus resistance as virus from same family or even from the same genus can have very different behaviour in environment or during treatment. In the preparation of

this report, it was concluded that there are currently no useful surrogates for HAV or HEV, and few, if any, publications have proposed their use. Cultivatable strains of HAV (CF13 or HM-175) are currently used for the estimation of natural HAV strains. For HEV, there is no validated virus / cell culture system, and consequently information on virus survival and inactivation is currently sparse.

In this report, information on hepatitis virus survival first, followed by information on elimination, in an encyclopaedic format, i.e. by topic (matrix, procedure). Elimination procedures are listed alphabetically. Some procedures (such as heat, disinfection by chlorine-based substances) are in widespread use within the food industry whereas others (such as ultrasound or ozonisation) are not widely applied or are currently only being examined in academic proof of concept studies. Information from studies conducted within other contexts (e.g. elimination of HAV from therapeutic products) has also been reviewed as the data accrued should be of relevance to food production contexts.

Details of how the review was performed are given in Annex 2.

2. Survival of hepatitis A and E viruses

2.1 In water

An early indication of the robust survival capacity of HAV in the environment was provided by the study of Sobsey *et al.* (1986) where the time taken for a 2 log decline in the number of infectious virus units in various water and wastewater samples was monitored. In each instance, several weeks were necessary for this scale of reduction to be observed. The presence of viable microorganisms negatively affected survival, but their effect was mitigated by the presence of clay particles, perhaps by adsorption of the viruses conferring protection from predation. In a subsequent study carried out by Sobsey *et al.* (1988) samples of groundwater, raw wastewater, sea water and estuarine water were artificially contaminated with ~ 1 x 10^5 infectious units HAV and incubated at 5°C and 25°C for up to 84 d. At 25°C, infectious virus remained detectable at the end of the experiment in all the samples, with HAV in groundwater showing the highest survival. No direct correlation was made between water types and microbial counts, but it may be that there were fewer inhibitory / predatory microorganisms in the groundwater samples than in samples taken from other sources. Little decline in infectivity was reported in samples stored at 4°C (the data was not given). Similar results were obtained by Nasser and Oman. (1999). They expressed the data they obtained as inactivation rate (log d^{-1}). In groundwater, HAV was inactivated at a rate of approximately -0.06 log d^{-1} at 10°C, and approximately -0.02 and -0.03 log d^{-1} at 20°C and 30°C respectively. Although the inactivation rate at 10°C is anomalous (the rate of decline is greater than at higher temperatures) overall and the indication is that many days would be required for infectious HAV to decline by 1 log in groundwater. Analysing data from these prior studies, John and Rose (2005) predicted a T90 value for HAV in groundwater of 100 d. In wastewater the inactivation rates were less than -0.02 log d^{-1} at 5°C, 10°C and 20°C, and approximately -0.04 log d^{-1} at 30°C.

The survival of HAV in seawater can be influenced by temperature. Bosch (1995) reported less than 1 log reduction of infectious HAV after storage at 5°C for 30 d, whereas at 25°C for 30 d a reduction between 1 and 2 log was observed (the presence of sediment material having no effect). Crance *et al.* (1998) stored samples of artificially contaminated sterile seawater at 4°C, 19°C, and 25°C, for up to 92 d; they observed little decline in infectivity of HAV in seawater at 4°C, and even at 25°C several days elapsed before 1 log infectivity was lost. The rates of decline of detectable HAV antigen were considerably less than the rates of decline in

infectivity. As in the study of Biziagos *et al.* (1988), detection of antigenicity will have signified that the viral capsid proteins remained in a detectable state (see below); whether this represented capsid stability was not determined as no genome detection was performed. In both studies the water was filtered before addition of HAV, so decline in infectivity and viral antigen concentration was not due to a predation effect of other microorganisms. Substances produced by marine microorganisms may however have been responsible; Patti *et al.* (1987) demonstrated the presence in estuarine water of a biological heat-labile factor active against HAV.

The survival of HAV in mineral water was examined by Biziagos *et al.* (1988). Viral infectivity was determined by cell culture; the presence of HAV antigen was monitored by radioimmunoassay-endpoint titration. After 360 days storage at 4°C, HAV was still infectious, whereas no infectivity could be detected after 330 days storage at 23°C. At both temperatures, the infectivity of HAV declined at a faster rate than the antigenicity. This could imply that the virus capsid remained intact but lost features determining infectiousness; however there are several possible interpretations. At 4°C the difference in the decline rates was less pronounced. The persistence of infectivity was enhanced by the protein concentration of the mineral water: at each temperature survival was greater when the water contained 50 mg ml^{-1} protein than when it contained 5 mg ml^{-1} protein (proteins derived from cell culture supernatant). Studies performed to ascertain virus survival in water (Espinosa *et al.,* 2008; Gassilloud *et al.*, 2003) found that the decline in genome copy number as measured by qRT-PCR was less than the decline in infectivity, indicating that viral capsids could remain intact after infectiousness has been lost.

Table 2.1 shows information from studies on HAV survival in water.

Table 2.1: Survival of HAV in water

Water type	Temperature (°C)	Initial decrease in viral load (days)	Approximate overall log decline in infectivity	Reference
Groundwater	25	ND	2 in 84 d	Sobsey *et al.* (1988)
Seawater	25	ND	> 3 in 84 d	Sobsey *et al.* (1988)
Estuarine water	25	ND	> 3 in 84 d	Sobsey *et al.* (1988)
Estuarine water,	24	1*	3 in 5 d	Sobsey *et al.* (1988)
Mineral water	4	519†	< 1 in 360 d	Biziagos *et al.*, (1988)
Mineral water	23	89†	5 in 330 d	Biziagos *et al.*, (1988)
Seawater	4	-	< 1 in 30 d	Bosch (1995)
Artificial seawater	4	-	< 1 in 85 d	Crance *et al.* (1998)
Seawater	25	21*	1.5 in 30 d	Bosch (1995)
Artificial seawater	19	24†	3 in 92 d	Crance *et al.* (1998)
Artificial seawater	25	11†	5 in 55 d	Crance *et al.* (1998)

* TFL: time to reduce first $\log_{10}$
†T90: time to reduce 90 percent of initial load
ND: not determined.

The data on initial decrease of viral load obtained by the above studies epitomise what has been observed in studies of survival in other environmental matrices, and in studies on the effect of elimination procedures: both linear and non-linear declines in infectivity are observed. This variability may be due to differences in experimental procedure, in number of replicates, or in differences between strains, and indicates the difficulty in producing comprehensive statistical models on HAV (and HEV) survival and elimination.

Costafreda *et al*. (2014) postulated that the outstanding physical stability of HAV in the environment is mediated by a folding-dependent highly cohesive capsid. HAV strains whose capsids differ in such folding show significant differences in resistance to temperature and acid pH (Costafreda *et al.*, 2014).

2.2 In soil and organic wastes

HAV in sewage or sewage by-products such as activated sludge could contaminate agricultural land through their application as fertiliser. This could be a concern if the virus is able to survive long enough to contaminate food crops subsequently grown on the land (Gerba *et al.*, 2002). Sobsey *et al.* (1988) reported that several weeks elapsed before HAV spiked into sewage effluent declined by greater than 1 log. HAV was found to decline by less than 1 log when sewage sludge was stored at 4°C or 28°C for 24 h (Katz & Margolin, 2007). When calcium hydroxide was added to the sludge samples to mimic alkaline (pH 12) treatment of wastes, less than 5 log reduction in infectious virus was observed over 24 h at 4°C, and less than 6 log reduction over 12 h at 28°C (Katz & Margolin, 2007); this treatment would thus appear effective in eliminating infectious HAV from sewage sludge prior to land application. Wei *et al.* (2010) also reported that HAV degraded immediately in high pH biosolids. Desiccation of faecally-related waste will also likely have a negative effect on the persistence of HAV infectivity (Stauber *et al.*, 2005).

There may be a potential for HAV to contaminate the environment via the disposal of contaminated babies' nappies in landfill. Gray *et al.* (1993) modelled the survival of HAV in soiled nappies and found that at 5°C a 1 log reduction in infectivity was achieved after 30 d. At 40°C less than 4 log virus was inactivated by at least 30 d.

HAV spiked into septic tank effluent declined only by less than 1.5 log after 70 d at 5°C, and by ~ 2 log after 70 d at 22°C (Deng & Cliver, 1995b). Mixing the effluent with swine manure enhanced decline, possibly through increased microbial activity, or by the action of microbial metabolites in the waste (a similar effect was seen when effluent was mixed with filtered waste). Deng and Cliver (1995a) subsequently found that several strains of bacteria (*Bacillus*, *Enterococcus* and *Pseudomonas*) isolated from manure possessed anti-HAV properties, in most cases via extracellular substances. Extracellular compounds (not further identified) of *Pseudomonas aeruginosa* were found to reduce HAV infectivity by ~1.5 log over 400 min (Nasser *et al.*, 2002).

Wei *et al.* (2010) spiked swine manure with 10^{7} $TCID_{50}$ HAV and reported declines in infectivity up to 3 log over 60 d when the waste samples were stored at both 4°C and 20°C. From the graphical data presented, at day 10 there had been a 2 log reduction in infectivity,

but thereafter the decline was less pronounced, and infectious virus was still detectable at day 60. These findings suggest that HEV could behave similarly in stored animal manure; however this information awaits specific studies.

In contaminated soil and sediments, the persistence of infectious HAV may be prolonged. In samples of saturated sand, clay and organic soil saturated with HAV-contaminated primary sewage and stored at 25°C, 3.2%, 0.8% and 0.4% of the original number of infectious units remained at 84 d (Sobsey *et al.*, 1988). In marine sediment saturated with HAV-contaminated seawater, 1.7% of their original infectious HAV was detected after 56 d storage at 25°C (Sobsey *et al.*, 1988). Blanc and Nasser (1996) examined the survival of HAV in both loamy and sandy soil types at 10°C and 23°C. They saturated the experimental samples with groundwater, and primary and secondary effluent from a wastewater treatment plant and stored them at the temperatures mentioned for 20 d. In none of the samples containing the soil / groundwater / effluent / temperature combinations did HAV infectivity decline by more than 1 log. The greatest decline in infectivity was observed in some control samples (presumably containing groundwater or effluent alone, although it was not stated): in groundwater at 10°C and 23°C infectious HAV declined by 1.66 and 1.54 log respectively, and in tertiary effluent at 10°C infectious HAV declined by 1.44 log (the secondary effluent control at 10°C was not performed, but at 23°C HAV infectivity declined by 0.22 log, and the tertiary effluent control at 23°C was not performed). Examining the persistence of HAV in spiked soil samples (type not given, possibly taken from a riparian site), Parashar *et al.* (2011) monitored persistence of HAV RNA, and observed that it could be detected up to and including the 13th week in samples stored at 37°C, 5 weeks after it could not be detected in samples placed in a location where they were exposed to environmental temperatures. This may indicate that temperature fluctuations may affect persistence of intact virus. However it should be noted that this study was conducted in Pune, India, where diurnal temperatures are higher than those normally encountered in the UK and Europe; similar environmentally-relevant studies in the UK and Europe would provide useful information on HAV survival. Parashar *et al.* (2011) also examined the persistence of HEV genotype (gt)I under the same conditions as described above. Again, HEV RNA was detected for longer (week 10) at 37°C than in samples exposed to environmental temperatures (week 9), although the difference was not pronounced.

Table 2.2 shows information from studies on HAV survival in soil and organic wastes.

Table 2.2: Survival of HAV in soil and organic waste

Sample type	Temperature (°C)	Initial decrease in viral load (days)	Approximate overall log decline in infectivity	Reference
1° sewage effluent		ND	2 log in 84 d	Sobsey *et al.* (1988)
2° sewage effluent		ND	2 log in 84 d	Sobsey *et al.* (1988)
Septic tank effluent	5	58.5*	< 1.5 log in 70 d	Deng and Cliver (1995b)
Septic tank effluent	22	35.1*	< 1.5 log in 70 d	Deng and Cliver (1995b)
Corolla sand saturated with 1° effluent	25	ND	2 in 84 d	Sobsey *et al.* (1988)
Cecil clay saturated with 1° effluent	25	ND	< 2 in 84 d	Sobsey *et al.* (1988)
Ponzer organic muck saturated with 1° effluent	25	ND	2.5 in 84 d	Sobsey *et al.* (1988)
Corolla sand saturated with 1o effluent	25	ND	< 2 in 84 d	Sobsey *et al.* (1988)
Loamy soil saturated with groundwater	10	ND	0.02 in 20 d	Blanc and Nasser (1996)
Loamy soil saturated with groundwater	23	ND	0.2 in 20 d	Blanc and Nasser (1996)
Loamy soil saturated with 2° effluent	10	ND	0.5 in 20 d	Blanc and Nasser (1996)
Loamy soil saturated with 2° effluent	23	ND	0.1in 20 d	Blanc and Nasser (1996)
Loamy soil saturated with 3° effluent	10	ND	0.6 in 20 d	Blanc and Nasser (1996)
Loamy soil saturated with 3° effluent	23	ND	0.2 in 20 d	
Sandy soil saturated with groundwater	10	ND	0.3 in 20 d	Blanc and Nasser (1996)
Sandy soil saturated with groundwater	23	ND	0.47 in 20 d	Blanc and Nasser (1996)
Sandy soil saturated with 2° effluent	10	ND	0.03 in 20 d	Blanc and Nasser (1996)
Sandy soil saturated with 2° effluent	23	ND	0.03 in 20 d	Blanc and Nasser (1996)
Sandy soil saturated with 3° effluent	10	ND	0.2 in 20 d	Blanc and Nasser (1996)
Sandy soil saturated with 3° effluent	23	ND	0.31 in 20 d	Blanc and Nasser (1996)

* TFL: time to reduce first $\log_{10}$

1°: primary sewage effluent; 2°: secondary sewage effluent; 3°: tertiary sewage effluent.

ND: not determined

2.3 On fomites

An early study on the potential for survival of HAV on surfaces was conducted by Sobsey *et al.* (1988). They dried virus suspensions (in PBS or a 20% faecal suspension) in the wells of polystyrene tissue culture plates, stored the plates at 5°C and 25°C and recovered infectious virus at various time points up to 30 d. Survival was more pronounced at the lower temperature, with 60% and 10% of infectious virus remaining after 30 d in the dried PBS and faecal suspensions respectively. At 25°C it took over two weeks for a 1 log decline in infectivity of virus dried in PBS, and over one week for a similar reduction in infectious virus in dried faecal suspension. This demonstrated the potential for HAV to survive on surfaces over a prolonged period of time which could be sufficient to allow the virus to pass from an infected person to a new contact. To examine whether relative humidity (RH) has any effect on persistence of HAV infectivity, Mbithi *et al.* (1991) prepared HAV suspensions in a medium comprising 10% faeces in saline, then placed 10 ml drops onto stainless steel disks. The discs were then stored at various temperatures and RHs up to 96 h (see table 2.3), and remaining infectious virus counted by plaque assay. Persistence of infectivity was inversely proportional to RH and temperature level. Infectious virus was detectable up to 96 h at 20°C, with log reductions of 1 or less under RHs up to 80%. Tests at 5°C were only conducted over 4 h and little reduction in infectivity was observed: it is likely that survival would have been at least as prolonged as at 20°C. At 35°C at 95% RH, infectious HAV was undetectable after 4 h; as reduction was reported as percentage loss of infectivity, and no details of virus titre are given it cannot be seen what log reduction this represented. At 30°C, data was only reported for time points up to 4 h, but infectious virus still remained on discs stored at RHs of 80% or lower. The drops of virus suspension on the discs became dry in 20 min or less at RHs of 25% and 55%, but infectious HAV remained; this demonstrates the resistance of the virus to desiccation, confirmed by several other studies. Terpstra *et al.* (2007), for instance, observed no loss of infectious virus after 4 h drying on stainless steel; after 28 d at room temperature the log reduction was high, although infectious HAV could still be detected after this time. An earlier study, carried out by McCaustland *et al.* (1982), showed that HAV in dried faeces from marmosets (species of monkey) was still infectious after 30 d at 25°C.

Abad *et al.* (1994) found that HAV could survive for up to 60 days on a variety of surfaces. HAV was inoculated onto several materials which were either porous (paper and cotton cloth) or non-porous (aluminium, china, glazed tile, latex, and polystyrene), in either PBS or a 20% faecal suspension to produce a contamination of approximately 5 x 10^6 infectious units

per cm, dried for 3-5 h at room temperature, then stored under various temperatures and humidities (see table 2.3) for 30 or 60 d. The initial drying step produced a decline of approximately 1 log, but thereafter on each material (both porous and non-porous types) HAV infectivity declined at most by approximately 3 log, and infectious virus could still be recovered at the end of the experiment. Decline in infectivity was less pronounced on non-porous materials (aluminium, china, glazed tile, latex, and polystyrene) than porous (paper and cotton cloth); this may have been due to viruses being better retained by the porous materials and thus not being recovered for the infectivity assay. Generally, the presence of faecal material made no difference to HAV survival rates; although some protection was observed, it was not consistent or significant. Abad *et al.* (2001) performed a series of additional tests to monitor HAV survival on china and paper each day for 7 d and found similar enhanced survival of HAV on the non-porous china surface. In these experiments higher rates of decline were observed than in their previous tests, although infectious virus was still present at the last time point. Liu (2007) examined the survival of HAV on ceramic and formica surfaces, following artificial contamination of the virus in a faecal suspension; HAV RNA levels were monitored at 6 weeks and an approximately 2 log decline in 6 weeks was observed, but no correlation with infectivity was made.

In contrast, a study by Kim *et al.* (2012) indicated that HAV may survive more robustly on porous than non-porous surfaces. HAV was spiked onto samples of stainless steel and wood, which were then stored at various temperatures and humidities (see table 2.3). The infectivity of the virus declined faster on steel than on wood, and the decline was enhanced by increasing temperature. However, RH had no significant effect. Kim *et al.* (2012) statistically analysed their data using both a linear model and Weibull model with the latter being used to describe non-linear survival patterns. The Weibull model was used as the authors stated that tailing occurred – a rapid fall in infectivity followed by a less pronounced decline. The Weibull distribution, which is based on two parameters, is more versatile than the memory-less exponential failure distribution to describe the survival of microorganisms. Indeed, the time-dependent hazard rate function, through its shape parameter, allows the Weibull to fit a wide variety of inactivation profiles in comparison with the more classic (usual) exponential model. However, examining the data provided in the figures of the publication it is not clear whether tailing was pronounced. It appeared to occur only in the low RH tests, and this may have been due to an initial drying of the virus inoculum, as seen in the study of Abad *et al.* (1994). The T90 and 1-log reduction times are predicted in different ways by the two models,

and with the data from this study the values calculated by the Weibull model were lower than those calculated by the linear model. In Table 2.3 only the T90 values of the Kim *et al.* (2012) data calculated by the linear model are shown; this at least will give worst-case information if the Weibull interpretation proves valid.

Table 2.3 shows information from studies on HAV survival on fomites.

Table 2.3: Survival of HAV on various fomites and material surfaces

Fomite / material	**Temperature (^{o}C)**	**Relative Humidity (%)**	**Initial decrease in viral load (days)**	**Approximate overall log reduction in infectivity**	**Reference**
Aluminium	4	90	~40*	1.5 over 60 d	Abad *et al.* (1994)
Aluminium, faecal material present	4	90	~ 55*	1 over 60 d	Abad *et al.* (1994)
Aluminium	20	85	60*	1 over 60 d	Abad *et al.* (1994)
Aluminium, faecal material present	20	85	45*	1.5 over 60 d	Abad *et al.* (1994)
Aluminium	20	50	15*	2 over 60 d	Abad *et al.* (1994)
Aluminium, faecal material present	20	50	30*	2 over 60 d	Abad *et al.* (1994)
China	4	90	NA	< 1 over 60 d	Abad *et al.* (1994)
China	4	90	3*	2 over 7 d	Abad *et al.* (2001)
China, faecal material present	4	90	NA	< 1 over 60 d	Abad *et al.* (1994)
China, faecal material present	4	90	NA	< 1 over 7 d	Abad *et al.* (2001)
China	20	90	1*	2.5 over 7 d	Abad *et al.* (2001)
China, faecal material present	20	90	2*	1.5 over 7 d	Abad *et al.* (2001)
China	20	85	60*	1 over 60 d	Abad *et al.* (1994)
China, faecal material present	20	85	60*	1 over 60 d	Abad *et al.* (1994)
China	20	50	40*	2 over 60 d	Abad *et al.* (1994)
China, faecal material present	20	50	30*	2 over 60 d	Abad *et al.* (1994)

Latex	4	90	10*	1.5 over 60 d	Abad *et al.* (1994)
Latex, faecal material present	4	90	ND	< 1 over 60 d	Abad *et al.* (1994)
Latex	20	85	10*	2 over 60 d	Abad *et al.* (1994)
Latex, faecal material present	20	85	50*	2 over 60 d	Abad *et al.* (1994)
Latex	20	50	15*	3 over 60 d	Abad *et al.* (1994)
Latex, faecal material present	20	50	15*	1.5 over 60 d	Abad *et al.* (1994)
Paper	4	90	ND	< 1 over 60 d	Abad *et al.* (1994)
Paper	4	90	1*	~ 1.5 over 7 d	Abad *et al.* (2001)
Paper, faecal material present	4	90	ND	< 1 over 60 d	Abad *et al.* (1994)
Paper, faecal material present	4	90	< 1*	3.5 over 7 d	Abad *et al.* (2001)
Paper	20	90	ND	< 1 over 7 d	Abad *et al.* (2001)
Paper, faecal material present	20	90	1*	2 over 7 d	Abad *et al.* (2001)
Paper	20	85	5*	2 over 60 d	Abad *et al.* (1994)
Paper, faecal material present	20	85	5*	2 over 60 d	Abad *et al.* (1994)
Paper	20	50	5*	2 over 60 d	Abad *et al.* (1994)
Paper, faecal material present	20	50	5*	1.5 over 60 d	Abad *et al.* (1994)
Polystyrene	5	ND	ND	< 1 in 30 d	Sobsey *et al.* (1988)
Polystyrene, faeces material present	5	ND	30*	1 in 30 d	Sobsey *et al.* (1988)
Polystyrene	25	ND	ND	2 in 30 d	Sobsey *et al.* (1988)
Polystyrene, faeces material present	25	ND	ND	2.5 in 30 d	Sobsey *et al.* (1988)
Stainless steel	5	25	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	5	55	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	5	80	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	5	95	ND	< 1 over 4 h	Mbithi *et al.* (1991)

Stainless steel	15	30	5.4 [†]	4 in 30 d	Kim *et al.* (2012)
Stainless steel	15	50	4.4[†]	4 in 20 d	Kim *et al.* (2012)
Stainless steel	15	70	8.4[†]	3 in 30 d	Kim *et al.* (2012)
Stainless steel	20	25	ND	< 1 over 96 h	Mbithi *et al.* (1991)
Stainless steel	20	55	ND	< 1 over 96 h	Mbithi *et al.* (1991)
Stainless steel	20	80	ND	1 over 96 h	Mbithi *et al.* (1991)
Stainless steel	20	95	ND	1 over 96 h	Mbithi *et al.* (1991)
Stainless steel	25	30	2.2[†]	4 in 10 d	Kim *et al.* (2012)
Stainless steel	25	50	0.78[†]	4 in 3 d	Kim *et al.* (2012)
Stainless steel	25	70	1.68[†]	4 in 7 d	Kim *et al.* (2012)
Stainless steel, inoculum dried in cell culture medium	RT	ND	7*	3.5 over 28 d	Terpstra (2007)
Stainless steel dried in plasma	RT	ND	7*	6 over 28 d	Terpstra (2007)
Stainless steel	32	30	0.72	4 in 1 d	Kim *et al.* (2012)
Stainless steel	32	50	0.24[†]	4 in 1 d	Kim *et al.* (2012)
Stainless steel	32	70	0.78[†]	4 in 3 d	Kim *et al.* (2012)
Stainless steel	32	55	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	32	80	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	32	95	ND	< 1 over 4 h	Mbithi *et al.* (1991)
Stainless steel	40	30	0.06[†]	> 4 in < 1 d	Kim *et al.* (2012)
Stainless steel	40	50%	0.06[†]	> 4 in < 1 d	Kim *et al.* (2012)
Stainless steel	40	70	0.2[†]	> 4 in 1 d	Kim *et al.* (2012)
Wood	15	30	9[†]	3 in 30 d	Kim *et al.* (2012)
Wood	15	50	5.2[†]	4 in 20 d	Kim *et al.* (2012)
Wood	15	70	12[†]	< 2 in 30 d	Kim *et al.* (2012)

Wood	25	30	7.2†	3 in 30 d	Kim *et al.* (2012)
Wood	25	50	1.54†	4 in 7 d	Kim *et al.* (2012)
Wood	25	70	9†	2 in 30 d	Kim *et al.* (2012)
Wood	32	30	2.4†	4 in 10 d	Kim *et al.* (2012)
Wood	32	50	0.66†	4 in 3 d	Kim *et al.* (2012)
Wood	32	70	4.8†	3 in 20 d	Kim *et al.* (2012)
Wood	40	30	4.4†	4 in 20 d	Kim *et al.* (2012)
Wood	40	50	0.2†	4 in 1 d	Kim *et al.* (2012)
Wood	40	70	1.54†	4 in 7 d	Kim *et al.* (2012)

*TFL: time to reduce first log_{10}
†T90: time to reduce 90 percent of initial load
ND: not determined
RT: room temperature

2.4 On hands

HAV can survive in an infectious state for at least several hours on hands. Mbithi *et al.* (1992) contaminated finger pads of volunteers with ~ 6 x 10^4 plaque-forming units (pfu) HAV suspended in a 10 % faecal suspension. The finger pads were rinsed after 4 h, and virus eluted and assayed for infectivity. Less than 1 log infectivity was lost, the amount of infectious HAV detectable being 16% and 30% of that inoculated onto the finger pads.

2.5 On berry fruit

The evidence from several outbreaks of hepatitis A implicated to frozen fruit (Hutin *et al.*, 1999; Ramsay & Upton, 1989) strongly indicates that HAV can survive, i.e. persist in an infectious state, for several months in frozen berries. The acidity of the fruit will have little effect on HAV, as, like other enteric viruses it is tolerant of low pH (Scholz *et al.*, 1989).

Butot *et al.* (2008) examined the persistence of HAV in berries (blueberries, raspberries and strawberries) by both viral culture and quantitative reverse transcription-PCR (RT-PCR), and found very limited, if any, decline over 90 days of storage at -20°C. Freeze-drying, such as is commonly performed to dry berry fruit for subsequent incorporation in e.g. confectionary and cereals, reduced the infectious titre of HAV by less than 2.5 log on berry fruits (Butot *et al.*, 2009), the process involved freezing at -20°C followed by a heating cycle during which the

berries reached an internal temperature of 55°C. The reduction was greater in samples of blueberries than in blackberry, raspberry and strawberries possibly due to a protective effect of the more textured surfaces of the latter berries. In the above studies, approximately 1×10^6 infectious HAV had been used to contaminate the sample; therefore the inference is that considerable numbers of infectious virus may still have remained on the berries after the treatments applied. The results of the studies by Butot *et al*. (2009) are summarised in Table 2.4.

The decline in infectivity of HAV on stored berry fruit is mirrored by the decline in detectable viral RNA. A study by de Paula *et al.* (2010) observed less than 2 log decline in genome copies (as detected by qRT-PCR) up to 7 days after storage of artificially contaminated strawberries at 4°C (they did not perform any infectivity assays).

HAV is capable of tolerating the low pH of berry fruits: Provost *et al.* (2011) found no reduction in infectivity after suspending virus in buffer solutions at pH 1, 2 and 3 for 30 min at 37°C.

It is clear from the above studies that HAV can survive from a contamination event to point of consumption.

Table 2.4: Survival of HAV on berry fruit and effect of freeze drying (from Butot *et al.*, 2008 and 2009)

Berry fruit	**Treatment / storage condition**	**Temperature (°C)**	**Approximate overall log decline in infectivity**
Blackberries	Freeze drying		< 2
Blueberries	Freeze drying		< 2.5
Blueberries	Frozen storage	-20	< 1 after 90 days
Raspberries	Freeze drying		~ 1.5
Raspberries	Frozen storage	-20	< 1 after 90 days
Strawberries	Freeze drying		< 1.5
Strawberries	Frozen storage	-20	< 1 after 90 days

2.6 On vegetables

In a study designed to simulate pre-harvest conditions in the USA and Central America, Stine *et al.* (Stine *et al.*, 2005) contaminated iceberg lettuce leaves, bell peppers and cantaloupe

melons with HAV, and stored them in controlled environment chambers at mean air temperatures of approximately 25°C under low (45% to 48%) and high (85% to 90%) relative humidities. They did not show numerical or graphical data, but calculated from their presented rates of inactivation that it could take 822 days in pre-harvest conditions for HAV to decline by 3 log under these conditions. It may be postulated that under growing conditions encountered in the UK the survival of HAV could be similarly robust.

Bidawid *et al.* (2001) contaminated portions of Romaine lettuce leaves with approximately 1 x 10^6 pfu HAV and then incubated the samples for 12 days either exposed to air or placed in sealed bags containing air or modified atmospheres mimicking bagged fresh-cut produce sold at retail. The incubations were carried out at room temperature and at 4°C. Virus infectivity declined significantly at room temperature under all storage conditions, except for 70:30 CO_2:N_2), although infectious virus could be detected in all samples after 12 days. The authors postulated that the enhanced HAV survival under high levels of CO_2 may have been due to an inhibitory effect of the gas on enzymes present in lettuce, although a further explanation may be that inhibitory microflora on the plant leaves may have been suppressed. There was little decline in HAV infectivity on lettuce kept at 4°C, the modified atmospheres having no significant effect on survival. The overall conclusion is that while modified atmospheres may in some circumstances (e.g. storage at ambient temperatures) prolong virus survival, as refrigeration temperatures are generally used for storage using a modified atmosphere will not result in a decline in virus infectivity to any extent.

Croci *et al.* (2002) found a less than 2-log reduction of HAV on fresh carrot and fennel after 9 days at 4°C, indicating that the virus can persist under normal refrigeration conditions between purchase and consumption. HAV survived on spinach leaves at 5°C, with a T90 value of 28.6 days (Shieh *et al.*, 2009). Sun *et al.* (2012) examined survival of HAV on green onions at various temperatures from 3°C to 23°C. It was found that survival was lowest at the higher temperature; even so the indication was that HAV would lose only 1 log infectivity after 5 days at 23°C. Sun *et al.* (2012) performed regression analyses of the data from each temperature test; in Table 2.5 below where the correlation coefficient was above 0.9 the data have been deemed linear, where below 0.9 non-linear.

Shieh *et al.* (2009) reported that HAV on spinach leaves declined by less than 1 log over 1 week storage at refrigeration temperatures and by less than 2 log over 6 weeks. They

estimated the D-value at 28.6 d (Sun *et al.*, 2012) obtained similar findings using green onions, and at room temperature storage D-values were still around a week, although at the higher temperatures the foodstuff perished more quickly.

Table 2.5 shows information from studies on HAV survival on vegetables. The overall message from these results is that HAV will remain infectious on contaminated vegetables from contamination through purchase to consumption.

Unsurprisingly, the small decline in infectivity of HAV on stored vegetables is mirrored by the decline in detectable viral RNA. de Paula *et al.* (2010) observed less than 2 log decline in genome copies (as detected by qRT-PCR) up to 7 d after storage of artificially contaminated lettuce at $4^{o}C$ (they did not perform any infectivity assays).

Table 2.5: Survival of HAV on vegetables

Fomite / material	**Temperature (^{o}C)**	**Initial decrease in viral load (days)**	**Approximate overall log decline in infectivity**	**Reference**
Green onions	3.1	29.3[†]	< 2 log over 29 d	Sun *et al.* (2012)
Green onions	3.4	30*	< 2 log over 28 d	Sun *et al.* (2012)
Green onions	10.3	14.4[†]	< 2 log over 16 d	Sun *et al.* (2012)
Green onions	14.2	11.4[*]	1 log over 12 d	Sun *et al.* (2012)
Green onions	20.2	6.6[†]	< 2 log over 9 d	Sun *et al.* (2012)
Green onions	20.7	7.3[*]	< 1 log over 5 d	Sun *et al.* (2012)
Green onions	22.4	6.0[†]	< 2 log over 9 d	Sun *et al.* (2012)
Green onions	23.4	5.4[†]	< 1 log over 8 d	Sun *et al.* (2012)
Lettuce leaf in dish	RT	3*	4.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf in dish	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in air, room temperature	RT	9*	1.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in air	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 30:70 CO_2:N_2	RT	9*	1.0 over 12 d	Bidawid *et al.* (2001)

Lettuce leaf bagged in 30:70 CO_2:N_2, C	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 50:50 CO_2:N_2, room temperature	RT	12*	1.0 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 50:50 CO_2:N_2,	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 70:30 CO_2:N_2, room temperature	RT	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 70:30 CO_2:N_2,	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 100 % CO_2:N_2, room temperature	RT	-	0.5 over 12 d	Bidawid *et al.* (2001)
Lettuce leaf bagged in 100 % CO_2:N_2,	4	-	0.5 over 12 d	Bidawid *et al.* (2001)
Spinach	5.4	-	< 1 over 1 week	Shieh *et al.* (2009)
Spinach	5.4	-	< 2 over 6 weeks	Shieh *et al.* (2009)

*TFL: time to reduce first $\log_{10}$
†T90: time to reduce 90 percent of initial load
RT: room temperature
-: no detectable initial decrease

2.7 In pork products

Schielke *et al.* (2011) attempted to determine the infectivity of HEV in suspensions derived from centrifuged and filtered homogenates of infected wild boar liver by incubating them on various cell lines (PLC/PRF/5, A549 and HepG2) followed by immunofluorescence-based antigen detection, but this proved unsuccessful. As an alternative approach, they treated the homogenates with RNase prior to nucleic acid extrication, to detect intact virus particles. They pointed out that the correlation with infectivity may not have been exact and thus HEV survival would have been overestimated, nevertheless considered that this would provide a conservative estimate of survival and elimination. By this means they reported that storage at various temperatures resulted in an initial decline in detectable genome equivalents followed by several weeks where no decline was observable (Table 2.6); the log reductions were not high, indicating that considerable numbers of HEV particles remained intact.

Table 2.6: Decline in detectable HEV genome equivalents in liver suspensions on storage at various temperatures (from Schielke *et al.*, 2011)*

Storage temperature (^{o}C)	Initial log decline*	Number of days where no further decline was observed^{+}
4	0.34 in 1 h	70
22	0.45 in 3 d	47
37	1.24 in 7 d	43

*The initial log titre of HEV in each experiment was ~ 5.4.
$^{+}$ the duration of the experiment was 70 d at 4°C, 50 d at 22°C, and 50 d at 37°C

Jones and Muelhauser (2014) examined the potential for persistence of detectable HEV in pig oral fluid by artificially contaminated samples of pig saliva with HEV particles purified from pig faeces. Each sample was contaminated with ~10^{6} genome copies of the virus and then the samples were stored at -20°C for 30 d, 4°C for 1h, 4°C for 24 h and 37°C for 24 h. After storage at each of the colder temperatures, ~10^{5} genome copies HEV could be detected in the samples. The reduction of genome copies from the original number may have been due to loss during the extraction procedure; however no control was performed to check this. After incubation at 37°C a 3 log loss of genome copies was observed; however this reduction was probably due to the experimental procedure used for treatment of these samples and it is likely that the actual loss was ~ 1 log or less. As study did not attempt to determine infectivity the data should be interpreted with caution, but nonetheless give an indication of the potential stability of HEV at the temperatures examined.

2.8 In shellfish

To investigate whether HAV could survive in marinated mussels, Hewitt and Greening (2004) performed a study mimicking commercial marination. The process was simulated by immersing the mussels in boiling water for 37 sec, steaming for ~ 3 min, then shucking them and immersing them in an acidic marinade (pH 3.75) before storage at 4°C for up to 4 weeks. Infectious HAV and RT-PCR-detectable units (RT-PCRU) were quantified at time 0 and at each week. There was a 0.6, 0.8 and 1.7-log reduction in infectivity after 1, 2 and 4 weeks respectively, with infectious virus still detectable at the last time point, but no significant reduction in RT-PCR titre at any time. It was concluded that HAV could survive in marinated mussels thus constituting a potential health risk if shellfish are contaminated prior to processing.

2.9 In other foods

Sobsey *et al.* (1988) examined the potential for HAV to survive on foodstuffs after contamination by an infected handler by artificially contaminated biscuits with 1 x 10^5 infectious units of the virus, and stored them at 21°C and 49°C over a period of 2 months, taking samples at various time interim points. At the end of the 2 month period, infectious virus had declined by ~ 4 and ~ 5 log at 21°C and 49°C respectively, but was still detectable. At both storage temperatures it took over 14 d for infectious HAV to decline by more than 2 log.

The use of contaminated water for irrigation is considered a potential route of virus contamination of produce. Stine *et al*. (2005) found that HAV is able to survive under pre-harvest conditions on bell peppers, cantaloupe and lettuce, and modelling suggests that it could take 822 days to achieve a 99.9% reduction of HAV.

Wang *et al.* (2013) examined the potential for HAV to survive in an infectious state on alfalfa seeds. They inoculated 1 g seeds with ~ 7 log $TCID_{50}$ HAV, then dried them for 1 h and stored them for up to 50 d at 22°C. Although drying achieved ~ 1 log inactivation of HAV, there was no further significant decline in HAV infectivity up to 20 d. Approximately 2 log reduction in HAV infectivity was observed between 20 and 50 d. In a separate test, some batches of seeds were allowed to germinate for up to 15 d with watering. After germination the sprouts were examined for infectious virus on d 0, 2, 5, 10 and 15: no significant reduction in infectious HAV was observed, although numbers were approximately 2.5 log lower than the initial titres on the inoculated seeds.

2.10 The effect of pH

Scholz *et al.* (1989) observed high stability of HAV at low/acidic pH (1.0) at room and physiological temperatures (24°C and 38°C); in their tests, an initial slow rate of decline in infectivity was followed by a rapid rate, as seen by the TFL and total decline values in Table 3.6 (see Section 3.8 below). The survival of HAV over a pH range from 3 to 11 had previously been observed by Siegl *et al.* (1984). Like other enteric viruses, HEV needs to be able to withstand the acidity of the stomach in order to reach the lower intestinal tract in an infectious state. The HEV major capsid protein undergoes conformational changes when subjected to low pH, which may provide more stability and allow the virus to retain its infectivity (Zafrullah *et al.*, 2004).

3. The effect of elimination procedures

3.1 Chlorine

In an early study using cultivatable HAV Grabow *et al.* (1983) examined the effect of chlorine on HAV (10^3 $TCID_{50}$) suspended in phosphate buffer. HAV was completely inactivated within 1 min by ~ 0.4 mg l^{-1} free chlorine at pH 6 and 8. Bosch (1995) reported that the effect of chlorine was not enhanced by the presence of copper and silver ions, in these tests an initial rapid decline in infectivity was observed, followed by a less pronounced decline, possibly indicating the presence of a resistant fraction of virus particles within the overall population.

Li *et al.* (2002) reported that the infectivity of HAV suspended in PBS pH 7 could be reduced by nearly 6 log with a free chlorine concentration of 10 mg l^{-1} or higher and a contact time of 30 min or longer. In contrast to the findings of Grabow *et al.* (1983), Li *et al.* (2002) reported that 5 mg ml^{-1} could only inactivate 1 log or less HAV even with a contact time of 60 min. They suggested that the effect of chlorine was exerted primarily on the genome, as they found that a region of the 5' non-translated regions (5'NTR) could not be detected following exposure. This effect was observed to be concurrent with the complete loss of infectivity at 10 mg l^{-1} chlorine for 10 min, whilst virus antigenicity was still detectable; it was undetectable after exposure for 60 min. Other areas of the genome could be amplified, which indicates that chlorine exerted a specific effect on the 5'NTR, and also that the chemical could penetrate the virus capsid while leaving it intact and capable of protecting the rest of the genome. Nuanualsuan & Cliver (2003a) reported that chlorine appeared to inactivate HAV primarily through damage of the capsid region interacting with cellular receptors on the capsid which mediate infectivity. Treating HAV suspensions with ~ 1.25 mg ml^{-1} free chlorine until no infectivity could be detected (a contact time of at least 7 min; the virus titre of the suspension was not given), they found that inactivated virus could not attach to cell monolayers but could be captured by anti-HAV antibodies. RNA could be amplified from inactivated virus by RT-PCR, but not after treatment with RNase (Nuanualsuwan & Cliver, 2003b), indicating that the chlorine-damaged capsids still retained some ability to protect the genome from degradation. Martin *et al*. (2013) reported that active chlorine concentrations of 8.73 mmol l^{-1} had no impact on detectable HAV RNA.

Bigliardi and Sansebastiano (2006) performed a series of tests using hypochlorous acid, and displayed the results obtained as regression plots. From these plots they calculated that doses of 0.4 and 1 mg l^{-1} at temperatures of greater than $20^{o}C$ could inactivate 2 log HAV in 35 and 12 min respectively. The level of free chlorine was not calculated. The pH of the suspending medium had no influence on the inactivation time.

Data from studies on inactivation of HAV by chlorine is shown in Table 3.1.

Table 3.1: Inactivation of HAV by chlorine

Concentration*/time (mg l^{-1})	**CT values (mg.l^{-1}.min^{-1})**	**Approximate overall log reduction in infectivity**	**Reference**
0.4 / 1 min	0.4	3	Grabow *et al.* (1983)
0.5 / 120 min	60	2	Bosch (1995)
1 / 120 min	120	2.5	Bosch (1995)
5 / 10 min	50	< 1	Li *et al.* (2002)
5 / 30 min	150	< 1	Li *et al.* (2002)
5 / 60 min	300	1	Li *et al.* (2002)
10 / 10 min	100	1	Li *et al.* (2002)
10 / 30 min	300	5.75	Li *et al.* (2002)
20 / 10 min	200	2	Li *et al.* (2002)
20 / 30 min	600	5.75	Li *et al.* (2002)

*Free chlorine

In a series of experiments, Butot and colleagues (2008; 2009) examined the effectiveness of commercial processes for the elimination of HAV from berries and other produce types. To simulate post-harvest conditions, fresh berries were artificially contaminated with HAV and then washed with 200 ml tap, warm or chlorinated water, or with a chlorine dioxide (ClO_2) solution, followed by rinsing with 200 ml tap water as recommended by the US FDA. Washing for 30 sec in tap water at $18^{o}C$ or $43^{o}C$ reduced HAV in raspberry and strawberry samples by less than 1 log. Both infectivity and genome number were reduced equally, indicating virus particles were physically removed by the process. Treatment with chlorine at an available concentration of 200 ppm reduced HAV in raspberry and strawberry samples by less than 2 log; the reduction in blueberry samples was greater (2.4 log), although possibly not significantly so. Immersing raspberry samples in ClO_2 at 10 ppm for 10 min had a similarly limited effect as the above treatments. In the above studies, approximately 1 x 10^{6} infectious virus had been used to contaminate the sample, therefore the inference is that

considerable numbers of infectious virus may still have remained on the berries after the treatments applied. The authors concluded that current sanitation treatments will not completely ensure that HAV will be eliminated from berry fruits.

Casteel *et al.* (2008) artificially contaminated strawberries, then immersed them in solutions containing various concentrations of free chlorine. The experimental procedure (addition of virus followed by immersion in chlorine solution and decanting of wash liquid) resulted in 3.6 – 4 log infectious virus remaining on the treated strawberry samples. At 10 ppm and 20 ppm chlorine, 10 min was necessary to achieve an approximately 2 log reduction in infectious HAV; with 200 ppm chlorine at 5 min immersion resulted in a 2.6 log reduction. With all treatments, infectious virus remained detectable on the samples. Washing strawberries in tap water is probably just as effective as using chlorinated water for elimination of virus; just over 2 log virus could be removed by stirring artificially HAV-contaminated 100 g strawberries in 300 ml tap water for 30 sec, as determined by qRT-PCR (de Paula *et al.*, 2010).

Data from studies on elimination of HAV on berry fruit by chlorination are summarised in Table 3.2.

Table 3.2: Elimination of HAV on berry fruit by disinfectant wash

Berry	Treatment	Time	Temperature (°C)	Approximate overall log decline in infectivity	Reference
Blueberries	water	30 sec	18	< 1	Butot *et al.* (2008)
Blueberries	water	30 sec	43	< 1	Butot *et al.* (2008)
Blueberries	200 ppm chlorine	30 sec	18	~ 2.5	Butot *et al.* (2008)
Raspberries	water	30 sec	18	< 1	Butot *et al.* (2008)
Raspberries	water	30 sec	43	< 1	Butot *et al.* (2008)
Raspberries	200 ppm chlorine	30 sec	18	< 1	Butot *et al.* (2008)
Raspberries	ClO_2	10 min	18	< 1	Butot *et al.* (2008)
Raspberries	ClO_2	10 min	18	< 1	Butot *et al.* (2008)
Strawberries	water	30 sec	18	< 1	Butot *et al.* (2008)
Strawberries	water	30 sec	43	< 1	Butot *et al.* (2008)
Strawberries	200 ppm chlorine	30 sec	18	< 2	Butot *et al.* (2008)
Strawberries	10 ppm chlorine	30 sec	RT	0.7	Casteel *et al.* (2008)
Strawberries	10 ppm chlorine	1 min	RT	1.4	Casteel *et al.* (2008)
Strawberries	10 ppm chlorine	3 min	RT	1.7	Casteel *et al.* (2008)
Strawberries	10 ppm chlorine	5 min	RT	1.5	Casteel *et al.* (2008)
Strawberries	10 ppm chlorine	10 min	RT	2.2	Casteel *et al.* (2008)
Strawberries	20 ppm chlorine	30 sec	RT	0.6	Casteel *et al.* (2008)
Strawberries	20 ppm chlorine	1 min	RT	0.7	Casteel *et al.* (2008)
Strawberries	20 ppm chlorine	3 min	RT	1	Casteel *et al.* (2008)
Strawberries	20 ppm chlorine	5 min	RT	1.2	Casteel *et al.* (2008)
Strawberries	20 ppm chlorine	10 min	RT	2.3	Casteel *et al.* (2008)
Strawberries	200 ppm chlorine	30 sec	RT	0.5	Casteel *et al.* (2008)

Strawberries	200 ppm chlorine	1 min	RT	0.6	Casteel *et al.* (2008)
Strawberries	200 ppm chlorine	3 min	RT	1.2	Casteel *et al.* (2008)
Strawberries	200 ppm chlorine	5 min	RT	2.6	Casteel *et al.* (2008)

RT: room temperature

Casteel *et al.* (2008) artificially contaminated heads of iceberg lettuce, with HAV, then immersed them in solutions containing either 10 ppm or 200 ppm free chlorine. The experimental procedure (addition of virus followed by immersion in chlorine solution and decanting of wash liquid) resulted in approximately 2 log infectious virus remaining on the treated lettuce heads, so only up to approximately 2 log reduction could be monitored. Even so, up to 5 min washing in each chlorine concentration was necessary to remove detectable virus from the lettuce heads. Fraise *et al.* (2011) found a similar reduction using 15 ppm chlorine on butter lettuce artificially contaminated by HA. Hirneisen and Kniel (2013) compared the effect of chlorine against HAV on the surface of green onions, and against the virus internalised within the plants. They sprayed the onions for 40 sec with 150 ppm calcium hypochlorite at 4°C pH 7.5. The internalised HAV was less susceptible to inactivation by chlorination (0.4 log inactivation of internalised virus against 2.6 log inactivation of virus located on the surface of the green onions). Although the difference appears pronounced, the authors stated that it was not significant; however only three replicates of each test were performed, which may not have allowed an effective statistical comparison.

Data from studies on elimination of HAV on lettuce by chlorination are summarised in Table 3.3.

Table 3.3: Elimination of HAV on lettuce by disinfectant wash

Leafy green vegetable	Treatment	Time	Temperature (°C)	Approximate overall log decline in infectivity	Reference
Iceberg lettuce	10 ppm chlorine	30 sec	RT	0.8	Casteel *et al.* (2008)
Iceberg lettuce	10 ppm chlorine	1 min	RT	1.3	Casteel *et al.* (2008)
Iceberg lettuce	10 ppm chlorine	3 min	RT	1.4	Casteel *et al.* (2008)
Iceberg lettuce	10 ppm chlorine	5 min	RT	>2.2	Casteel *et al.* (2008)
Iceberg lettuce	10 ppm chlorine	10 min	RT	> 2.2	Casteel *et al.* (2008)
Iceberg lettuce	20 ppm chlorine	30 sec	RT	0.8	Casteel *et al.* (2008)
Iceberg lettuce	20 ppm chlorine	1 min	RT	1.0	Casteel *et al.* (2008)
Iceberg lettuce	20 ppm chlorine	3 min	RT	0.8	Casteel *et al.* (2008)
Iceberg lettuce	20 ppm chlorine	5 min	RT	>1.7	Casteel *et al.* (2008)
Iceberg lettuce	20 ppm chlorine	10 min	RT	>1.7	Casteel *et al.* (2008)
Butter lettuce /	Bubbled tap water	2 min	NG	0.62	Fraise *et al.* (2011)
Butter lettuce	15 ppm chlorine	2 min	NG	1.89	Fraise *et al.* (2011)
Butter lettuce	Bubbled tap water + ultrasound	2 min	NG	0.82	Fraise *et al.* (2011)
Butter lettuce	100 ppm peroxyacetic acid-based biocide	2 min	NG	0.66	Fraise *et al.* (2011)

NG: not given
RT: room temperature

Butot and colleagues (Butot *et al.*, 2008; 2009) examined survival of HAV in fresh herbs, and the effectiveness of commercial processes for the elimination of HAV from samples of the herbs. They measured the effect of each process on virus infectivity (using cell culture assay) and on the viral genome (using qRT-PCR). Very limited, if any, decline in HAV infectivity was observed over 90 days of storage of basil and parsley at -20°C. Freeze-drying, which is commonly performed to dry berry fruit for subsequent incorporation in confectionary and cereals, reduced the infectious titre of HAV by less than 2 logs. To simulate post-harvesting

conditions, fresh herbs were washed with 200 ml tap, warm or chlorinated water, followed by rinsing with 200 ml tap water as recommended by the US FDA. Washing for 30 sec in tap water at 18°C reduced HAV in basil and parsley samples by less than 1 log whereas at 43°C the reduction was slightly greater. Both infectivity and genome number were reduced equally indicating virus particles were physically removed by the process. As approximately 1 x 10^{6} infectious virus had been used to contaminate the sample the inference is that considerable numbers of infectious virus may still have remained on the herbs after washing. Treatment with chlorine at an available concentration of 200 ppm reduced HAV in basil and parsley samples by less than 2.5 log.

Casteel *et al.* (2008) artificially contaminated cherry tomatoes with HAV, then immersed them in solutions containing either 10 ppm or 200 ppm free chlorine. The experimental procedure (addition of virus followed by immersion in chlorine solution and decanting of wash liquid) resulted in approximately 2 log infectious virus remaining on the treated tomatoes, so only up to approximately 2 log reduction could be monitored. Even so, up to 5 min washing in each chlorine concentration was necessary to remove detectable virus from the tomatoes.

Data from the study of Casteel *et al.* (2008) on elimination of HAV on tomatoes by chlorination at room temperature are summarised in Table 3.4.

Table 3.4: Elimination of HAV on tomatoes by chlorination (from Casteel *et al.*, 2008)

Chlorine treatment (ppm)	Time	Approximate overall log decline in infectivity
10	30 sec	0.6
10	1 min	1.3
10	3 min	1.1
10	5 min	>2.3
20	30 sec	0.8
20	1 min	1.4
20	3 min	>2.4

Alternatives to the use of chlorine as a disinfectant in the food industry are being investigated due to issues such as corrosion of surfaces caused by chlorination, and the formation of hazardous by-products. Fraise *et al.* (2011) examined the use of ultrasound, and a peroxyacetic acid-based biocide to remove HAV from lettuce. The samples were treated for 2 min (no temperature cited). Viruses inoculated onto and recovered from lettuce samples,

were quantified by cell culture and qRT-PCR. No greater reduction of infectivity or genome copy number than a mean of less than 1 log could be achieved by either treatment.

Van der Poel and Berto (2013) exposed suspensions of HEV to sodium hypochlorite, and then inoculated them into a cultured cell system (see section 3.8 below). No cytopathic effect could be observed using this system, but virus replication could be inferred from detection of HEV RNA. In cultures inoculated with untreated HEV suspensions, viral RNA could be detected to day 33, whereas in cultures inoculated with hypochlorite-treated HEV suspensions RNA could be detected only to day 7. Nonetheless, this indicates that replication of virus occurred, and therefore the elimination of HEV infectivity was not complete.

3.2 Chlorine dioxide

Bigliardi and Sansebastiano (2006) calculated that doses of 0.4 and 0.6 mg l^{-1} chlorine dioxide (ClO_2) at temperatures of over 20°C could inactivate 2 log HAV in 4 and 2 min respectively. In suspension in a "physiological solution", HAV could be rapidly inactivated by concentrations of 0.6 mg l^{-1} and higher (Zoni *et al.*, 2007). On surfaces higher concentrations may be necessary for similar effects: Sabbah *et al.* (2010) reported that 500 and 1,000ppm ClO_2 at 500 – 1,000ppm could reduce the infectivity of HAV dried onto stainless steel disks by approximately 4 log after 5 min contact time in the presence of organic material.

Data from studies on elimination of HAV by chlorine dioxide are summarised in Table 3.5.

Table 3.5: Inactivation of HAV by chlorine dioxide

Concentration/time (mg l^{-1})	CT values (mg.l^{-1}.min^{-1})	Approximate overall log reduction in infectivity	Reference
0.4 / 2.5 min	1	2	Zoni *et al.* (2007)
0.4 / 4 min	1.6	2	Bigiardi and Sansebastiano (2006)
0.4 / 7 min	2.8	3	Zoni *et al.* (2007)
0.4 / 19.5 min	7.8	4	Zoni *et al.* (2007)
0.6 / 32 sec	0.32	2	Zoni *et al.* (2007)
0.6 / 51 sec	0.51	3	Zoni *et al.* (2007)
0.6 / 1.5 min	0.9	4	Zoni *et al.* (2007)
0.6/ 2 min	1.2	4	Bigiardi and Sansebastiano (2006)
0.8 /16 sec	0.21	2	Zoni *et al.* (2007)
0.8 /21 sec	0.28	3	Zoni *et al.* (2007)
0.8 / 26 sec	0.35	4	Zoni *et al.* (2007)

Immersing parsley samples in ClO_2 at 10 ppm for 10 min reduced infectious HAV in basil and parsley samples by less than 2 log (Butot *et al.*, 2009).

3.3 Commercial disinfectants

Abad *et al.* (1997a) tested several commercial disinfectant formulations against HAV, both in suspension and against the virus dried onto a polystyrene surface. Against HAV in suspension, only on sodium hypochlorite and diethylenetriamine-based disinfectants achieved a greater than 3 log reduction in HAV after 10 min contact; chlorhexidine gluconate, phenol, and sodium chlorite based disinfectants were unable to reduce HAV infectivity by more than 0.33 log. On a polystyrene surface, the sodium hypochlorite-based disinfectant inactivated 3.7 log infectious HAV, while phenol, the sodium chlorite-based disinfectant and the diethylenetriamine-based disinfectant effected a greater than 2 log reduction in infectivity. The chlorhexidine gluconate-based disinfectant was unable to reduce HAV infectivity on the polystyrene surface by more than 1 log in 10 min. The effect of the disinfectants was not significantly lessened by the presence of faecal material. Testing six commercial disinfectants (not named) with different active compounds, Jean *et al.* (2003) found only a formulation containing sodium hypochlorite to be able to consistently eliminate > 5 log HAV infectivity on when used at 1,000 ppm to 3,000 ppm with 5 min contact time. The commonly-used commercial formulation Virkon® (Antec Inc., Sudbury, UK) at 1% was found able to reduce greater than 3 log HAV dried onto glass discs after 10 min exposure (Solomon *et al.*, 2009); at 0.5% and below less than 1 log infectious HAV could be eliminated. Sabbah *et al.* (2010)

reported that domestic bleach (sodium hypochlorite) at concentrations between 2,500 ppm and 5,000ppm could reduce the infectivity of HAV dried onto stainless steel disks by approximately 4 log after 5 min contact time in the presence of organic material.

Mbithi *et al.* (1993a) examined the effectiveness of nine commercially available hand washing agents against HAV contaminated hands. They contaminated six finger pads of volunteers with virus suspended in a 10% faecal suspension, then exposed three of the finger pads to the agents for 10 sec. The finger pads were then rinsed, and remaining virus eluted and assayed for infectivity. A whole-hand test was also performed in which virus was spread over the hands, then rubbed with the agent and rinsed with lukewarm tap water before drying with a paper towel. The treatments were also performed using 70% ethanol, and tap water. Results were given as percentages reduction in pfu; the highest percentage reduction (~95%) was achieved by an antibacterial soap, the lowest (~78%) by one of the other commercial hand washing agents. Most of the treatments achieved less than 1 log reduction either on finger pads or whole hands.

Martin *et al.* (2013) examined the effectiveness of two commercial disinfectants, one based on peracetic acid and the other on potassium monopersulphate, against HAV in suspension and on polypropylene and stainless steel surfaces, and found both to be effective in eliminating 4 log HAV.

Gerba *et al.* (2007) determined whether HAV could survive on cotton cloth subjected to common household washing practices. Detergent with and without bleach was added to washing machines containing sterile and virus (~ 6 log) inoculated pieces of cotton, plus other items (e.g. cotton T-shirts) designed to simulate the conditions encountered in soiled laundry (pH, organic load, etc.). The tests were performed in a 69-liter capacity washing machine with a wash cycle of 12 min followed by a 3 min rinse; the temperature was 20°C - 23°C. Bleach and/or household washing powder were added. After washing, the fabrics were tumble-dried at 55°C for 28 min. Washing with powder plus bleach resulted in a ~ 6.5 log reduction in infectious virus; using washing powder alone the reduction was only ~2.7 log. Drying resulted in a small (less than 1 log) reduction in infectivity of the recovered virus. Interestingly, HAV could be transferred to uninoculated pieces of cotton during the process. This study strongly indicates that common household washing or laundering practices may not eliminate HAV contaminating clothing or articles such as tablecloths. The use of bleach

in such procedures would constitute an effective measure if items are suspected of being heavily contaminated.

The precise effects of disinfectants on HAV structure and function await elucidation. This may be achieved by advanced tools such as cryo-electron microscopy and X-ray crystallography, and allow the development of specific and highly efficient disinfectants (Wigginton & Kohn, 2012).

3.4 Depuration

Depuration is a purification process used by the shellfish industry to remove microbiological contamination found in oysters and other shellfish however, it is not fully effective against viruses such as HAV. Sobsey *et al.* (1988) found that the elimination of HAV accumulated in oysters was slow, with 18% of the initial infectious virus load remaining in the shellfish after 5 d. Abad *et al* (1997b) showed a reduction of 2 logs of bioaccumulated HAV occurred after 4 d of depuration. Kingsley and Richards (2003) reported that depuration of oysters for 3 weeks was unable to remove all infectious HAV from shellfish which had been previously exposed to the virus. After 1 week of depuration, infectious virus had declined by ~ 1 log, and after 3 weeks a mean of 500 pfu could be detected per oyster from an initial load of 3.4×10^4 pfu. A similar reduction was observed after HAV-contaminated mussels were depurated for 7 days (Enriquez *et al.*, 1992). Kingsley and Richards (2003) reported that HAV RNA could be detected after 60 d depuration (similar results were obtained by Provost *et al.* (2011) and McLeod *et al.* (2009) found no significant reduction in infectious HAV in oysters after depuration for 24 h. Kingsley and Richards (2003) concluded that depuration, as it is performed by industry, would be ineffective to eliminate HAV, and that relaying may have to be performed for at least 1 month to be effective. UV disinfection of depuration water may be effective against HAV, but this has not been thoroughly examined (de Abreu Correa *et al.*, 2012).

3.5 Ethanol

Ethanol is not very effective against HAV. Abad *et al.* (1997a) reported that HAV displayed high resistance to treatment with 70% ethanol. Less than 0.5 log reductions in infectivity were obtained against HAV in suspension after 1 min, although when virus suspensions were dried on a polystyrene surface and exposed to the disinfectant reductions of ~ 1.3 log were obtained. Bae *et al.* (2010) found a reduction of 1.85 % HAV infectivity on artificially contaminated dermal material (derived from human skin) after treatment for 20 min (2.5 min,

5 min, and 10 min treatments resulted in ~ 0.3, ~ 0.5, and ~ 1 log reductions respectively). Two commercial ethanol-based disinfectants were tested for their efficacy against HAV by Wolff *et al.* (2001) with each achieving only a 1 – 2 log reduction in infectivity after a contact time of 10 min.

Alcohol-based hand rubs can be limited in their ability to inactivate non-enveloped viruses such as HAV. Kramer *et al.* (2006) reported a US FDA-registered formulation (NDC-6673-1230-(I)-(9)) containing 55% ethanol in combination with propam-1.2-diol, butan-1.3-diol and phosphoric acid, could inactivate greater than 4 log HAV in a 30 sec contact time in the presence of organic material (serum). A weakness of this study is that they demonstrated this using a standard suspension test, but not actually on hands themselves.

3.6 Gamma and E-beam irradiation

In cell culture supernatant, Pruss *et al.* (2001) observed that 4 and 6 log inactivation could be achieved by doses of 21.2kGy and 31.8kGy respectively (D-value = 5.3kGy). Examining the effectiveness of procedures used for inactivating viruses in the context of bone tissue transplantation, Pruss *et al.* (2001) found that greater than 7 log infectious virus in bone tissue could be inactivated by a dose of 33.7kGy; lower doses (~ 12kGy) in similar circumstances produced lower (~ 2.5 log) inactivation (Moore, 2012). Using electron beam irradiation (which has a comparable impact to γ-irradiation but has less penetrative power) on frozen suspensions of HAV in cell culture supernatant, Schmidt *et al.* (2012) reported that 4 log HAV inactivation in tendon transplant containers could be achieved by doses of ~ 30kGy (D-value ~ 8kGy). Praveen *et al.* (2013) proved that a dose of 4.83KGy of electron beam irradiation was required for a 1 log reduction of HAV in oysters. In the UK, the maximum overall average dose which can be used for processing shellfish is 3kGy, for fruit 2kGy and for vegetables 1kGy (information from the UK Food Standards Agency), which may not be fully effective in the virological safety of a food if HAV is contaminating at high levels.

3.7 Glutaraldehyde

Mbithi *et al.* (1990) tested a range of commercial disinfectants against HAV dried onto steel discs. They reported that only 2 disinfectant formulations (one containing 2% glutaraldehyde and another containing a quaternary ammonium compound) could reduce infectious virus by greater than 4 log, the other 18 tested being unable to achieve a 1 log reduction. They did not provide numerical or graphical data however; Mbithi *et al.* (1993b) reported that 2% – 2.5%

of alkaline glutaraldehyde could reduce at least 3 log HAV dried onto a glass surface, after 10 min contact. Again, no numerical or graphical data was provided.

3.8 Heat

Early experiments (Peterson *et al.*, 1978; Provost *et al.* 1975) using infection of laboratory animals (simians) indicated that HAV was relatively heat-resistant compared to other picornaviruses. The development of cell-culture adapted HAV allowed the survival characteristics of the virus to be studied in a precise manner. Parry and Mortimer (1984) infected cultured mammalian cells with HAV suspended in PBS and heated at various temperatures. They measured infectivity by the quantity of HAV antigen that could be detected by radioimmunoassay in the cell culture medium to day 34 of incubation of the culture (the cumulative counts of antigen were stated to be directly proportional to the dilution of the inoculum); detection of antigen in the cell monolayer at 7 weeks was the final check on infectivity. By this means it was determined that HAV could be reduced in a time/temperature dependant manner, with complete elimination of infectivity being achieved at 75°C for 30 sec or at higher time/temperature combinations. They also reported that pasteurisation conditions of 30 min at ~ 63°C and 15 sec at ~ 72°C were insufficient to eliminate all infectivity from milk samples artificially contaminated with ~ 10^5 tissue culture infectious doses of virus.

Borovec *et al.* (1998) found that infectious HAV could still be detected in saline solution at 60°C after 8 min and after 16 min in one replicate test. Bhattacharya *et al.* (2004) showed that heating HAV in cell culture medium at 70°C for 15 min resulted in approximately 94% reduction in infectivity, but that heating at 95°C for 3 min did not result in complete inactivation (as they measured infectivity using enzyme immunoassay detection of antigen it is not possible to precisely determine the degree of inactivation that was achieved in their test).

Schwab and Gibson (2007) reported that the infectivity of HAV was minimally reduced (0.5 log or less) at 37°C; the suspending medium used in their study was not given.

Data from studies on the effect of heat on HAV in suspension are summarised in Table 3.6.

Table 3.6: Effect of heat on hepatitis A virus in suspension

Suspension	Temperature (^oC)	TFL (min)	Approximate total log decline in infectivity	Reference
PBS	20	ND	0	Parry and Mortimer (1984)
PBS, pH 1.0	24	210	6.6 in 5.5 h	Scholz *et al.* (1989)
PBS, pH 1.0	38	90	6.6 in 2 h	Scholz *et al.* (1989)
NG	50	729	ND	Schwab and Gibson (2007)
cell culture medium	55	ND	1 in 2 min	Parry and Mortimer (1984)
cell culture medium	55	ND	1 in 4 min	Parry and Mortimer (1984)
0.05 M NaCl	60	ND	3.3 in 10 min	Anderson (1987)
PBS + fetal calf serum	60	60	ND	Shimasaki *et al.* (2009)
aqueous solution	60	ND	4.1 in 10 h	Schaefer *et al.* (2001)
cell culture medium	60	8	4 – 5.5 in 16 min	Borovec *et al.* (1998)
aqueous solution	60	20	5.8 in 600 min	Barrett *et al.* (1996)
cell culture medium	60	ND	1 in 2 min	Parry and Mortimer (1984)
cell culture medium	60	ND	2 in 4 min	Parry and Mortimer (1984)
PBS	62.8	ND	> 5 in 30 min	Parry and Mortimer (1984)
cell culture medium	65	ND	2 in 2 min	Parry and Mortimer (1984)
cell culture medium	65	ND	4 in 2 min	Parry and Mortimer (1984)
purified suspension	65	10	ND	Siegl *et al.* (1984)
NG	60	104	ND	Schwab and Gibson (2007)
NG	70	3.8	ND	Schwab and Gibson (2007)
cell culture medium	70	ND	5 in 2 min	Parry and Mortimer (1984)
cell culture medium	70	ND	> 5 in 4 min	Parry and Mortimer (1984)
1 M $MgCl_2$	70	ND	2.1	Anderson (1987)
PBS	71.6	ND	3 in 15 sec	Parry and Mortimer (1984)
cell culture medium	75.1	ND	4 in 10 sec	Parry and Mortimer (1984)
cell culture medium	75	ND	> 5 in 30 sec	Parry and Mortimer (1984)
cell culture medium	75.1	ND	4 in 10 sec	Parry and Mortimer (1984)
cell culture medium	80	ND	> 5 in 5 sec	Parry and Mortimer (1984)
cell culture medium	85	ND	> 5 in ~1 sec	Parry and Mortimer (1984)

TFL: time to reduce first log_{10}
ND - not determined
NG - not given
PBS - phosphate-buffered saline

To try to elucidate how temperature exerts an inactivating effect on HAV, Nuanualsuwan and Cliver (2003b) heated virus suspensions at 37^oC and 72^oC until no infectivity could be detected (they did not state how long this required). They found that inactivated virus could not attach to cell monolayers but could be captured by anti-HAV antibodies. Viral RNA could be amplified by RT-PCR from viruses treated at 37^oC but not from those treated at 72^oC. These results indicate that temperature exerts an effect on HAV by damaging the virus capsid, particularly the capsid region binding with the cellular receptors which mediates

infectivity but at lower temperatures the damage is not sufficient to disrupt the capsid. The presence of organic materials may have a protective effect: Barrett *et al.* (1997) observed a lower level of inactivation in 25% human serum albumin (HSA) pasteurised at 60°C for 10 h (4.3 log) than in 3.5% HSA treated similarly (5.8 log).

Different HAV strains display differing resistance to heat (Anderson, 1987). Shimasaki *et al.* (2009) observed that heat treatment at 60°C for 10 h reduced infectivity by 3 log among some strains of HAV tested and by 5 log among others. All strains were inactivated by 1 log after 1 h at 60°C. Farcet *et al.* (2012) noted that the log reductions of variants of the cell culture adapted strain HM175 achieved by pasteurisation in HSA varied over a 2 log range with variants of the original strain being more resistant (the mean reductions from this study are given in Table 3.7). This may have been due to repeated passage in cell culture, although Barrett *et al.* (1996) reported no difference in heat sensitivity between culture-adapted and wild-type HAV. Tuladhar *et al.* (2012) cautioned that, as reduction in virus infectivity on heating may not follow linear kinetics and tailing can occur reflecting the survival of resistant virus fractions, care should be taken in using T90 values as they may over-predict complete inactivation.

The effect of heating is manifested by unfolding of the viral capsid at high temperatures (Volkin *et al.*, 1997); Siegl *et al.* (1984) reported that at temperatures above 60°C HAV RNA starts to be released into the surrounding medium. In the presence of salts, ($MgCl_2$), the capsid becomes more heat-stable (Anderson, 1987; Siegl *et al.*, 1984; Volkin *et al.*, 1997).

Additional information on the effect of heat on HAV can be acquired from studies investigating the efficacy of heating regimes applied to inactivate the virus in products such as blood plasma-derived coagulation factors. Table 3.7 summarises the results of several such studies. From data obtained (Savage *et al.*, 1998) it appears that the presence of moisture potentiates the effect of heat.

Table 3.7: Data from studies on HAV inactivation in therapeutic products

Matrix	Temperature (^{o}C)	TFL (min)	Approximate total log decline in infectivity	Reference
HSA, pH 9.4	37	> 300	1.9 in 24 h	Roberts *et al.* (2011)
HSA, pH 10	37	300	3.5 in 24 h	Roberts *et al.*(2011)
3.5% HSA	58	ND	5.2 in 600 min	Farcet *et al.*(2012)
5% HSA	58	ND	3.1 in 600 min	Farcet *et al.*(2012)
25% HSA	58	ND	4.5 in 600 min	Farcet *et al.*(2012)
urokinase solution	59	100	4 in 600 min	Kim *et al.* (2002)
3.5% HSA	60	20	5.8 in10 h	Barrett *et al.* (1997)
HSA, pH 7.0	60	2	4.8 in 5 h	Roberts *et al.* (2011)
HSA, pH 9.5	60	< 2	>5 in 10 min	Roberts *et al.* (2011)
HSA, pH 10	60	< 2	>5 in 10 min	Roberts *et al.* (2011)
5% HSA	60	20	4.8 in 10 h	Barrett *et al.* (1997)
25% HSA	60	20	4.3 in10 h	Barrett *et al.* (1997)
protein solution^{+}	60	ND	3.6 in 6 h	Murphy *et al.* (1993)
protein solution^{+}	60	ND	≥ 6.4	Groener *et al.* (2005)
protein solution^{+}	60	ND	3.5 in 6 h	Hilfenhaus and Nowak (1994)
protein solution^{+}	60	ND	5 in 8 h	Hilfenhaus and Nowak (1994)
coagulation factor	63	ND	> 6.4	Biesert *et al.* (1995)
lyophilised coagulation factor, residual moisture ≥ 0.8 %	80	ND	≥ 4.5 in 72 h	Savage *et al.* (1998)
lyophilised coagulation factor, residual moisture ≤ 0.8 %	80	ND	0.1 in 72 h	Savage *et al.* (1998)
bone tissue	≥82.5	ND	≥ 4.5 in 15 min	Pruss *et al.* (2001)
coagulation factor solution	90	ND	2.6 in 2 h	Hart *et al.* (1994)
coagulation factor solution	90	ND	≥ 3.9 in 6 h	Hart *et al.* (1994)
lyophilised coagulation factor	99	ND	≥ 5.6 in 10 min	Shin *et al.* (2006)
lyophilised coagulation factor	> 100	ND	≥ 5.7 in 2 h	Biesert *et al.*(2005)
reconstituted lyophilised coagulation factor	100	ND	> 5.3 in 5 min	Dichtelmueller *et al.*(1996)

TFL: time to reduce first log_{10}
HSA: human serum albumin
ND: not determined
NG: not given
PBS: phosphate-buffered saline
$^{+}$: protein solutions used to stabilize blood plasma products

Bidawid *et al.* (2000) contaminated three types of dairy products containing different fat contents with HAV (0% fat skimmed milk, 3.5% homogenised milk, and 18% fat table cream) and heated them in microcapillary tubes for various temperature/time combinations. Heating at 85°C caused a 6-log reduction in infectious HAV titre in all 3 dairy products within 30 seconds. Lowering the temperature increased the TFL and a lower overall decline in infectivity was observed the lower the temperature. This study demonstrated that the fat content of dairy products can play a protective role and allow contaminating HAV to become more heat-stable.

Data on the elimination of HAV in milk and fresh herbs are given in Table 3.8.

Table 3.8: Effect of heat on HAV in various foodstuffs

Foodstuff	**Temperature** (^{o}C)	**TFL**	**Approximate overall log decline in infectivity**	**Reference**
Milk	20	ND	0	Parry and Mortimer (1984)
Milk	62.8	ND	3 in 30 min	Parry and Mortimer (1984)
Milk	71.6	ND	2 in 15 sec	Parry and Mortimer (1984)
Skimmed milk	65	2.7	2 in 16 min	Bidawid *et al.* (2000)
Skimmed milk	67	1.0	3 in 16 min	Bidawid *et al.* (2000)
Skimmed milk	69	0.73	4 in 16 min	Bidawid *et al.* (2000)
Skimmed milk	71	0.16	5.5 in 14 min	Bidawid *et al.* (2000)
Skimmed milk	73	0.16	5 in 12 min	Bidawid *et al.* (2000)
Skimmed milk	75	0.13	5.5 in 8 min	Bidawid *et al.* (2000)
Skimmed milk	80	0.1	5.5 in 1 min	Bidawid *et al.* (2000)
Skimmed milk	85	<0.5	6 in 1 min	Bidawid *et al.* (2000)
Homogenised milk	65	6.2	1.5 in 16 min	Bidawid *et al.* (2000)
Homogenised milk	67	1.19	3 in 16 min	Bidawid *et al.* (2000)
Homogenised milk	69	0.79	3.5 in 16 min	Bidawid *et al.* (2000)

Homogenised milk	71	0.18	5 in 16 min	Bidawid *et al.* (2000)
Homogenised milk	73	0.17	5.5 in 12 min	Bidawid *et al.* (2000)
Homogenised milk	75	0.15	5.5 in 14 min	Bidawid *et al.* (2000)
Homogenised milk	80	0.1	5.5 in 8 min	Bidawid *et al.* (2000)
Homogenised milk	85	<0.5	6 in 1 min	Bidawid *et al.* (2000)
Cream	65	7.34	1 in 16 min	Bidawid *et al.* (2000)
Cream	67	2.82	2 in 16 min	Bidawid *et al.* (2000)
Cream	69	1.11	3 in 16 min	Bidawid *et al.* (2000)
Cream	71	0.52	4 in 16 min	Bidawid *et al.* (2000)
Cream	73	0.36	5 in 14 min	Bidawid *et al.* (2000)
Cream	75	0.29	5.5 in 14 min	Bidawid *et al.* (2000)
Cream	80	0.12	5.5 in 2 min	Bidawid *et al.* (2000)
Cream	85	<0.5	6 in 1 min	Bidawid *et al.* (2000)
Basil	-20	-	< 1 log after 90 days	Butot, *et al.* (2008)
Basil, freeze drying	-	ND	< 2 log	Butot, *et al.* (2009)
Basil, steam blanching for 2.5 min	75	ND	< 2	Butot, *et al.* (2009)
Basil, steam blanching for 2.5 min	95	ND	> 3	Butot, *et al.* (2009)
Chives, steam blanching for 2.5 min	75	ND	> 3	Butot, *et al.* (2009)
Chives, steam blanching for 2.5 min	95	ND	> 3	Butot, *et al.* (2009)
Mint, steam blanching for 2.5 min	75	ND	< 2	Butot, *et al.* (2009)
Mint, steam blanching for 2.5 min	95	ND	> 3	Butot, *et al.* (2009)

Parsley	-20	-	< 1 log after 90 days	Butot, *et al.* (2008)
Parsley, freeze drying	-	ND	< 1.5 log	Butot, *et al.* (2009)
Parsley, steam blanching for 2.5 min	75	ND	~ 2	Butot, *et al.* (2009)
Parsley, steam blanching for 2.5 min	95	ND	> 2	Butot, *et al.* (2009)

TFL: time to reduce first $\log_{10}$
ND: not determined

In a study to characterise the effects of a food ingredient on the thermal inactivation of HAV, Deboosere *et al.* (2004) inoculated synthetic formulations designed to model various parameters of berry fruit (pH, sugar content, calcium concentration) and subjected them to heating. Increasing the sugar content noticeably increased the resistance of the virus, while lowering the pH increased its heat susceptibility (Table 3.9). Calcium concentration had no noticeable effect. Test using fruit products (mashed strawberries and stabilizers) gave similar results. The initial inoculum used in these experiments gave a virus concentration in the samples of 10^7 pfu ml^{-1} and 10^2 (the apparent limit of detection used) infectious virus were still detectable after 4 min, 11 min and 30 min at 28%, 40% and 52% sugar content respectively. Cooking berries if they are suspected of contamination with HAV may be an option for food processors.

Table 3.9 summarises the results from this study.

Table 3.9: Effect of physical and chemical parameters on heat inactivation of HAV in a berry fruit model system (from Deboosere *et al.*, 2004)

Matrix	**Parameter**	**Temperature (°C)**	**T90 (min)**	**Virus undetectable* after**
Synthetic medium	28 % sucrose	80	1.73	Not shown
Synthetic medium	52 % sucrose	80	12.22	Not shown
Fruit products	28 % sucrose	80	1.22	Not shown
Fruit products	52 % sucrose	80	8.94	Not shown
Synthetic medium	28 % sucrose	85	0.80	4 min
Synthetic medium	40 % sucrose	85	1.88	11 min
Synthetic medium	52 % sucrose	85	6.28	30 min
Fruit products	28 % sucrose	85	0.96	Not shown
Fruit products	52 % sucrose	85	4.98	Not shown
Synthetic medium	28 % sucrose	90	0.22	Not shown
Synthetic medium	52 % sucrose	90	2.87	Not shown
Fruit products	28 % sucrose	90	0.32	Not shown
Fruit products	52 % sucrose	90	3.00	Not shown
Synthetic medium	pH 3.3	85	1.52	Not shown
Synthetic medium	pH 3.8	85	1.88	10 min
Synthetic medium	pH 4.3	85	2.87	15 min

T90: time to reduce 90 percent of initial load

*: The initial concentration of HAV was ~ 10^7 pfu ml^{-1} and the limit of detection of the method used appeared to be ~ 10^2 pfu ml^{-1}.

Deboosere *et al*. (2010) artificially contaminated purees of various fruits (bilberries, strawberries and raspberries) with 10^6 – 10^8 pfu ml^{-1} HAV and heated them for various time periods at pH 2.5 – 3.3; the results were reported in graphical format. At 65°C and 70°C, they found the decline in virus infectivity to be non-linear, in that no decrease in infectious titre was observed until 1 min to 4 min after target temperature was reached. For fruit purees heated at 75°C and acidified to pH 2.5, virus inactivation from 1.5 log to 4 log was observed.

They produced a statistical model which they claimed can be used to reliably predict inactivation of HAV in berry fruit by heat.

Butot *et al.* (2009) reported that after heating freeze-dried blueberries at 100°C for 20 min, infectious HAV could be detected; heating at 120°C for 20 min completely eliminated virus infectivity. The survival of HAV at 100°C is surprising and appears anomalous; in the same study this temperature eliminated HAV infectivity in blackberries, raspberries and strawberries. No discussion of this was made in the publication, and in the view of the authors of this report it may be that some experimental error occurred.

Steam blanching at 95°C for 2.5 min resulted in a 2.5 to 3 log reduction of HAV on leafy greens (Butot, *et al.*, 2009).

Millard *et al.* (1987) examined the effect of heating on HAV in shellfish. Artificially contaminated (~ 5 log HAV) cockles were either immersed in water at temperatures of 85°C and above, or steamed, and examined at various time points for infectious virus. Table 3.10 shows the results obtained.

Table 3.10: Heat inactivation of HAV in artificially contaminated cockles (from Millard *et al.*, 1987)

Treatment	Temperature (^{o}C)	Duration of treatment							
		0.25	0.5	0.75	1	1.5	2	2.5	3
Immersion in water	85	ND	ND	ND	+	ND	ND	ND	-
	90	ND	+	ND	+	ND	ND	ND	-
	95	+	+	+	+	+	-	-	-
Steaming	100/101	ND	ND	ND	+	ND	-	-	-

+: infectious virus detected

-: infectious virus not detected

ND: not determined

After 2 min at each treatment, the cockles reached an internal temperature of 91.5 to 94°C. No infectious virus was detected in the cockles after immersion in water at 95°C after 90 sec, or after steaming for 1 min. Subsequently, the then UK Ministry of Agriculture recommended for commercial cooking operations that cockles be cooked to an internal temperature of 85-

90°C for 1.5 min, and this resulted in a decrease in cockle-associated illness outbreaks (Lees, 2000). Abad *et al.* (1997b) performed a study to determine the inactivation of HAV in artificially contaminated mussels, after cooking by steaming. At the opening of the molluscs' bivalves (2.5 min steaming) a reduction of 2.2 log of infectivity was observed but after an additional 2.5 min of boiling the reduction did not reach 3 log. Considering that the temperatures required to fully inactivate viruses will cause an unacceptably overcooked product, and that these temperatures are seldom used in preparation of shellfish dishes in restaurants or domestically, Hewitt and Greening (2006) examined a series of cooking scenarios likely to be found in these settings. Only when mussels were boiled for 3 min, steamed for 3 min, or boiled for 37 sec then steamed for 3 min, were significant reductions (>1, 1.5 and 2 log respectively) in infectious HAV titre observed. No effect was seen on RT-PCR-detectable units (RT-PCRU), indicating that capsid disruption did not occur even when infectivity was reduced. Hewitt and Greening (2006) recommended that immersion for at least 3 min in boiling water rather than steaming should be performed to reduce the risk of viral illness from contaminated shellfish. Sow *et al.* (2011) made a similar recommendation - that heating at 90°C for 180 sec should be used for commercial shellfish products. However such treatments may not result in a product which is acceptable to consumers due to adverse changes in its organoleptic properties.

Table 3.11 summarises data from studies on elimination of HAV from shellfish by heat.

Table 3.11: Elimination of HAV in shellfish by heat

Treatment	**Time**	**Approximate overall log decline in infectivity**	**Reference**
90°C	180 sec	> 5	Sow *et al.* (2011)
Steaming	37 sec	0	Hewitt and Greening (2006)
Boiling	37 sec	0	Hewitt and Greening (2006)
Steaming	3 min	1.5	Hewitt and Greening (2006)
Boiling	3 min	> 3.1	Hewitt and Greening (2006)
Boiling 37 sec then steaming 3 min		2.1	Hewitt and Greening (2006)
Steaming	2.5 min	2.2	Abad *et al.* (1997b)
Steaming then boiling	2.5 min each	2.9	Abad *et al.* (1997b)

Dehydrated vegetables are commonly used in food production; drying can generally be achieved by heating at 40°C – 60°C up to 24 h. To examine whether such treatment had any effect on infectivity of HAV, Laird *et al.* (2011) artificially contaminated green onion pieces with HAV, then dehydrated them for 20 h at various temperatures. They reported that inactivation increased linearly with increasing temperature, from 0.84 log at a mean temperature of 42.7°C to >3.9 log at a mean temperature of 65.9°C. The water content of the onions was identical after treatment at the lowest and the highest temperature indicating that the inactivating effect on HAV was primarily due to the temperature. This finding is similar to those of Butot *et al.* (2008; 2009) (see section 2.5) and prompts the recommendation that dehydration should be performed at temperatures greater than 60°C. Laird *et al.* (2011) performed qRT-PCR on nucleic acids extracted from viruses subjected to dehydration on the onion pieces at 60.7°C for 20 min and, although a 2.64 log reduction in infectivity had been recorded, there was no reduction in the qRT-PCR cycle threshold (Ct) values obtained.

Nguyen-The (2012) compared data on heat inactivation of HAV and *Enterococcus faecalis*, which is a non-spore forming bacterium used to evaluate the impact of food processes involving heat-treatment. The study concluded that above 65°C, the use of *Enterococcus faecalis* would be unable to predict the impact of temperature on HAV as the virus is considerably more heat-resistant. A previous statistical analysis performed on inactivation data of enteric viruses in food and water showed a similar resistance of HAV and bacteriophages PhiX174 and PRD1 at temperatures below 50°C but HAV seemed to be less persistent than these bacteriophages at higher temperatures (Bertrand *et al.*, 2012). A systematic review comparing data on inactivation by chemical disinfection of several enteric viruses as surrogates for norovirus (Hoelzer *et al.*, 2013) did not find that HAV was more resistant than poliovirus or rotavirus, although they stated that data allowing this comparison was scarce.

An indication of the possibility for HEV to remain infectious after exposure to temperatures used in some cooking procedures (e.g. meat dishes cooked rare) was provided by Emerson *et al.* (2005) who monitored the infectivity of faecal suspensions of 3 strains of the virus (two gt1 and one gt2) after incubation for 1 h at temperatures between 45°C and 50°C. The treated suspensions were inoculated onto cultured HepG2/C3A cells, which after 5-6 d were stained with fluorescent anti-HEV antibodies. Detection of fluorescence was taken as indicative of viral replication within the cells, and by counting the number of fluorescing cells a

quantitative comparison of the effect of the treatments could be attempted. By this means Emerson *et al.* (2005) estimated that 95% of infectious HEV was inactivated by heating at 56°C for 15 min, but that 1% infectivity remained after 60 min at this temperature; no infectivity was detected after heating at 66°C or 70°C for 1 h. They used only one of the strains for this latter determination, and the other two strains appeared slightly more heat resistant according to the number of fluorescing cells observed after 60°C for 1 h (these strains were not tested at higher temperatures). Incubating suspensions of the latter two HEV strains on ice for 1 h resulted in less fluorescing cells being observable than after the suspensions were heated at 50°C or 56°C; this odd finding possibly reveals the limitations of using the fluorescence-based method to try to obtain quantitative data. Interestingly, the faecal samples from which the viruses were derived had been stored for over 10 years with several rounds of freezing and thawing; that infectious HEV remained is an indication of the robustness of this virus.

Tanaka *et al.* (2007) used an infectivity in cell culture-PCR (ICC-PCR) type approach to determine the effect of heat on infectious HEV gt3. This approach involved challenging cultured PLC/PRF/5 cells with virus suspensions, untreated or after being subjected to heat, and monitoring viral RNA levels by qRT-PCR immediately after inoculation and up to 50 d incubation. When virus suspensions were heated at 25°C for 30 min, HEV RNA could be detected from day 16, while after 56°C for 30 min, HEV RNA could be detected from day 20; this longer time to reach a detectable titre after treatment at the higher temperature may indicate that the heat treatment had some effect on infectious titre but was unable to inactivate all HEV particles in the treated suspensions. After heating at 70°C for 10 min, 95°C for 1 min, and 95°C for 10 min, no HEV RNA was detected in the inoculated cell cultures at any time.

A similar approach was used by Yunoki *et al.* (2008): here, A549 cells were inoculated with heated and untreated virus suspensions, and monitoring viral RNA levels by qRT-PCR immediately after inoculation and after 7 days incubation. Infectious titres were determined by diluting suspensions before inoculation of the cells. After heating of suspensions of HEV gt3 and gt4 in PBS at 60°C for 30 min, no viral RNA could be detected after 7 d incubation of inoculated cells, indicating that this treatment was effective in eliminating 2.4 – 3.7 log virus (based on the titre of the untreated suspensions). When the virus was suspended in a 25%

human serum albumin solution all the virus isolates showed heat resistance, with log reductions now between 1.0 and 2.2 after 5 h at 60°C; gt3 infectivity was not completely eliminated. This indicates that the surrounding matrix can be strongly protective of this virus. Dry heating of virus in a freeze-dried fibrinogen formulation at 80°C for 24 h completely eliminated (≥ 4 log reduction) HEV gt3 isolates (gt4 was not tested).

Using a 3-D cell culture system for propagating HEV which they had previously developed (Berto et al., 2013), Berto *et al.* (unpublished) studied the effect of heating on HEV following an approach similar to that used by Tanaka *et al*. (2007) and Yunoki *et al*. (2008). After heating an HEV suspension at 56°C for 1 h, viral RNA could be detected in the cell culture system to 62 d post-inoculation, albeit to a lesser extent than was observed with unheated cultures. No viral RNA was detected at any time in the cells infected with HEV suspension heated at 100°C for 15 mins. Jones and Muelhauser (2014) artificially contaminated pig oral fluid with HEV (at 10^{3} genome copies ml^{-1}) and heated the samples at 60°C for 15 min after which no HEV RNA could be subsequently detected.

To determine whether commonly used cooking procedures are effective in inactivating HEV in infected pig livers, Feagins *et al.* (2008) intravenously inoculated pathogen-free pigs with the supernatant from centrifuged homogenates of naturally contaminated pigs livers. When homogenates were incubated at 56°C for 1 h, four of five pigs became infected with HEV. When liver cubes were stir-fried at 191°C for 5 min in canola oil or boiled in water for 5 min, none of the pigs inoculated with the subsequently-prepared suspensions showed any sign of infection. In each of these latter tests, the internal temperature of the liver samples reached 71°C, although it was not stated how long this was for.

To model the effect of cooking on HEV, Barnaud *et al.* (2012) obtained HEV-infected liver from an experimentally infected pig, and processed it into a pâté-like preparation by homogenisation and the addition of various ingredients including salt and spices. Then 25 g, 2 mm thick portions of the preparations were heated to nine time/temperature combinations. Aliquots of the treated preparations were then inoculated into pathogen-free pigs, which were subsequently monitored for evidence of HEV-infection by serology and analysis of their faeces. The outcome is shown in Table 3.12.

Table 3.12: Effect of heating on HEV, as monitored by development of infection by pigs after intravenous inoculation with contaminated liver (from Barnaud *et al.*, 2012)

Temperature (^{o}C)	Time (min)	Excretion of HEV by pigs	Serological conversion
No treatment	-	Yes	Yes
62	5	Yes	Yes
62	20	Yes	Yes
62	120	Yes	Yes
68	5	Yes	Yes
68	10	Yes	No
68	20	Yes	Yes
71	5	Yes	Yes
71	10	Yes	Yes
71	20	No	No

Source: (Barnaud et al., 2012)

The authors concluded that heating to an internal temperature of 71°C for 20 min was necessary to completely inactivate the virus. However, there is a possibility that the time/temperature combination for HEV inactivation was over-estimated in this experiment. The pigs inoculated with viral suspensions from liver pâté treated at 71°C for 10 min were kept in the same pen as animals inoculated with viral suspensions from liver pâté treated at 62°C for 10 min; the latter animals were excreting virus 9 days earlier than the former, and therefore likely infected them through proximity. Thus the reliable inference from this study is that HEV could survive heating to 71°C for at least 5 min but not 20 min in contaminated liver. Fat in the liver may have had a protective effect on the virus. Subsequently Barnaud and colleagues (Rogee *et al.*, 2013) tested the effect of heat on an HEV gt3 strain while developing two cell lines for HEV propagation. After treating virus in a filtered faecal suspension at 56°C for 60 min or 95°C for 5 min, no replication was observed on either cell line.

Schielke *et al.* (2011) attempted to determine the infectivity of HEV in suspensions derived from centrifuged and filtered homogenates of infected wild boar liver by incubating them on various cell lines (PLC/PRF/5, A549 and HepG2) followed by immunofluorescence-based antigen detection, but this proved unsuccessful. As an alternative approach, they treated the homogenates with RNase prior to nucleic acid extrication, to detect intact virus particles. They pointed out that the correlation with infectivity may not have been exact and thus HEV survival would have been overestimated, but considered that this would allow a conservative estimate of survival and elimination. By this means they reported that storage at various temperatures resulted in an initial decline in detectable genome equivalents followed by several days where no decline was observable: (Table 3.13); the log reductions were not high, indicating that considerable numbers of HEV particles remained intact. Heating at 56°C for 15 min resulted in less than 1 log reduction in detectable genome copies; at 56°C for 30 min and at higher time / temperature combinations reductions of at least 3 log were obtained (Table 3.14). Heating for 1 min at various temperatures produced the following mean log genome reductions: <1 at 70°C, <1 at 75°C, ~2.5 at 80°C, ~2.6 at 85°C, ~3.6 at 90°C and ~3.7 at 95°C. The variability in reduction at temperatures of 80°C and above was high; the authors linked this to changes in the appearance of the liver suspensions at these temperatures. They had found that the number of HEV genome equivalents in the original liver suspension was ~ 10^8, therefore it might be inferred from the results of their studies that short term heating procedures may not be sufficient to inactivate all HEV present in a liver portion. Shielke *et al.* (2011) referred to the evidence of cases of hepatitis E occurring following consumption of grilled wild boar meat as confirmation of this possibility.

Table 3.13: Decline in detectable HEV genome equivalents in liver suspensions on storage at various temperatures* (from Schielke *et al.*, 2011)

Storage temperature (°C)	**Initial log decline**	**Number of days where no further decline was observed⁺**
4	0.34 in 1 h	70
22	0.45 in 3 d	47
37	1.24 in 7 d	43

*The initial log titre of HEV in each experiment was ~ 5.4.
⁺ the duration of the experiment was 70 d at 4°C, 50 d at 22°C, and 50 d at 37°C

Table 3.14: Reduction in detectable HEV genome copies in liver suspensions after treatment at various temperature/time combinations (from Schielke *et al.*, 2011)

Temperature (^{o}C)	Time (min)	Log reduction
56	15	<1
56	30	4
56	60	3
60	60	3-4
95	1	3-4

3.9 High hydrostatic pressure

High hydrostatic pressure (HHP) is increasingly used in the food industry to reduce microbial load in some foodstuffs while preserving their organoleptic properties; it may not be suitable for some delicate foods however. Kingsley *et al*. (2002) showed that HAV suspended in tissue culture medium was reduced from 7 log pfu ml-1 to undetectable levels following exposure to higher than 450 MegaPascals (MPa) for 5 min. They reported that suspending the virus in seawater reduced the effect of the high pressure treatment, possibly by stabilising viral capsid proteins. After incubating the inactivated HAV with RNase then performing RT-PCR, amplification of viral RNA was observed, indicating that the high pressure treatment did not disrupt the viral capsid. Grove *et al.* (2008) obtained similar findings, with 600 MPa reducing ~ 3.5 log ml-1 HAV in tissue culture medium below the limit of detection of the assay by 90 sec. In a subsequent study, Grove *et al.* (2009) confirmed that increasing the salt concentration of the medium increased the resistance of the virus to the pressure treatments. The effect of HHP on HAV does not appear to be linear. Kingsley *et al.* (2006) found that extending treatment times at a range of temperatures from -10°C to 50°C did not result in complete elimination of activity from HAV suspensions; an initial rapid reduction was followed by a long tail-off where only small reductions in infectivity were observed at the different time points. This indicates that a proportion of a population of HAV virions could resist inactivation by pressure (at least up to 400 MPa), although the mechanism for this remains unclear. Shimasaki *et al.* (2009) found that some strains of HAV were more resistant to HHP than others (although all could be inactivated by several logs by pressures of 400 MPa). Kingsley *et al*. (2006) also found that low temperatures reduced HAV sensitivity to high pressure and suggested that mild heating, e.g. at >30°C, may be required for effective treatment of samples by HHP. There can be a protective effect of food components on HAV subjected to HPP: Hirneisen *et al.* (2012) found that suspending the virus in mayonnaise resulted in 2 log lower reduction at 500 MPa than if the virus was suspended in cell culture medium.

Table 3.15 summarises data from studies on the effect of heat on HAV in suspension.

Table 3.15: Effect of high hydrostatic pressure on HAV in suspension

HHP treatment (MPa)+	**Temperature (°C)**	**TFL (sec)**	**Approximate overall log decline in infectivity**	**Reference**
300	RT	~ 100	1 in 5 min	Grove *et al.* (2008)
300*	RT	> 300	< 1 in 5 min	Grove *et al.* (2009)
400	ND	ND	3 in 5 min	Kingsley *et al.* (2002)
400	10	ND	2 in 1 min	Kingsley *et al.* (2002)
400	RT	~ 90	2 in 5 min	Grove *et al.* (2008)
400*	RT	> 300	< 1 in 5 min	Grove *et al.* (2009)
400	30	ND	2.5 in 1 min	Kingsley *et al.* (2006)
400	40	ND	3.5 in 1 min	Kingsley *et al.* (2006)
400	50	ND	4.5 in 1 min	Kingsley *et al.* (2006)
450	ND	ND	7 in 5 min	Kingsley *et al.* (2002)
500	RT	~ 30	3 in 5 min	Grove *et al.* (2008)
500*	RT	~ 100	2.5 in 300 sec	Grove *et al.* (2009)
600	RT	NG	> 3 in 90 sec	Grove *et al.* (2008)

TFL: time to reduce first $\log_{10}$
ND: not determined
NG: not given
RT: room temperature
+: experiments where viruses were suspended in cell culture medium unless stated by *
*: experiments where 30 g l^{-1} marine salt mixture was the suspending medium

Following their work on the effect of HHP on foodborne viruses particularly in shellfish, Kingsley and co-workers (2005) wished to determine whether this process could also be effective as an intervention strategy for fresh produce. HAV artificially contaminated into strawberry puree could be reduced in infectivity by almost 4 log by a 5 min treatment at 350 MPa; at 375 MPa the virus was reduced beyond detectable limits indicating at least a 4 log reduction.

Table 3.16 summarises data from studies on elimination of HAV from berry fruits and products by high hydrostatic pressure.

Table 3.16: Effect of HHP on HAV infectivity in strawberry puree (from Kingsley *et al.*, 2005)

Foodstuff	HHP Treatment (MPa)*	Approximate log reduction in infectivity
Strawberry puree	250	1
Strawberry puree	275	1.75
Strawberry puree	300	2.75
Strawberry puree	325	3.5
Strawberry puree	350	3.75
Strawberry puree	375	> 4

*: all treatments for 5 min

Kingsley *et al.* (2005) examined the potential for HHP to inactivate HAV on green onions (Table 3.17). HAV artificially contaminated into green onion slices could be reduced in infectivity beyond detectable limits by a 5 min treatment at 350 MPa indicating at least a 4 log reduction. Hirneisen and Kniel (2013) compared the effect of HHP against HAV on the surface of green onions, and against the virus internalised within the plants (Table 3.17). They subjected the contaminated vegetables to 500 MPa for 2 min at 20°C. The internalised HAV was less susceptible to inactivation by HHP, although some reduction in infectivity was observed (2.5 log inactivation of internalised virus against 5.5 log inactivation of virus located on the surface of the green onions). Although the difference appears pronounced, the authors stated that it was not significant; however only three replicates of each test were performed, which may not have allowed an effective statistical comparison to be made.

Table 3.17: Effect of HHP on HAV infectivity on green onion surfaces and internalised within green onions

Location of HAV	HHP Treatment	Approximate log reduction in infectivity	Reference
Surface	250 MPa 5 min	0.3	Kingsley *et al.* (2005)
Surface	275 MPa 5 min	0.8	Kingsley *et al.* (2005)
Surface	300 MPa 5 min	1.5	Kingsley *et al.* (2005)
Surface	325 MPa 5 min	3.25	Kingsley *et al.* (2005)
Surface	350 MPa 5 min	> 4	Kingsley *et al.* (2005)
Surface	500 MPa 2 min	5.5	Hirneisen and Kniel (2013)
internalised	500 MPa 2 min	2.5	Hirneisen and Kniel (2013)

Calci *et al.* (2005) demonstrated directly that HHP could inactivate HAV in shellfish. They immersed live oysters (*Crasseotrea virginica*) in seawater artificially contaminated with 40 pfu ml^{-1} HAV. After 24 h, they were shucked and treated at various pressures for 1 min at ~ 9°C in water/ethylene glycol 50/50. The final temperature of the samples was between 17 °C and 20°C. Viruses were quantified by plaque assay. After accumulation, the oysters contained approx. 5.8 log pfu. The highest reduction (~ 3 log) was achieved by processing at 400 MPa for 1 min. They also processed oysters containing lower titres of HAV, approximately 10^3-10^4 pfu, and (although not showing the data) reported that no virus could be detected after HHP treatment at 400 MPa for 1 min, indicating that HAV at low concentrations in shellfish could be completely inactivated. The inactivation of HAV was not as great as was seen using similar pressures in cell culture medium (Kingsley *et al.*, 2002), indicating a possible protective effect of the shellfish tissue, although the treatment time used in that study was longer at 5 min. In contrast, Grove *et al.* (2009) found that inactivation of HAV in homogenised shellfish tissue was greater than in tissue culture medium alone and suggested that released cellular enzymes enhanced virus degradation. They produced a mathematical model predicting HHP inactivation of HAV in various salt concentrations but they found that it under-predicted the inactivation of HAV in shellfish, possibly due to the effects of components of shellfish tissue enhancing elimination of virus infectivity.

Table 3.18 summarises data from studies on elimination of HAV from shellfish by high hydrostatic pressure.

Table 3.18: Elimination of HAV in shellfish by high hydrostatic pressure

Shellfish	HHP Treatment	Log reduction in infectivity	Reference
Clams	400 MPa 5 min	3.4	Hirneisen *et al.* (2012)
Oysters	375 MPa 1 min	2.3	Calci *et al.* (2005)
Oysters	375 MPa 5 min	1.9	Grove *et al.* (2009)
Oysters	350 MPa 1 min	1.3	Calci *et al.* (2005)
Oysters	325 MPa 1 min	0.8	Calci *et al.* (2005)
Oysters	300 MPa 1 min	0.2	Calci *et al.* (2005)
Oysters	400 MPa 1 min	3.1	Calci *et al.* (2005)
Shrimp	400 MPa 5 min	1.7	Hirneisen *et al.* (2012)

Sharma *et al.* (2008) performed a study examining the potential for HHP and high dynamic pressure (HDP; where detonation of an explosive produces a shockwave in a container) to inactivate infectious HAV artificially contaminating sausage meat; although it was not the aim of the study, the information may be relevant to the inactivation of HEV in pork

products. HHP at 500 MPa could inactivate > 3 log virus, whereas HDP effected only an approximately 1 log reduction.

3.10 Hydrogen peroxide

Hall and Sobsey (1993) reported that 1 mg l^{-1} hydrogen peroxide (H_2O_2) at pH 8.2 could inactivate 1.3 log HAV after 90 sec contact time at 6^oC.

3.11 Iodine

Examining the effectiveness of iodine as a disinfectant for water supplies, Sobsey *et al.* (1991) found that iodine could rapidly inactivate HAV under neutral to alkaline conditions (Table 3.19). Organic matter and turbidity reduced the effect of the disinfectant, except when the pH was high and with the higher tested dose.

Table 3.19: Inactivation of HAV by iodine (from Sobsey *et al.*, 1991)

Iodine Dose (mg l^{-1})	Temperature (^oC)	pH	Time to 4 log inactivation (min)
8*	5	4.5	37
8^{+}	5	4.5	140
8*	5	7.0	1.1
8^{+}	5	7.0	1.8
8*	5	9.5	0.4
8^{+}	5	9.5	< 0.4
8*	25	4.5	7.2
8*	25	7.0	< 0.4
8*	25	9.5	< 0.3
16*	5	4.5	60
16^{+}	5	4.5	104
16*	5	7.0	< 1.1
16^{+}	5	7.0	0.8
16*	5	9.5	< 0.3
16^{+}	5	9.5	< 0.4
16*	25	4.5	7.8
16*	25	7.0	< 0.4
16*	25	9.5	< 0.4

*: experiments performed in biological oxygen demand-free water
$^{+}$: experiments performed at 5^oC in biological oxygen demand-free water containing 10 mg l^{-1} organic acids and 5 turbidity units of bentonite clay

3.12 Ozone

Ozone or trioxygen (O_3) is a gas formed from dioxygen (O_2) by UV or electrical discharge and appears quite effective against HAV in suspension. Vaughn *et al.* (1990) found that initial concentrations of at least 1 mg l^{-1} could reduce the infectivity (as measured by radioimmunofocus units in infected cell culture) of treated HAV by 4 log in less than 1 min. Survival of HAV was slightly enhanced at lower ozone levels by increasing alkalinity. A

study by Hall and Sobsey (1993) found similar reductions could be obtained by lower ozone doses in less time; the differences may have been due to dissimilar ways of calculating residual O_3. Herbold *et al.* (1989) also found that an O_3 concentration of 0.38 mg l^{-1} could continuously inactivate ~ 3 log HAV in a steady-flow system. Hirneisen and Kniel (2013) compared the effect of ozone against HAV on the surface of green onions and against the virus internalised within the plants. They placed the contaminated vegetables in a continuous flow system where they were exposed to 6.25 ppm O_3 for 10 min. The internalised HAV was less susceptible to inactivation by HHP, although some reduction in infectivity was observed (1.5 log inactivation of internalised virus against 2.9 log inactivation of virus located on the surface of the green onions; the authors stated however that this difference was not statistically significant). Ozone can be used in modified atmosphere packaging but there is no data on its effect on HAV in such circumstances.

Table 3.20 summarises data from studies on the effect of ozone on HAV.

Table 3.20: Inactivation of HAV by ozone

Ozone concentration (mg l^{-1})	pH	TFL (sec)	Approximate total log reduction in infectivity	Reference
0.1	6.0	165	4 in > 3 min	Vaughn *et al.* (1990)
0.1	7.0	> 180	4 in > 3 min	Vaughn *et al.* (1990)
0.1	8.0	> 180	4 in > 3 min	Vaughn *et al.* (1990)
0.4	6.0	ND	> 6 in 5 sec	Hall and Sobsey (1993)
0.4	8.1	ND	> 4.6 in 5 sec	Hall and Sobsey (1993)
0.4	8.3	ND	> 4.2 in 5 sec	Hall and Sobsey (1993)
0.4	9.9	ND	> 4.2 in 5 sec	Hall and Sobsey (1993)
0.5	6.0	3	4 in > 3 min	Vaughn *et al.* (1990)
0.5	7.0	60	4 in > 3 min	Vaughn *et al.* (1990)
0.5	8.0	65	4 in > 3 min	Vaughn *et al.* (1990)
1.0	6.0	10	4 in 24 sec	Vaughn *et al.* (1990)
1.0	7.0	10	4 in 24 sec	Vaughn *et al.* (1990)
1.0	8.0	20	4 in 52 sec	Vaughn *et al.* (1990)
2.0	8.0	5	4 in 24 sec	Vaughn *et al.* (1990)

TFL: time to reduce first $\log_{10}$
ND: not determined

Hall and Sobsey (1993) tested ozone in combination with hydrogen peroxide (H_2O_2), against HAV. Similar inactivation levels were seen as with ozone (O_3) alone (Table 3.21)

Table 3.21: Inactivation of HAV by ozone/hydrogen peroxide combinations (from Hall and Sobsey, 1993)

O_3/H_2O_2 Concentration (mg l^{-1})	pH	Approximate total log reduction in infectivity after 5 sec at $4^oC – 5^oC$
2.0/0.6	6.3	> 4.0
2.0/0.6	8.2	> 3.
2.0/0.6	10.0	> 4.1
2.0/1.0	6.3	> 4.8
2.0/1.0	8.1	> 3.92
2.0/1.0	10.0	> 4.0
2.0/1.6	6.2	> 3.2
2.0 / 1.6	8.1	> 4.3
2.0 / 1.6	10.0	3.5

3.13 Peracetic acid

This agent does not appear to be very effective against HAV. Bigliardi and Sansebastiano (2006) calculated that at a temperature of "+20"^{o}C, the doses of 480 mg l^{-1} and 640 mg l^{-1} took 52 and 36 min respectively to inactivate 2 log HAV. Sabbah *et al.* (2010) found peracetic acid at 500 ppm and 1000 ppm to be ineffective against HAV dried onto stainless steel disks, mediating less than 1 log reduction after 5 min contact. Pruss *et al.* (2001) noted that even after 4 h suspension in a peracetic acid/ethanol solution, only a 3.7 log reduction of HAV could be obtained.

3.14 Plant extracts

Grape seeds contain phenolic compounds, and extracts have been shown to have antimicrobial activity. Testing a commercial preparation containing grape seed extract on HAV in suspension Su and D'Souza (2011) found it capable of inactivating up to ~ 3 log infectious virus. They highlighted the inexpensive availability of this material and considered that it may be a promising alternative as a disinfectant in food settings. Su and D'Souza (2013) reported more recently that grape seed extract in a commercial preparation could inactivate ~ 1 log HAV on lettuce and jalapeno peppers after 1 min contact. As the treatment did not affect the appearance of the vegetable, the authors considered that it could be used within hurdle approaches containing other novel processes with potentially mild effects on foodstuffs, such as ultrasound. Extracts of *Scutellaria baicalensis* Georgi (SBG), a plant used in Chinese herbal medicine which could have applications as a food additive, were ineffective (maximum reduction 0.1 log) in reducing HAV activity (Lu *et al.*, 2011).

3.15 Pulsed light

Pulsed UV light produces high energy UV light pulses with a low energy input requirement (Elmnasser *et al.*, 2007). Jean *et al.* (2011) tested it on suspensions of HAV in PBS and PBS plus 5% bovine serum (to mimic an organic load). Reductions of ~ 5 log could be achieved by fluences of 0.05 and 0.091 W s /cm^2 when the virus was suspended in PBS. When serum was present only a 2 log reduction was achieved; this might have been due to the proteins limiting the penetrative power of the UV pulses. The authors suggested that since pulsed UV is prone to such interference its use would be limited to disinfection of clear fluids or surfaces. Suspending the lamp 10.5 cm from PVC and stainless steel discs contaminated with ~ 5 x 10^3 pfu HAV, they exposed the virus to a 3 sec treatment, representing 0.091 W s /cm^2. Viruses were deposited on the discs in PBS and PBS with 5% bovine serum to represent an organic load. The authors reported that this treatment resulted in total reduction of HAV, as the virus could not be detected by plaque assay after recovery from the discs. Pulsed UV might be useful as a surface decontamination procedure in e.g. catering establishments, although more studies would be necessary, e.g. using greater distances between the lamp and the surface, to demonstrate that it could be applied practically. Roberts and Hope (2003) investigated the potential of a commercial system (PureBright®, Pulsed Light technologies, San Diego, USA), which produces flashes of high-intensity broad-spectrum light, to inactivate HAV in suspension. With fluences of 0.5 J/cm^2 and higher (to 2.0 J/cm^2) > 5.9 log infectivity could be reduced. The effect was less pronounced when the virus was suspended in a serum-containing medium, but only at the lower fluences. Such a system may be effective for disinfection of e.g. food preparation surfaces.

3.16 Quaternary ammonium compound (QAC)

Jean *et al.* (2003) tested a commercial disinfectant containing 10 % quaternary ammonium against HAV in suspension. They diluted the disinfectant to obtain various concentrations of the active ingredient, added 10^6 pfu HAV to each dilution (final volume 300 µl) and measured viral infectivity after 5 min contact time at 2 temperatures. Table 3.22 shows the results obtained.

Table 3.22: Inactivation of HAV in suspension by quaternary ammonium (from Jean *et al.* 1993)

Quaternary ammonium concentration (ppm)	Temperature (°C)	Log reduction in infectivity after 5 min
200	4	1.2
	20	1.1
500	4	1.6
	20	1.8
1000	4	2.0
	20	2.0
3000	4	2
	20	2.8

Testing a commercial QAC-based formulation, Solomon *et al.* (2009) reported that active concentrations of 8480 ppm active quaternary ammonium and below inactivated less than 1 log HAV dried onto glass discs after 10 min contact.

3.17 Smoking

Richards *et al.* (2010) considered that HAV might survive smoking of shellfish. No studies appear to have been done however to determine this.

3.18 Sodium hydroxide

Examining the potential for removal of viruses from blood products, the effect of temperature on HAV alone and in the presence of sodium hydroxide (NaOH) was studied by Borovec *et al.* (1998) (Table 3.23). At 15°C, 0.5 and 1.0 M NaOH inactivated greater than 5.5 log virus (no virus was detected above the limit of detection of the infectivity assay) after 10 min, whereas in the presence of 0.1 M NaOH only a 2.5 log reduction was observed. At 60°C in saline solution plus 0.1 M NaOH no infectious virus was detectable after 2 min. A synergistic effect of heat and chemical was observed; with Borovec *et al*. (1998) hypothesising that heating mediates a relaxing of the viral capsid allowing penetration of the chemical disinfectant. In a review of procedures used in disinfection of equipment in the production of plasma-derived products, Groner (2008) (providing unpublished data of Behring, C.) reported

that 0.1 M NaOH at room temperature inactivated 4 log HAV in 2 h, but gave no further experimental details.

Table 3.23: Inactivation of HAV by sodium hydroxide (from Borovec *et al.*, 1998)

Sodium hydroxide disinfection (M)	Temperature (°C)	TFL (min)	Approximate overall log decline in infectivity
0.1	60	< 0.5	> 5.5 after 2 min
0.1	15	~ 10	> 5.5 after 125 min
0.5	15	~ 1	> 5.5 after 10 min
1.0	60	~ 1	> 5.5 after 10 min

TFL: time to reduce first log_{10}

To study the effectiveness of disinfection procedures against virus contaminating surfaces after spillage of contaminated fluids in the laboratory or hospital, Terpstra *et al.* (2007) tested 0.1 N NaOH and 0.1% hypochlorite solution on HAV suspended in blood plasma and dried onto stainless steel discs. Each disinfectant was applied for 10 min. Each treatment could inactivate between 3-4 logs HAV.

3.19 Solar disinfection

Examining the effect of this simple disinfection procedure on HAV in a model system, Heaselgreave and Kilvington (2012) reported a ~ 2.5 log reduction in activity after an irradiance of 550 W/m^2 for 1 h, and a 4 log reduction after 2 h. They concluded that the technique should be useful, but recommended that this be verified using natural sunlight in the regions where solar disinfection is used for disinfection of drinking water supplies.

3.20 Ultraviolet irradiation

Wang *et al.* (1995) reported that UV irradiation with a dose of 736 J m^{-2} could completely inactivate HAV suspended in cell culture medium but that infectivity remained after doses of 552 J m^{-2} or less; as they did not provide numerical data it cannot be determined what log reduction this represented. They found that the detection of HAV RNA mirrored the detection of infectivity, while antigenicity remained unaffected; this indicates that the principle effect of UV at these doses is on the HAV genome. Bhattacharya *et al.* (2004) observed a slight degradative effect of UV on the viral genome by lower UV doses, indicated by the inability to amplify sequences by RT-PCR after irradiation of 2 pfu HAV for 480 mJ/cm^2 and 600 mJ/cm^2 (RNA could be detected from irradiated suspensions containing 20 pfu and 200 pfu).

UV inactivation of HAV can be said to show "one-hit kinetics", according to Nuanualsuan *et al.* (2002), i.e. a single photon can inactivate an infectious virus particle by hitting a critical target following absorption. In their tests on HAV in suspension in PBS, they observed approximate log reductions of 3 and 4 following irradiation by 75 mW s/cm^2 and 125 mW s/cm^2 respectively and calculated that the decimal reduction UV dose for HAV to be 36.5 mW s/cm^2 (although their tabulated results (see Table 3.22) would appear to indicate that this was an underestimate). Chin *et al.* (1995) considered that short-wavelength UV (UVC) is highly effective at eliminating single-stranded nucleic acid viruses such as HAV and demonstrated that a dose of ~ 0.1 J/cm2 could inactivate ~4.5 log virus in blood plasma products in a continuous-low irradiation system. In similar studies, Wang *et al.* (2004) and Bae *et al.* (2009) respectively observed 4 log and > 5 log reductions of HAV using later generation continuous-flow irradiation systems.

Table 3.24 summarises data from studies on the effect of ultraviolet irradiation on HAV.

Table 3.24: Inactivation of HAV by ultraviolet irradiation

Ultraviolet irradiation dose	TFL (sec)	Approximate overall log reduction in infectivity	Reference
6.25 mW s/cm^2	ND	1.2	Nuanualsuan *et al.* (2002)
12.5 mW s/cm^2	ND	1.3	Nuanualsuan *et al.* (2002)
25 mW s/cm^2	ND	2.0	Nuanualsuan *et al.* (2002)
50 mW s/cm^2	ND	2.5	Nuanualsuan *et al.* (2002)
75 mW s/cm^2	ND	2.9	Nuanualsuan *et al.* (2002)
100 mW s/cm^2	ND	3.5	Nuanualsuan *et al.* (2002)
125 mW s/cm^2	ND	4.1	Nuanualsuan *et al.* (2002)
197 µW / cm^2	30	3.5 in 3 min	Meng *et al.* (1987)

TFL: time to reduce first $\log_{10}$
ND: not determined

To try to elucidate how UV exerts an inactivating effect on HAV, Nuanualsuan and Cliver (2003b) exposed virus suspensions to doses of approximately 1.6 - 1.7 mW/cm^2 until no infectivity could be detected (the titre of the virus suspension was not given). They found that inactivated virus could be captured by anti-HAV antibodies, and partially retained the ability

to attach to cell monolayers. Viral RNA could be amplified by from UV-treated viruses, but not after RNase treatment (Nuanualsuwan & Cliver, 2003b). This indicated that UV can partially damage the viral capsid. However, Han *et al.* (1997) found that a dose of 300 mW/cm^2 for 5 min (producing a ~95% inactivation) resulted in a fragmentation of the HAV RNA while antigenicity and capsid polypeptides remained unaltered, and considered therefore that the genome was the attack target of UV.

The effect of UV irradiation on HAV may be potentiated by the addition of porphyrins, which can act as photosensitizers, to the suspending medium (Casteel *et al.*, 2004); this may have use in e.g. field disinfection of water supplies.

Examining the potential for UV light as a disinfection approach for fresh produce, Fino and Kniel (2008) artificially contaminated pieces of Romaine lettuce leaves and portions of green onions with HAV, then treated them with various doses (40, 120, and 240 mW s/cm^2) of UV (see Table 3.25). Reductions of 4 to 5 logs in virus infectivity could be achieved. The effect was not linear, as 40 mW s/cm^2 produced a ~ 4 log reduction, with the other doses achieving only a further 1.5 log reduction at most. UV technology might be useful to control virus contamination, if it could be ensured that the UV light could hit all the surfaces of the foodstuff; otherwise shading effects and surface topography of leafy greens would reduce the effectiveness of the disinfection. Hirneisen and Kniel (2013) compared the effect of UV against HAV on the surface of green onions, and against the virus internalised within the plants. They treated the contaminated vegetables with UV at 240mW s/cm^2. The internalised HAV was much less susceptible to inactivation by UV (0.4 log inactivation of internalised virus against 5.2 log inactivation of virus located on the surface of the green onions). Although the difference appears pronounced, the authors stated that it was not significant; however only three replicates of each test were performed, which may not have allowed an effective statistical comparison to be made.

Table 3.25: Effect of ultraviolet irradation on HAV on vegetables (from Fino and Kniel, 2008)

Vegetable	Ultraviolet irradiation dose (mW s/cm^2)	Approximate overall log decline in infectivity
Green onions	40	4.16
Green onions	120	5.31
Green onions	240	5.58
Lettuce	40	4.29
Lettuce	120	4.45
Lettuce	240	4.62

Berto *et al.* (unpublished) examined the effect of UV treatment on HEV. Virus suspensions (homogenate of HEV-infected pig liver) were exposed to UV by a lamp producing 30 W UVC for 20 min, 30 min and 50 min at 20cm distance, then inoculated into a 3-D cell culture system which was then monitored for evidence of viral replication through detection of viral RNA (see Section 3.9). Viral RNA could be detected in the cultured cells to 60 d post-inoculation (the duration of the experiment) with all treated and untreated suspensions. This finding could indicate that UV is not wholly effective against HEV, although the shading effect of the liver suspension could have been involved, where particulate matter occluded the virus particles and prevented them being exposed to an inactivating dose of UV.

4. Conclusions

Survival of hepatitis A virus

Hepatitis A virus (HAV) is one of the most, if not the most, resistant human enteric virus. Its outstanding physical stability in the environment is mediated by the properties of its capsid. In water at ambient temperature, it can remain infectious for months.

In soil and organic wastes, the persistence data are very variable depending on the type of soil or waste. Dried on fomites, including china, tile, aluminium, paper and cotton clothes, HAV is capable of surviving for several months, depending on temperature and relative humidity. The virus may resist desiccation; this is critical for survival on environmental fomites. Dried on finger pads, HAV may survive for several hours, thus contributing to its person-to-person spread. HAV decay is negligible in frozen berry fruits which supports the vehicular transmission of hepatitis A through berry consumption. HAV may retain its infectivity in leafy greens for the time elapsing from harvest to fork. In bivalve shellfish, the virus can remain infectious from harvest to the consumer.

Survival of hepatitis E virus

It is not known how long hepatitis E virus (HEV) can survive in any environment or food product. Acquisition of the necessary data awaits the development of an effective, easy to perform, and reproducible cell culture system which will allow the determination of HEV infectivity to be readily performed.

The effect of elimination procedures on HAV

Treatment with chlorine at concentrations of at least 10 mg^{-1} for 30 min can achieve a reduction of >5 log of infectious HAV in aqueous suspension, however, rinsing contaminated berry fruits or leafy greens with chlorinated water is much less effective. Chlorine dioxide is also highly efficient in inactivating HAV suspended in water. Water ozonation is also capable to efficiently inactivate HAV. Among commercial products for the disinfection of surfaces, including clothes, the most effective in reducing HAV are those containing sodium hypochlorite. Glutaraldehyde compounds and sodium hydroxide are also highly efficient for HAV inactivation. QACs may be useful for eliminating HAV in liquids, but not on dry surfaces.

Regarding processing of food, commercial shellfish depuration fails to be effective for the elimination of HAV in the edible bivalve molluscs tissues. High hydrostatic pressure seems to be adequate for the inactivation of HAV in bivalve molluscs.

Gamma irradiation and electron beam exposure are only effective for the inactivation of HAV in food matrices at doses above 10 KGy, which is allowed limit for application in food products. Pulsed light and UV irradiation have been reported to be effective for HAV inactivation, although both systems need to overcome the problems associated with matrix composition hindering exposure of virus particles by shadowing.

HAV is relatively resistant to high temperature. Commercial heat-treatment of shellfish, where the internal temperature is raised to 90°C for 90 sec, is effective for elimination of infectious HAV in these products. However there have not been sufficient studies performed using at-risk foods such as berry fruits or their products to precisely determine the effect of heating procedures used in the food industry.

The effect of elimination procedures on HEV

The limited number of studies which have been performed indicate that HEV could remain infectious at temperatures used in some cooking regimes (e.g. 75°C for 5 min). It may also survive refrigeration at 4°C, and freezing at -20°C. Not enough data has been acquired however for a precise determination to be made of the effect of heat.

More research is required in the area of HEV inactivation to improve our understanding of the transmission routes of HEV and also to develop control strategies of HEV contaminations and infections. However, HEV propagation in cell culture has shown to be very difficult and testing of HEV viability *in vivo* requires non-standard laboratory animal experiments. The failure to develop a pragmatic and efficient *in vitro* propagation system for HEV has hampered environmental survival studies. To assess HEV inactivation, most studies currently rely on qRT-PCR with standard curves generated by using serial dilutions of known amounts of RNA controls. Others have used *in vivo* testing to determine infectivity after the inactivation treatment but such methods are not well standardized and relatively expensive. To effectively study viability of different HEV strains *in vitro* the current culture methods in monolayers or three dimensional bioreactor systems will have to be optimized.

Statistical analysis

There is insufficient data on different strains of HAV available to get a statistical conclusion on whether the effect of heat is the same for all strains of the virus.

5. Recommendations for further research work

- More studies examining the effects of heating regimes used in the food industry on HAV in at-risk foods such as berry fruits and their products (e.g. fruit puree) should be performed, to provide a precise determination of the virological safety of these procedures. These studies should model all features of the industrial process as closely as possible.

- Experiments conducted with other strains than vaccine HM-175 are needed.

- A robust cell culture system for HEV is urgently required; the studies on this virus recommended below cannot be easily performed until such a system is identified.

- More studies are required to determine the effect of heat on HEV, in free suspension and in food products derived from pork meat or pig offal.

- The survival of HEV on fomites should be determined, especially those which can be found in food production facilities. This will give a better understanding of the risk of cross-contamination in food supply chain premises.

- The effect of disinfectants on HEV needs to be fully elucidated.

- Further evaluation of the effect of handwashing on HAV would be beneficial to complement the limited number of studies so far performed on this. For HEV no such studies have been performed, but should be done when appropriate methods become available.

- Freeze-thawing may have a negative effect on HEV (van der Poel, unpublished observations); a study should be performed to determine this.

- Experiments comparing the inactivation of HAV, HEV, and highly resistant bacteriophages such as PhiX174 or PRD1 should be performed to assess the comparative effects of various parameters, and to obtain relatively easier to use indicators of the efficacy of HAV/HEV elimination regimes.

More surveillance is required to generate data on the prevalence and levels of HAV and HEV contamination naturally in both UK and imported foods (such as pork products and fresh produce) and food supply chain environments. This will help to establish the extent of the issue and improve our understanding of which type and level of disinfection procedure would be appropriate in actual practice.

Annex 1: Statistical analysis of the TFL data for HAV

The data were given in a spreadsheet initially provided by FERA and adapted in a new table for statistical analysis by the University of Lorraine. In this new table the following information were included: Taxonomy (strain), detection method (cell culture or RT-PCR), kind of matrices (complex or simple), pH, temperature, TFL, and other additional information (Total Log decrease, number of experimentation days, initial viral load, reference).

Simple and complex matrices were defined as previously given in the work by Bertrand *et al.* (2012) Three types of matrices were considered as simple: (i) synthetic and sterile media without suspended matter (e.g. phosphate-buffered saline, cell culture medium, artificial seawater, artificial groundwater), (ii) drinking water (dechlorinated tap water, bottled water, filtered water, sterilized water, distilled or deionized water), and (iii) groundwater (filtered or non-filtered groundwater or well water). The seven other types of matrices did not correspond to the above criteria and were considered as complex: (i) freshwater (water from river, stream...), (ii) natural seawater (seawater an estuarine water), (iii) sewage (primary and secondary sewage), (iv) soil, (v) dairy product (e.g. milk, skim milk, cream...), (vi) food (e.g. vegetables, fruits, mussels, meat...) and (vii) urine (reuse in agricultural purpose). TFL represents the time to reduce the first log of initial viral load with no additional hypothesis than log-linearity of the decrease.

To achieve the statistical analysis, we considered a total of 155 TFL values for HAV with 144 (93%) data on infectivity and 11 (7%) data on genome degradation. T90 values have also been included. However, all values are termed as TFL for purposes of uniformity.

Here, it must be emphasized that the experimentations (i.e. data/papers), which have been considered in the present study, were not submitted to a random procedure of selection for their inclusion. This may have undesirable consequences, in the sense that it may introduce some kind of bias, which therefore requires the results of the statistical analysis to be examined with caution. Moreover, data in this area are quite sparse with only a few papers looking at certain variables (e.g. pH, strain, etc.). As a consequence of this paucity, it has been decided to conduct the statistical analysis without these variables.

Eventually, log-linear regression analysis was performed on the TFL data and the following explanatory variables: temperature, detection method and matrix, including all the possible interactions among these variables. The computation of a reduced model to fit the experimental data was carried out by following a backward step-wise procedure. All the calculations were performed by using the statistical package R (version 2.12.2; Bell Laboratories, Lucent Technologies, http://www.r-project.org). Only the temperature (Fisher test; P-value <10-5), the type of matrix (Fisher test; P-value = 0.019) and the interaction between temperature and matrix (Fisher test; P-value = 0.032) showed a significant effect.

As for the method of detection (cell culture or RT-PCR), it showed no significant difference. Note that such an outcome does not necessarily mean that there is none. Indeed, this result may be attributed to a lack of power in the testing procedure since the table includes only 11 data for RT-PCR and 144 for cell culture. By reviewing all the published data on viral inactivation and genome degradation for enteric viruses in food and water, the log10TFL values obtained for genome was similar or higher than those observed by cell culture (Bertrand *et al*., 2012). The strain could not be included in the statistical analysis since vaccine strain HM-175 represents 85% of the 155 data. Similarly, the pH has been mentioned in a few studies, only. In this regard, only two studies (Deboosere *et al*., 2004; Scholz *et al*., 1989) providing a total of 17 data were performed at acidic pH (i.e., between 3.4 and 4.3 for the first study and at pH=1 for the second one). Moreover, the pH was around 7 for 14 data and was not mentioned for the 124 remaining data.

The type of matrix has a significant influence on virus survival. This effect is either direct or expressed in terms of an interaction with temperature, which means in other words that the temperature does not have the same effect according to whether the matrix is simple or complex. On one hand, at low temperature, near 0°C, survival is about 8.3 times higher in a simple matrix compared to a complex one (TFL simple = 912 days vs TFL complex = 110 days). On the other hand, at high temperature, near 80°C, survival is about 1.7 times higher in a complex matrix compared to simple one (TFL complex = 64 s vs TFL simple = 38 s). The estimated equilibrium (i.e. the point where the temperature effect may be considered similar in both complex and simple matrices) is situated near 64°C.

For simple (resp. complex) matrices the reduced fitted model is expressed as:

$$\log_{10}(TFL) = 2.96 - 0.0789\,T \text{ (simple matrix)} \qquad \text{(Eq. 1)}$$

$$\log_{10}(TFL) = 2.04 - 0.0646\,T \text{ (complex matrix)} \qquad \text{(Eq. 2)}$$

where T is the temperature (°C) and TFL the time to reduce first log (days)

Note that $r^2 = 0.8656$, which means that these models fit the data quite satisfactorily.

Finally, temperature has unsurprisingly the highest influence on virus survival. In this regard, it is interesting to note that if the type of matrix is removed from the previous model, the loss of explained variability is less than 1% ($r^2 = 0.8567$), which means that Eqs. 1 and 2 could simplify to the following expression, i.e. a function of the temperature, only:

$$\log_{10} TFL = 2.20 - 0.0662\,T \qquad \text{(Eq. 3)}$$

where T and TFL are the temperature (°C) and time to first log (days), respectively.

The simplified reduced fitted model, as expressed in Eq.3, including both a confidence interval for the mean and a prediction zone calculated at the 95% probability level, are illustrated in Figure 1. This final model can be used to assess the required temperature, that is, the temperature which will reduce HAV by any pre-specified given amount.

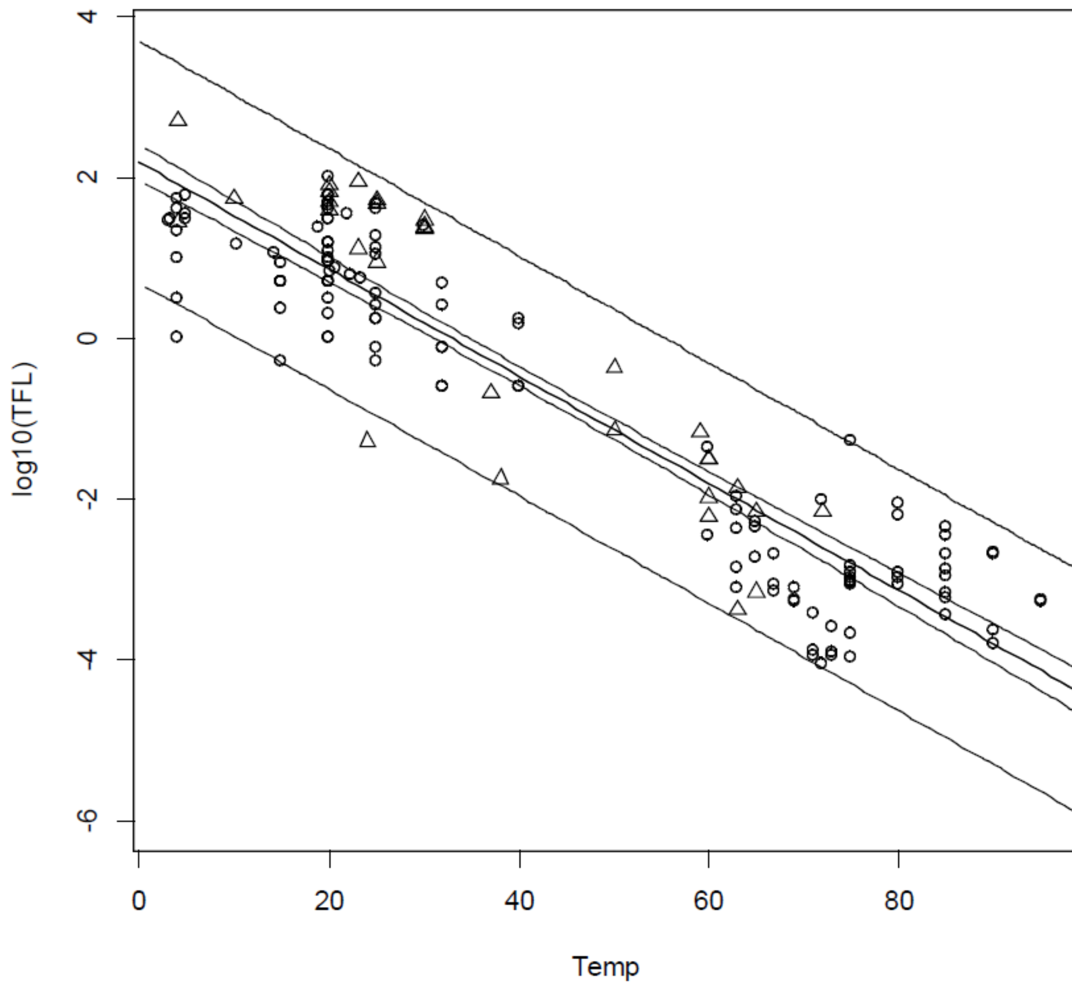

Figure 1 Time to first log10 reduction (TFL; days) as a function of the temperature (Temp: °C) by using a linear regression model. The figure includes confidence lines for the mean and predictive band, both at 95%. The fitted regression line has been obtained by means of Eq.3, which is given in the main text of Appendix 1. Observed data points are indicated for complex matrices (circles) and simple matrices (triangles).

Annex 2: How the review was performed

The project commenced with an inception teleconference involving all partners, at which a list of key words to be used in the literature search was discussed. Project partners completed the refinement of the terms, then a database search was performed.

The databases searched on the OVID host were:

- Medline
- CAB Abstracts
- Biosis
- Food Science and Technology Abstracts

The search terms used included: “hepatitis A virus” “hepatitis E virus”, “survival”, “stability” “persistence”, “food”, “surfaces”, “elimination”, "fruit", "surface decontamination", “temperature”, ”heat”, “pH”, “water activity” “UV”, “inactivation”, “pork”, “meat” and “swine”.

The initial search identified 487 publications. The references and abstracts were collated in an EndNote file.

An initial screen of the database was then performed; 132 abstracts were immediately excluded as their publications described work which was not relevant to the survival and elimination of HAV (e.g. the effect of heat on replication of the virus, effect of antivirals, etc). The database was sent to the project partners, who confirmed the selection of the references.

Drafting of the review had commenced in the interim. Scanning publications on murine norovirus or astrovirus, no reference to its use as a surrogate for HAV could be found. Project partners subsequently agreed that these viruses have not been used as a model for HAV and need not be included in the review, and that there were furthermore no useful publications on other viruses as surrogates for HAV or HEV. The relevant references were then excluded. One review of HAV inactivation written wholly in German was also excluded. Duplicated

references were also removed from the database. Finally, 130 publications from the initial search were considered suitable for inclusion in the review.

All 130 publications were reviewed at FERA, then the draft review was sent to the other project partners for comment and input. Subsequently the draft was sent to the Food Standards Agency for comment.

All FSA comments were responded to in the penultimate draft. This was presented to the FSA at a meeting, then the Report was finalised.

The statistical analysis was conducted as described in Annex 1.

In the course of the review, 59 further publications were identified, either general references for inclusion within the Introduction, or specific references on hepatitis virus survival or elimination which the initial search had overlooked or which had subsequently been published. Finally, 189 publications were included in the review.

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