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Thermal Inactivation Model for Hepatitis E Virus

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Contents

Glossary

- **Genotype:** Sub-classification of HEV, based on nucleotide differences in the genome
- **IPC:** internal positive control assay for PCR
- **HE:** Hepatitis E; a disease of the liver
- **HEV:** Hepatitis E Virus; causative agent of hepatitis E
- **ICC-PCR:** A method for monitoring virus infectivity based on detection of increasing amount or numbers of detectable genome material in infected cultured cells
- **IFA:** Immunofluorescence assay - method of visualising a cell or microorganism based on binding of fluorescently labelled antibodies
- **PBS:** phosphate-buffered saline solution
- **PCR:** Polymerase Chain Reaction; a NAA technique used as a detection assay. Can be performed in “conventional” or real-time” format
- **RNase:** an enzyme which degrades RNA
- **qRTPCR:** RTPCR where quantification has been performed using RNA standards the assay is thus fully actually quantitative
- **RTPCR:** Reverse Transcription PCR; RT transcribes RNA into complementary cDNA prior to PCR amplification
- **RTqPCR:** RTPCR where quantification has been performed using DNA standards, and therefore, only the PCR is actually quantitative

1. Lay Summary

Hepatitis E is an infection of the liver caused by the hepatitis E virus (HEV). HEV infection usually produces a mild disease, hepatitis E. However, disease symptoms can vary from no apparent symptoms to liver failure. The World Health Organisation estimates that, annually, there are 20 million hepatitis E infections, over 3 million acute cases of hepatitis E, and over 57,000 hepatitis E-related deaths. There are 4 main types of the virus that currently cause concern in humans. These are known as genotypes of which 1 and 2 are found mainly in developing countries, and 3 and 4 which are linked to infections in Europe, America and Asia. Hepatitis E genotypes 1 and 2 are transmitted mainly via the faecal/oral route through ingestion of contaminated drinking water. This mainly occurs in areas of poor sanitation. Genotypes 1 and 2 infections are manly restricted to humans but 3 and 4 can be identified in numerous other animal species including pigs.

Transmission routes of HEV genotypes 3 and 4 have been identified to include the ingestion of food products derived from infected animals and shellfish and via transfusion of infected blood products.

Hepatitis E infection is an emerging issue in the UK and there is evidence to suggest an association of this virus with undercooked pork and pork products. Currently, there is no standardized method for evaluating the stability of HEV that may be present in food during cooking processes. There is also lack of a suitable method that can detect only infectious HEV.

This has raised concerns as makes it extremely difficult to determine how cooking practices might affect the potential for exposure via pork products. There are also uncertainties due to the fact that, in the UK, detection of HEV in food has not been directly related to infections, nor has the detected virus been shown to be infectious. Developing a method to assess this has been technically difficult.

The published evidence suggests that heating does have an impact on the stability of HEV, but there is uncertainty on the survival of the virus over a range of cooking temperatures and in different foods therefore further work is needed in this area.

The aim of this project was to address this by generating a model that can be used to predict the amount of virus which will be degraded over a specific time and with cooking at a certain temperature. A user-friendly model and a guide to its use has

been generated which is flexible and can be updated at any time to reflect new information. This model will not only inform risk assessments, but will also provide an indication if cooking is sufficient to inactivate the virus in foods. Currently the model is limited by the methods available to generate data to use it, i.e., we cannot currently confirm if the virus present in a foodstuff is infectious, therefore the recommendation is to thoroughly cook food as advised by the FSA.

2. Executive Summary

The main objective from the FSA's Strategic Plan for 2015-2020 is to protect consumers from unacceptable risks which may arise in connection with the consumption of food (including risks caused by the way in which it is produced or supplied) and otherwise to protect the interest of consumers in relation to food. This includes reducing foodborne disease to ensure 'food is safe'. This research falls under the FSA's Foodborne Disease Research Programme which aims to provide data/evidence to address considerable gaps in our understanding of microbiological hazards including the foodborne viruses.

Hepatitis E is the most frequent cause of enterically transmitted hepatitis worldwide. Although HEV infection has traditionally been associated with poor sanitation conditions in developing countries, steadily increasing numbers of cases which are not associated with travel to endemic countries are being reported in developed countries, including continental Europe and the UK. The number of confirmed non-travel related hepatitis E cases in the UK has increased particularly since 2010; similar trends have also been observed in several European countries such as Germany, Netherlands, Spain and the Czech Republic.

Human cases of hepatitis E are caused by strains within species A, which comprises eight genotypes. Two of these (genotypes (gt) 1 and 2) only infect humans via the fecal-oral route. Gt 3 and 4 are endemic in animal species such as pigs and wild boar; these strains cause zoonotic infections in humans. In addition, Gt 3 and 4 are capable of infecting other species including rabbits, rats, goats, sheep and deer. HEV gt 1 and 2 usually cause a brief, self-limiting hepatitis in young adults that is clinically indistinguishable from other causes of acute viral hepatitis and does not

manifest as chronic infection. However, the mortality rate in pregnant women is approximately 25%. Acute HEV gt 3 infection is clinically silent in the vast majority of patients. Only a minority (probably less than 5%) develop symptoms of acute hepatitis with elevated liver enzymes, jaundice and non-specific symptoms such as fatigue, itching and nausea, however, progression to chronicity can occur in immune-suppressed individuals and other at risk populations.

An increasing number of animals have been found to carry HEV, most of which have little relevance to human infection. Animals carrying HEV that have implications for human health are more limited and pigs, wild boar and deer are acknowledged as the main reservoirs of foodborne HEV. A small study carried out by the Animal and Plant Health Agency (APHA) found HEV RNA to be present in the UK pork production chain with 10% of retail pork sausages testing positive by RT-PCR, although five of the six HEV positive sausages were from a single batch. It is also not known whether the virus detected in the samples was infectious (and likely to lead to illness) and the study was not representative of the UK market coverage. These findings have generated significant interest across Government, industry (particularly the pig sector) and in the media. However, these findings are not unusual as there are many published reports of HEV contaminated pork from other countries. There has been some epidemiological evidence of an association between genotype 3 (clade II) HEV confirmed cases in England and Wales and consumption of uncooked pork and pork products, although it was acknowledged that the study had limitations. This study also indicated that the HEV genotype present in human infections was not the same as that identified in pigs in the UK. Regardless, these studies generated significant media attention and scrutiny on whether the cooking advice and practical application of standards for temperature time combinations are likely to be sufficient to inactivate the virus in pork meat and pork products.

In February 2016, the FSA and the European Food Safety Authority (EFSA) jointly organised a foodborne viruses research workshop which also considered HEV. More recently, EFSA published in 2017 a scientific opinion on the public health risks associated with hepatitis E virus (HEV) as a foodborne pathogen. These reports and the virus workshop proceedings concluded that there remain significant evidence gaps concerning HEV and identified many HEV research priorities including an assay

to assess HEV infectivity in food and further research on the thermal stability of HEV in foods.

The impact of cooking on HEV inactivation in pork meat and pork products is clearly an area where further work is needed to inform our risk assessment but also the advice the FSA provides to consumers and caterers.

Published evidence suggests that heating does have an impact on the stability of HEV, but there is uncertainty on the survival of the virus at relevant temperatures and in different food matrices. Additional variables arise in that HEV exists in a number of states with respect to the viral envelope. What this essentially means is that the virus can exist with or without an envelope, or with a partial one; indeed, all could be present at any one time in a product. However, this has not been fully studied so the extent to which type predominates in a given product needs to be investigated.

Ideally, we would want to evaluate the effect that heat has on infectivity; however, the lack of a robust HEV infectivity assay which would allow us to compare infectious ability of the virus prior to and following intervention steps is currently not available. Developing a HEV infectivity assay at this time has proven technically difficult and, although it is not impossible to culture the virus, the current assays are unlikely to provide a robust system for evaluation.

To summarise, there is currently no complete and consistent experimental data for HEV thermal stability in pork / pork products; however, there are publications available which can be evaluated in order to select the most suitable for downstream applications and inform a model to predict thermal stability.

This study aimed to collate thermal stability data on HEV in the published literature and from other sources and to use this data to develop a model to describe the relationship between time and temperature and its impact on HEV. Account was also to be taken of data relating to different types of matrices as this is likely to influence the time/temperature relationships found. The second part was to apply the developed model to data on HEV titres which are typically found in different matrices including foods (including pork meat and pork products), water and plasma (blood) to predict the impact of different time temperature treatments on HEV titres.

One hundred and ninety-six potentially suitable references were identified by the literature search. The publications acquired had a second screen performed, which selected only those studies which contained usable data, for example quantitative information on infectivity or genome copy reduction. Temperature, source of HEV and duration of studies were also taken into account for generation of the data. Finally, 11 publications were accepted for data compilation based on the content as follows:

I. Temperature, Time, Log reduction
II. Matrix
III. Other Information
IV. Reference

To advise the model, a full literature review was carried out on the manuscripts and was used to assess the relevance and suitability of the data for the application. It was anticipated that there may be gaps in the literature due to the varying sources used to obtain HEV. Indeed, the data did not provide a full dataset and sufficient data points at the relevant time/ temperature parameters required. There was also no comparison of virus from different sources. Overall, the outcomes were;

1. From the limited literature data available, it is not possible to confirm an optimal time/temperature combination to assess the thermal degradation of HEV
2. Data in the literature is insufficient to create a consistent and robust model to assess the thermal stability of HEV over time at temperatures relevant to food and other matrices.
3. Further data using a consistent method of detection and varied viral sources for input over a relevant time/temperature setting is required.

As the available data in the literature at the higher end of temperature (70-95^{o}C) and those relevant to cooking were found to be much less than that described at the lower temperatures, this indicated a gap in the information and the ability to assess the real impact of thermal inactivation on HEV. It was determined that further data using a consistent method of detection and varied viral sources for input over a relevant time/temperature setting was required. As such, the next step sought to

complete the gap by providing a time series at cooking relevant temperatures using different sources of HEV.

To address this, a series of experiments were carried out using three viral sources, varying input quantity and utilising RT-PCR to detect the reduction in the amount of nucleic acid present after thermal treatment at relevant temperatures over a specified time period. No complex matrix background was used in the first instance. The caveat of this data is that we were unable to correlate the reduction in HEV by PCR directly with a reduction in infectivity due to lack of a suitable infectivity assay as mentioned previously. It was anticipated that the model should include a series of temperature-time profiles typically used by consumers and industry in relation to the cooking of pork and pork products. Given that the minimum advice is to cook food to an internal temperature of 70^{o}C for 2 minutes and the lack of information provided in the literature, temperatures applied were 70, 75, 80, 85, 90, 95 $^{\circ}$C with room temperature (RT 22-25^{o}C) and 100°C as controls. The time series ranged from 0-20 minutes.

Our data supported the observation that HEV is more thermal resistant than expected and that cooking at 70-75°C may not be sufficient to fully eliminate the virus. Overall, our data suggests that nucleic acid is detectable over a 20minute time period up to 95^{o}C, however, if that virus remains infectious is a question still to be answered. Utilising RT-PCR rather than a culture assay does create a caveat that although we can detect the virus nucleic acid, it may indeed be rendered non-infectious. Until we can replicate this in an infectivity assay which will allow the consistent assessment of any viral source, assumptions must be made and we can only predict the required thermal treatment and time to reduce the detection of virus. It was found that viral input may affect the reduction in detection of nucleic acid at higher temperatures; in addition, the viral source also appeared to influence the reduction in nucleic acid detection and it was agreed that viral source should be considered independently in the model as each source exhibited a different thermal profile.

This data provided the first consistent time series over temperatures from 70-95°C, encompassing those that are relevant to cooking, and compared differing viral sources to carry over to the model.

Mathematical models predicting the thermal inactivation of food-borne pathogens assist in developing adequate thermal processes. The generation of precise thermal process data and the establishment of proper thermal processes for inactivating HEV in food is important both for consumers and for industry.

Using the Weibull model; which allows us to estimate the probability of survival of the virus as a function of temperature and time, data from the literature and the experimental work were analysed to assess the performance of the model. The Weibull model assumes that the survival curve is a cumulative distribution of lethal effects where there is a constant frequency parameter and a constant shape parameter; both which are different for each temperature. As we have already indicated, the studies in the literature were not comparable and the literature data was unable to be fitted to the model due to the complexity and inconsistencies in methods for extraction and detection used.

As such, to develop a predictive model of thermal inactivation we were required to complete a time series dataset using consistent methods. Experimental data was generated at 70, 75, 80, 85, 90, 95 $^{\circ}$C for all three viral sources and was demonstrated to produce consistent values for parameters A and β , with excellent convergence and visually the fitted model curves were seen to fit the data to a high degree of agreement.

With respect to convergence, the Gelman-Rubin statistic, the “Rhat” value (a convergence diagnostic statistic) aims to quantify the convergence of the simulation that generated the fitted model results. If we have Rhat close to 1, this indicates the model has converged, whilst Rhat greater than 1.05 indicates we do not have good convergence. For all parameter estimates in the model we demonstrated high convergence with Rhat values close to, or equal to 1 in every single case. In Bayesian inference, the Effective Sample Size (ESS) of a parameter sampled from an MCMC is the number of effectively independent draws from the posterior distribution that the Markov chain is equivalent to. While 30 is a minimum, obviously we can generate many more. This should typically correspond to stability of the convergence. Again, we clearly demonstrated that we have good ESS for all the results, n.b: the model uses three “Markov chains”, in its simulations. These three chains simultaneously estimate the two model parameters, at each iteration. So

“convergence” refers to all three chains converging upon approximately the same value.

Overall, it was successfully demonstrated that the Weibull model is both a good fit to the available experimental data, and thus a suitable model for describing the thermal reduction of HEV within foodstuffs. This is clear from the convergence results shown. In addition, the model/application can easily be adapted to new data as this becomes available. This is a major advantage of developing the model into a user-upgradable application. As such, is anticipated that the model predictions will further increase in accuracy, as the dataset it uses develops and expands to include data from new studies. Given this flexibility, theoretically, users could replace the data contained in the apps .csv file with data for the thermal reduction of other similar viruses and, in theory, the model should be able to provide a reasonable fit, with little to no modification. Finally, as per the aim, to develop an interactive model, it is possible to predict the time temperature combination to reduce the detectable nucleic acid and therefore intact viral particles, by the log reduction required. The model has its limitations. The estimates it provides are only as good as the available data that they are based upon. Thus, the more data available, the better the model predictions will become. Similarly, if the quality of the data input into the model is not great, the model predictions will mirror this. The user guide provides full details on how the model may be used to predict HEV elimination in a range of food matrices under various time / temperature profiles.

Currently, the model outputs two unique parameters; A and β for each temperature that we have data on. Future steps could include evolving the model equation further, in order to incorporate temperature into the model equation itself. This would allow all data points, no matter what the temperature they correspond to, to be used to estimate model parameters for a particular source. For example, we could select all data from one source regardless of temperature treatment and the model would output the parameters for that one HEV source. Obtaining more experimental data, that can be added to the log-reduction dataset, will be extremely beneficial in further increasing the quality of the model predictions. It should also be noted that if the predicted time for cooking is greater than 20 minutes then more data will be required to predict reduction accurately.

Since we are currently unable to determine whether the HEV detected is infectious (viable) or not, the model can be adapted in the future when advancements have been made in measuring HEV infectivity in food.

Finally, verification and validation of the model was carried out to ensure that the model was fit-for-purpose and could establish a prediction of thermal reduction which was a reflection of the actual experimental data produced. Firstly, the model, user guide and input database were submitted to the FSA, prior to external peer review, for quality assurance purposes. Three FSA microbiologists verified the approach and the user guide, and were satisfied that the model was fit-for-purpose, user-friendly and conformed to the original specification and that results were reproducible. Secondly, predictions were made using specific temperature-time combinations which were subsequently tested experimentally with the expectation that the actual outcome should fall within the confidence intervals (2.5 and 97.5%) of the model.

The data used to validate the model all fell within the predicted confidence intervals during this quality assurance, however, as noted the model does not account for viral input. The model was confirmed to be fit for purpose and the agreement between the model's predicted log change values and the data from the validation experiments further strengthens the evidence that the Weibull model is a good choice for this application.

As many more parameters play a role in determining viral inactivation in meat and meat products, the results of predictive microbiology models are often designed to be very conservative. The situation in real life is often safer than the results the model suggests. Validation of the results or, in other words, the comparison of calculated with actual results is a major task. Some variables cannot be factored into mathematical models, such as variations in storage temperature and in general, the effects of a maximum of four environmental factors can be incorporated at any one time into most models. Predictive microbiology therefore has its limitations. However, these conservative estimates are equally likely to be the result of the limited available experimental data, used to inform the parameters that describe the thermal degradation. Thus, the estimates may become less conservative, and uncertainty reduced once more data is available to inform the model. The current model uses time as a parameter at a specific temperature and was designed to be the best fit

given the standard deviations; it was not designed to be conservative but as accurate as the data permitted.

Currently there is no validation data available on any HEV model to compare our outcome to but it is proposed to update the model as part of future work and create predictions prior to comparison with the actual experimental outcome. The next step is to have this model validated by a third party. Indeed, the addition of the thermal stability utilising virus derived from porcine liver (FSA project FS307033: Optimising Extraction and Detection of Hep E Virus from Pork Meat and Products) will also strengthen the model and its predictive capacity.

Further research is clearly required to investigate HEV inactivation within food matrices in a consistent manner as we have done here. This may require a more efficient cell culturing method and an assessment of different foods, cooking methods and HEV strains. Importantly, it will be very useful to correlate the PCR detection with infectivity as this has not yet been done.

3. Introduction

HEV infection occurs throughout the world but predominantly is associated with waterborne outbreaks in Asia and Africa (Par and Karna, 2020). There are seven recognised genotypes of HEV, HEV 1–7, but only a single serotype. HEV 1 and 2 are known to infect humans only, HEV 3 and 4 to infect humans and animals, while HEV 5–7 have animals as their host, albeit camelid HEV 7 has been identified in a human patient (Lee et al, 2016).

HEV genotypes 1 and 2 cause outbreaks, mostly in hyperendemic regions of the world. Mostly young adults are the target population, and the disease is particularly troublesome for pregnant women who develop a very severe form of the disease (Kamar et al., 2017). Genotype 1 causes outbreaks in Asia (south, southeast, and central) and North Africa while genotype 2 is usually located in Mexico and western parts of Africa (Goel et al, 2016). Using epidemiological tools and mathematical models, it has been estimated that HEV 1 and HEV 2 cause together approximately 20.1 million (95% credible interval 2.8–37.0 million) cases per year in Asia and Africa,

with acute hepatitis E accounting for 3.4 million cases (Hakim et al, 2017; Rein et al., 2012). HEV infection is also estimated to cause 70,000 deaths from acute liver failure and ~ 3000 stillbirths per annum (https://www.who.int/news-room/fact-sheets/detail/hepatitis-e). In the developed parts of the world, such as Europe, the USA, and some high-income areas in Asia, the infection is predominantly caused by HEV 3. HEV 4 usually causes infection in China and Japan (Par and Karna, 2020).

There are two different epidemiological patterns of HEV. Outbreaks from genotype 1 and 2 occur through the faecal-oral route due to faecal contamination of drinking water (Balayan et al, 1983). HEV 3 and HEV 4 cause only sporadic cases in less endemic parts of the world, mostly from direct or indirect animal contacts, and consumption of undercooked animal products such as pork, wild boar, deer and shellfish (Tei et al. 2003; Colson et al, 2010; Guillois et al. 2015; Riveiro-Barciela et al, 2015; Rivero-Juarez et al. 2017).

HEV genotypes 3 and 4 are zoonotic and can be transmitted via consumption of contaminated meat from a variety of animal species, predominantly domestic pigs, in which the virus is endemic. HEV RNA has been detected in pork products (liver, sausages) sold at retail, in several countries including the United Kingdom (Berto et al, 2012; Di Bartolo et al., 2012). HEV RNA detection rates ranging from 4% to 6.5% have been reported for commercially available pig liver (Bouwknegt et al., 2007; Wenzel et al., 2011). In pig liver sausages and raw sausages from Germany, HEV detection rates between 20% and 22% have been described (Szabo et al., 2015). Especially high detection rates (>57%) have been reported for a local liver sausage from France called “Figatelli” (Martin-Latil et al., 2014). Several reports have implicated the consumption of specific foodstuffs in the UK as being correlated with HEV infection, but to date, no food has been directly associated with infection (Said et al., 2009; Said et al., 2014; Said et al, 2017). Evidence has been questionnaire-based or circumstantial due to the lack of direct association of consumption and lack of testing for infectivity in the specific foodstuff. The reason for this is that detection of HEV is performed mainly by reverse-transcription polymerase chain reaction (RT-PCR) which detects the genomic RNA of the virus but gives no indication of its infectivity, and a reliable infectivity assay is currently lacking (Cook et al., 2017). Therefore, it is difficult to precisely determine the risk from consumption of HEV-contaminated pork products as the infectious dose cannot be determined.

Concomitantly, the lack of a reliable infectivity assay has hampered study of elimination procedures such as heat or chemical disinfection. Indeed, detection levels in food have been perceived as 'low' and the specific content of intact infectious virus is unknown.

The main concern associated with consumption, and based on the risk mainly being due to undercooked or raw food products, has led to the investigation of thermal inactivation on HEV. Some data suggest that 1-20% of the HEV virus can remain infectious after 15 min at 60˚C, indicating that a portion of the virus could remain infectious at temperatures used in some cooking regimes, albeit this was observed for genotype 1 and 2 (Emerson et al., 2005). Others have inferred that inactivation by heating at 71˚C for at for 20 minutes is required as assessed by experimental inoculation of pigs (Barnaud et al., 2012; reviewed in Cook and van der Poel, 2015). This is not the natural route of transmission. The study by Schielke et al, (2011) utilised a molecular approach assessing thermal inactivation in a liver suspension; according to the data, nucleic acid was still detectable at 56°C and 60°C after 1 hour and 95°C after 1 minute.

The main problem is that higher temperatures and long periods of cooking are unsuitable to allow certain food products to reach the recommended internal temperature and will affect the product quality. Johne et al, (2016), reported the first predictive model, based on a small sample size and cell culture adapted virus, for temperatures of 4˚C, room temperature, 37˚C and 70˚C (Johne et al.; 2016). Albeit a limited dataset, it would suggest that for delicatessen products potentially containing raw pork meat, the current prediction is that HEV would be stable for a period of >56 days at 4˚C (Johne et al.; 2016). This model is helpful for the lower temperatures presented which would represent storage, but the data only applies to liquid or transparent fluids (lacking the complicated content of a food matrix) and utilises a cell culture adapted virus that may not be representative of the wild type virus. There is also a model for 70°C but again, this is based on the same data and virus presented in the manuscript does not meet all the relevant factors that need to be addressed to present a suitable thermal inactivation model.

4. Project Research Question and Objectives

The reduction of foodborne disease has been an FSA priority since its inception in 2000 and foodborne disease incidence is generally considered to be preventable with the application of good personal hygiene and food safety practices. Cooking is recognised as a point in the food chain where risks to consumers can be minimised by thorough cooking according to the guidelines on products; however, for HEV, we do not know fully what cooking practices, both time and temperature, are sufficient.

The lack of extensive data on the effect of inactivation procedures on HEV constitutes a significant gap in our current knowledge (van der Poel et al., 2018); however, since it is considered that development of an infectivity assay is currently technically difficult (Cook et al, 2017), another approach to underpin an estimation of the effect of heat on HEV has to be envisaged.

The main aim of the project was to develop a model which will efficiently predict thermal inactivation for hepatitis E virus (HEV). There is currently no standardized method for evaluating thermal stability of HEV, and as mentioned above, there is a current lack of any validated infectivity assay. This has raised much concern in the pork meat industry for a number of years as it is extremely difficult to extrapolate the risk from pork products in relation to cooking practices.

This report and the model it describes fulfills the FSA's requirement specified for the research contract FS301062, by constructing a thermal inactivation model for HEV. Elimination signifies the removal of viral infectivity (reduction in genome copy will also be included, as this naturally corresponds to reduction in infectivity). The project comprises 2 phases: 1) acquisition of elimination data, and 2) construction of a mathematical model, which will predict elimination by temperature / time / matrix characteristics.

The objectives of the project were as follows:

1. By means of literature search and data collection, to identify literature containing information that can be utilised to build a suitable model of thermal inactivation of HEV. Surrogate data using models of HEV were also to be included in this search however not necessarily to have input to the model.

2. To perform relevant experimental work to support the literature findings and add data perceived to be missing to the model.
3. To prepare a predictive model in concordance with the FSA requirements and to prepare a user-friendly guide to model thermal inactivation of HEV from different sources.

5. Literature Review and Data Gathering

In order to create a database of information relevant to the construction of a thermal inactivation model; a literature search was performed utilising specific inclusion and exclusion criteria. A review article published six years ago (Cook and van der Poel, 2015) concluded that at that time, there was insufficient data available to calculate d- or z-values for the effect of heat on HEV. However, a preliminary literature search performed for this proposal (as requested in the FSA Specification – Appendix 1) identified 19 published studies which contain data on HEV inactivation by heat, 8 of which were published after 2015. Although none of these studies were performed using pork products, some, for example, Johne et al, (2016) and Imagawa et al, (2018), were structured to mimic the conditions encountered in cooking. As mentioned above, Johne et al, (2016) produced a predictive model based on their data, which is available online in Research Gate (http://dx.doi.org/10.13140/RG.2.1.1555.3044 and http://dx.doi.org/10.13140/RG.2.1.4701.0320). Other studies, for example Farcet et al. (2016) involved examination of the effect of heating on HEV in blood plasma; which could be considered to be relevant for food that has serum content. Three publications (Belda et al., 2014; Emmoth et al., 2017; Jones et al., 2015) were also found that described heat inactivation studies on viruses (feline calicivirus, murine norovirus, various bacteriophages and porcine teschovirus) explicitly used as models for HEV as they are all RNA viruses of similar size to HEV. These data were excluded from the final model as we wanted to focus explicitly on HEV but it is possible to add this if required at any point.

5.1 Materials and Methods

Academic and grey literature was searched using search terms agreed with the FSA as indicated below. The sourced literature was reviewed against inclusion/exclusion criteria.

5.1.1 Literature review search

Several databases containing scientific literature were searched as indicated below.

Food Science and Technology Abstracts (1969- to 2019)

FSTA® is a comprehensive source of worldwide information on food science, food technology and food-related human nutrition. FSTA covers literature on basic sciences relevant to food (biochemistry, microbiology, toxicology, etc.) as well as biotechnology, food safety, food processing, food products, patents, economics and legislation. FSTA is the most prestigious and comprehensive food database and is of interest to everyone working in the food sector, for example, at universities or colleges, in the food industry, at food research institutes, or in government departments.

Approximately 4,600 sources, including 4,500 journals, are indexed and abstracted for FSTA®. The sources originate from as many as 90 different countries and more than 40 languages are covered

Searches were conducted using the TOPIC field which covers:

- Title
- Abstract
- Descriptors (thesaurus used to identify appropriate terms (capitals below))
- Foreign Title
- Headings
- Keywords
- Commercial Names

Search terms used were:

Virus: (HEPATITIS E VIRUS OR HEV OR Hepatitis E) OR (Surrogates as agreed at initial meeting: cutthroat trout HEV OR rabbit HEV OR ferret HEV OR porcine teschovirus)

Action: deactivat* OR inactivat* OR reduc* OR death OR capsid integrity OR survival* OR viable OR viability OR eliminat*

Process: (thermal* OR heat* OR cook* OR ASEPTIC PROCESSING OR COOK CHILL PROCESSING OR TEMPERATURE OR STABILITY OR MICROWAVE COOKING OR STEAMING OR BAKING OR BOILING OR BRAISING OR FRYING OR GRILLING OR ROASTING OR SAUTEING OR SIMMERING OR SOUS VIDE OR STEAMING OR STEWING OR TOASTING

Matrix/Product listing: (pork OR pig meat OR boar OR swine OR deer OR game OR rabbit or plasma OR water* OR GROUNDWATER OR BLOOD OR LACON OR CHARCUTERIE PRODUCTS OR BACON OR BACONBURGERS OR DONER KEBABS OR MEAT PIES OR LIVER PATES OR LUNCHEON MEAT OR SAUSAGEMEAT OR SALAMI OR PEPPERONI OR FERMENTED SAUSAGES OR BRINED MEAT OR CEVAPCICI OR CURED MEAT OR DRIED MEAT PRODUCTS OR FRICADELLES OR GYROS OR HAM OR BOILED HAM OR DRY CURED HAM OR DRY HAM OR GAMMON OR IBERIAN HAM OR PARMA HAM OR SERRANO HAM OR KASSELER OR LIVER PATES OR MEAT BALLS OR MEAT DISHES OR MEAT LOAF OR MEAT PASTES OR RAGOUT OR RESTRUCTURED MEAT PRODUCTS OR SCHMALZFLEISCH OR SHASHLIK OR SPECK OR VENISON)

(shellfish OR crab OR CRUSTACEA OR MOLLUSCS OR CALLINECTES SAPIDUS OR CRAWFISH OR CRAYFISH OR KRILL OR LOBSTERS OR AMERICAN LOBSTERS OR NORWAY LOBSTERS OR LANGOUSTINES OR SCAMPI OR PRAWNS OR KURUMA PRAWNS OR SHRIMPS OR CRANGON CRANGON OR PANDALUS BOREALIS OR TIGER SHRIMPS OR BIVALVES OR ARKSHELLS OR CARPET SHELLS OR CLAMS OR FRESHWATER CLAMS OR PSEUDOCARDIUM SACHALINENSIS OR COCKLES OR MUSSELS OR BLUE MUSSELS OR GREEN MUSSELS OR MYTILUS EDULIS OR MYTILUS GALLOPROVINCIALIS OR MYTILUS TROSSULUS OR OYSTERS OR CRASSOSTREA GIGAS OR CRASSOSTREA VIRGINICA OR OYSTER SAUCES OR SCALLOPS OR PATINOPECTEN YESSOENSIS OR CEPHALOPODS OR CUTTLEFISH OR OCTOPUS OR SQUID OR GASTROPODS OR ABALONES OR LIMPETS OR PERIWINKLES OR SNAILS OR LAND SNAILS OR SNAIL MEAT OR WHELKS OR TOP SHELLS OR TRUMPET SHELLS OR TURBAN SHELLS)*

PubMed

PubMed includes more than 29 million citations for biomedical literature from MEDLINE, life sciences journals and book chapters including the fields of biomedicine and health, covering portions of the life sciences, behavioral sciences, chemical sciences, and bioengineering.
Searches were conducted using MESH and All fields.

Search terms used were:

Hepatitis E (MeSH term) OR HEV

Limited to “foodborne diseases/virology" OR pork OR swine OR deer OR venison OR shellfish OR game OR blood (MeSH term) OR plasma (MeSH term) OR thermal* OR cook* OR heat* OR temperature.

Filters (article type, species, subjects) were utilised to remove clinical/medical articles.

Current Contents (1998 to 2019)

Modules searched were the Life sciences (Includes over 1,370 journals and books); Agriculture, biology and environmental sciences (Includes over 1,040 journals and books). Contains complete tables of contents and bibliographic information from the world’s leading scholarly journals in these fields.

Searches were conducted using the TOPIC field which covers:

- Title
- Abstract
- Author Keywords
- Keywords Plus® (not available for separate searching)

Search terms to be used:

Virus: (Hepatitis E OR HEV)

AND RESEARCH AREAS**:** (FOOD SCIENCE TECHNOLOGY)

Scopus

Elsevier's Scopus covers peer-reviewed scientific journals, books and conference proceedings.

Search terms used were:

(Hepatitis E OR HEV) AND (deactivat* OR inactivat* OR death OR "capsid integrity" OR reduc* OR survival OR viable OR viability OR eliminat*)

AND (thermal* OR heat* OR cook*) AND NOT (hybrid AND electric* AND vehicle)

Web of Science Core Collection

This database includes over 21,100 peer-reviewed journals published worldwide in over 250 sciences, social sciences, and arts & humanities disciplines.

Search terms used were:

(Hepatitis E OR HEV) AND (Thermal* OR heat* OR cook*) AND (deactivat* OR inactivat* OR reduc* OR death OR capsid integrity OR survival OR viable OR viability OR eliminat*)

Weekly update searches were set on Elsevier ScienceDirect, Taylor and Francis Online and Wiley Online using Hepatitis E OR HEV as search terms.

Grey Literature sites were also searched:

System for Information on Grey Literature in Europe

Open access to **700.000 bibliographical references** of grey literature (paper) produced in Europe. OpenGrey covers Science, Technology, Biomedical Science, Economics, Social Science and Humanities

Government bodies:

- Canadian Public Health Agency
- CORDIS
- European Food Safety Authority
- Food and Agricultural Organization
- Food Safety Authority of Ireland

- Food Standards Agency (in particular report repository, including sections now with the National Archives)
- Food Standards Australia New Zealand
- Including Food Safety and Quality (AGFF) and Codex Alimentarius Commission
- Ministry for Primary Industries New Zealand
- Public Health England
- United States Department of Agriculture
- World Health Organisation

Search facilities on the website were utilised if available or Google Advanced search limiting to the appropriate website if not.

Search terms: Hepatitis E OR HEV OR virus*

University websites included:

- Colorado State University
- Illinois Institute of Technology, USA
- Johns Hopkins Bloomberg School of Public Health, USA
- Kansas State University, USA
- University of Guelph, Canada
- University of Nebraska, USA
- University of North Texas, USA
- Wageningen University and Research, Netherlands

Search facilities on the University website were utilised if available or alternatively Google Advanced search limiting to the appropriate website.
Search terms: Hepatitis E OR HEV or virus*

Other Organisations:

- Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), France.
- Bundesinstitut fur Risikobewertung (BFR), Germany and Veterinary Research Institute, Czech Republic
- CSIRO
- Institute of Food Technologists, USA
- International Life Sciences Institute
- National Institute for Public Health and the Environment (RIVM), Netherlands
- Norwegian Institute of Public Health, Norway

Thesis

- British Library EThOS
- Networked Digital Library of Theses and Dissertations (NDLTD) (Global search option)
- Open Thesis

Search terms: Hepatitis E OR HEV. Results limited to English language originals only.

5.1.2 Generation of Thermal inactivation database from the literature.

A full database was generated based on the literature screen prior to exclusion/inclusion criteria and is available in Appendix 1. A checklist was generated for the assessment of each publication (Table 1). Only abstracts in English are considered. Non-English papers with no English Abstract were rejected at Step "0".

Table 1. Checklist for screening references in the study. *Steps 1 and 2 performed on Abstracts. When a publication is accepted at Step 2, the full paper was acquired and assessed using the criteria in the subsequent steps.

Step	Question	Action if 'Yes"	Action if 'No"
1*	Is the study focussed on HEV?	Proceed to Step 2	Reject
2*	Does the study appear to contain information on thermal elimination of HEV?	Proceed to Step 3	Reject
3	Does the study actually contain information on thermal elimination of HEV?	Proceed to Step 4	Reject
4	Is the data quantitative?	Proceed to Step 5	Reject
5	Does the publication contain full details of methodology?	Accept	Proceed to Step 6
6	Can full details of study methodology be obtained from the author(s)?	Accept	Reject

5.1.3 Validation of publication database

The database was validated by both CBRI and GCU who checked the list of reviewed abstracts and papers, verified the selection of accepted studies, and agreed upon for the final literature to be considered in the predictive model.

5.2 Results for the literature screen

The abstracts obtained were scrutinized to screen out publications which actually do not contain relevant information. The basic inclusion / exclusion criterion applied are indicated in Table 1.
Only publications which clearly describe or refer to actual HEV elimination and the effect of decontamination procedures were included.

The initial screen was performed by JorFEV. One hundred and ninety-three potentially suitable references were identified by the literature search (Appendix 1). A further 3 references were identified from the reference lists of reviewed papers. All references were screened using the checklist in Table 1.

The abstracts, separated into "For Inclusion" and "Excluded", were sent to GCU, and CBRI for further scrutiny, and the final selection made according to combined expert judgment. The publications acquired had a second screen performed, which selected only those studies which contained usable data, for example quantitative information on infectivity or genome copy reduction. Temperature, source of HEV and duration of studies were also taken into account for generation of the data.

The reference list was populated at JorFEV and validated by CBRI. After validation, 6 references were finally rejected at Step 0, 25 at Step 1, 100 at Step 2, 21 at Step 3, and 21 at Step 4. Nine references were rejected at Step 5; all these papers were reviews of other published work which was available to the screen, so none of these were taken to Step 6.

Finally, 11 publications were accepted for data compilation.

A Thermal Inactivation Database spreadsheet was constructed, containing the following parameters:

I. Temperature
II. Time
III. Log reduction

IV. Matrix
V. Other Information
VI. Reference

This was populated with the quantitative information distilled from the studies selected by the second screen for G1/2 and G3/4 (Appendix 2 and 3).

The initial population of the database was performed at JorFEV, following scrutiny of the accepted publications (Appendix 1). For our purposes, given the lack of data available for surrogates and direct comparison with HEV proper, information from surrogate studies were not included in the final selection. It was proposed that G1/G2 could be appropriate surrogates for the zoonotic G3/4 strains.

The Database was validated by BRI. Any discrepancies identified were discussed with JorFEV and amended. Finally, 11 papers were deemed suitable to provide information for the model preparation (Table 2). Nine papers described studies using G3 strains, and 2 papers described studies using G3 strains. To advise the model and identify gaps, a full literature review was carried out on the manuscripts described in Table 2. The detail below describes the results taken from this review. This was used to assess the relevance and suitability of the data.

5.3 Critical review of selected publications for inclusion in the model.

The first study on the effect of heating on HEV was performed by Emerson et al. (2005). They tested various time / temperature combinations on two genotype (G) 1 strains (Akluj and Sar55) obtained from the faeces of infected patients, and a G2 strain (Mex14) from faeces of an experimentally infected primate. Infectivity was measured in HepG2/C3a cells by immunofluorescence assay (IFA). Only the data on the effect of heating at 56°C on the G1 Akluj strain can be regarded as fully quantitative however, as infected cell counts were given for this experiment but not for the others. It should also be considered that genotypes may vary in their response to thermal inactivation; in addition, this raises the question as to the relevance of G1

and 2 to food when they are more related to epidemic outbreaks. This data may be important when considering treatment of waste in the environment but for the purposes of food it could be excluded from the model.

Table 2: Accepted papers post literature screen. Detail provided in Appendix 2.

First Author	Date	Journal	Volume / Page Number
Johne R	2016	Applied Environmental Microbiology	82; 4225-4331
Barnaud E	2012	Applied Environmental Microbiology	78; 5153-5159
Schielke A	2011	Virol J	8; 487
Randazzo W	2018	Front Microbiol	9; 1973
Emerson S	2005	J Infect Dis	192; 930-933
Yunoki M	2016	Biologicals	44; 403-411
Farcet MR	2016	Transfusion	56; 383-391
Yunoki M	2008	Vox Sang	95;-94-100
Parashar D	2011	Clin Microbiol Infect	17; E1–E4

In 2008, Yunoki et al. tested four HEV strains isolated from the faeces of pigs: 1 x G3 strain (JPa) from an experimentally infected pig, 2 x G3 (SP and US) strains from naturally infected pigs, and a G4 strain (JP) from a naturally infected pig. The viruses were suspended in PBS and subjected to heating at 60˚C for 30 minutes (min), or suspended in PBS containing 25% fetal bovine serum and subjected to heating at 60˚C for 5 hours. An integrated cell culture (ICC) / RTPCR was used to assay infectivity: in brief, A549 cells were inoculated with heated and untreated virus suspensions, and viral RNA was monitored by RT-PCR immediately after inoculation and after 7 days of incubation. Infectious titres were determined by diluting suspensions before the inoculation of the cells, and calculated from the end-point dilution in which HEV RNA was detected. The temperatures and times described are not ideally conducive to cooking processes and how representative faecally derived virus is to food products can be called in to question. However, it is worth noting that virus present in faeces will have had its envelope removed while passaging the

biliary system so may be representative of virus in the liver which will have been exposed to bile.

In a study on the potential for persistence of HEV in the environment, Parashar et al. (2011) incubated a G1 strain (obtained from a human faecal samples) in soil at 37˚C. A quantitative (q)RTPCR assay was used to measure HEV genome equivalents (GE). When a GE count is reduced, it signifies destruction of encapsidated virus particles with subsequent degradation of viral RNA, and therefore loss of infectivity. GE reductions may however underestimate infectivity loss, as they do not take into account that particles may be intact but no longer infectious.

The above 3 studies, in general, do not really provide an insight into the thermal inactivation of HEV in food sources, but the information they produced could be utilised to determine survival in the environment. The two papers describing studies matching the relevant criteria, but focussed on G1 and G2, are described in Appendix 3. Although the data they contained were included in the Thermal Inactivation Database, lack of information relevant to thermal inactivation in a food matrix did not permit their inclusion in the final modelling data.

The first study to be more relevant to heat inactivation in food products was by Schielke et al. (2011), who examined the effect of heating on HEV G3 subtype 3i particles, obtained from the liver of a naturally infected wild boar. Liver may represent products such as pates, or those containing raw or undercooked meats that may contain HEV particles. The liver was homogenised, then filtered to reduce contaminants that may interfere with the assay. Aliquots of the resulting suspension, which contained 10^7 GE HEV ml-1, were subjected to various time / temperature combinations. A capsid integrity assay was used to detect intact virus particles. The virus suspensions were treated with RNase, so that only viral RNA protected by an intact capsid would be amplified. The rationale behind using capsid integrity assays is based on the assumption that intact particles are potentially infectious. This is not unambiguous however, as particles may be damaged but still intact and capable of producing a nucleic acid amplification signal, and thus capsid integrity results may overestimate infectivity (Walker et al., 2019). To date, no studies have been reported

which have demonstrated a relationship between HEV capsid integrity and infectivity. For the purposes of our study, it could be argued that the use of capsid integrity does not fully reflect the range of viral states present in food, i.e., raw or partially cooked food may contain a mix of intact and damaged viral particles due to the nature of the matrix suspension.

To examine the effect of cooking on HEV, Barnaud et al. (2012) obtained HEV G3 subtype 3e-infected liver (containing 10^8 GE HEV g-1) from an experimentally infected pig, and processed it into a pate-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. Nucleic acids were then extracted from aliquots of the treated preparations, and assayed for HEV RNA by qRTPCR. After 5, 10, and 120 min at 62°C, log reductions in HEV GE were 1.19, 1.83 and 2.17 respectively. This is comparatively low (with regard to effects recorded for similar temperatures and is likely due to the RTPCR data reflecting only those virus particles (with their RNA genome) which were completely disrupted by the temperature / time combination. At 68°C, Barnaud et al. (2012) observed log GE reductions of 2.28, 2.26 and 2.31 after 5, 10 and 20 min respectively. As with their data on the effect of heating at 62°C, these $\log_{10}$ reductions may be taken as reflecting a minimum loss of infectivity. A 2.93 log decline in GE after 5 min at 71°C, a 2.58 log GE decline after 10 min, and a 2.69 log GE decline after 20 min was also observed. Again, these log GE reductions, indicating a complete loss of intact HEV particles, may be taken as reflecting a minimum loss of infectivity produced at that temperature / times.

Farcet et al. (2016) subjected an HEV G3 isolated from naturally infected pigs to heating at 58°C for 1 h in PBS, and for 3 h in 12.5% human serum albumin. A suspension containing 3.8 $\log^{10}$ infectious units was used. Infectivity was measured in HepG2/C3a cells by immunofluorescence assay. At 58°C for 1 h, a reduction of HEV infectivity greater than 3.7 log was observed; the same log reduction was obtained after 10 h, possibly due to the limit of detection of the cell culture assay being reached.

Following on from the Schielke et al. (2011) study, and utilising a cell culture-adapted HEV G3 strain (47382c), Johne et al. (2016) examined the effect of heat on the virus suspended in cell culture supernatant. Suspensions containing between approx. 3.5 and 4.0 log infectious units were used. Various time / temperature combinations were tested. Infectivity was assayed by counting infected A549 cells by immunofluorescence microscopy after incubation on cell cultures.

Yunoki et al. (2016) suspended HEV G3b, obtained from the faeces of an experimentally infected pig, in buffer solutions containing various plasma derivatives, and heated the suspension at 60˚C for 1, 5 and 10 hours. HEV infectivity was assayed by ICC / PCR as in Yunoki et al. (2008). Inactivation/removal was found to be moderate in all cases albeit the properties of the virus varied depending on the HEV source; serum or faeces.

A suspension of HEV G3f obtained from the faeces of an infected patient was subjected to 15 minutes heating at various temperatures by Randazzo et al. (2018). the suspension contained approximately 6.7 GE ml^{-1}. A capsid integrity assay was used to measure intact HEV particles; in this assay, pre-treatment of the treated and untreated suspensions by platinum chloride (PtCl4) was performed. The platinum compound enters damaged capsids and interferes with amplification of RNA; Fraisse et al. (2018) found however that PtCl4 does not eliminate amplification completely, and hence loss of infectivity could be underestimated.

Another aspect that requires consideration in regard to HEV risk, is the effect of curing, preservation and fermentation. To study the stability of HEV in high-salt conditions which can apply during meat preservation, Wolff et al. (2020a) incubated suspensions of HEV G3b (47382c) (cell culture adapted virus) in PBS pH7.5 alone or supplemented with various combinations of NaCl (2%, 10% and 20%), 0.015% sodium nitrate, and 0.03% sodium nitrate, for 1d at 23˚C, 6d at 22˚C, and 8 weeks at 16˚C. Infectivity was assayed following the protocol described by Johne et al. (2016). Likewise, to evaluate the effect of lowering pH during the production of raw cured and fermented meat products, Wolff et al. (2020b) incubated suspensions of HEV G3b (47382c) in PBS adjusted to various pH levels with either HCL/NaOH or D/L-lactic

acid, the latter chosen to give pH values which could be found during fermentation of sausages. Infectivity was assayed following Johne et al. (2016).
Wolff et al. (2020b) found no reduction in infectivity after 7 d at 4˚C in PBS adjusted with D/L-lactic acid to pH values between 4.5 and 6.5. After a 56 day incubation at 16˚C in PBS pH7.5 supplemented with various salt combinations and concentrations, Wolff et al. (2020a) observed a decline in infectivity of 1.80 log, with no significant difference in mean decline between the various conditions tested.
Wolff et al.(2020a) observed a 1.60 log decline in infectivity, in PBS pH7.5 supplemented with various salt combinations and concentrations, for each condition tested. After 7 d in cell culture supernatant at 22˚C, Johne et al. (2016) observed a 1.70 log decline in infectivity; a 3.70 log decline was observed over 28 d.
At 23˚C for 1d in PBS supplemented with various salt combinations and concentrations, Wolff et al. (2020a) observed a mean 0.6 log decline in infectivity, for each condition tested. At 23˚C for 3 h in PBS adjusted to pH values between 1 and 10 with HCL/NaOH, Wolff et al. (2020b) observed 3-log reductions in infectivity only at pH1 and 10; a slight reduction (0.5 log) was observed at pH 2 to 5, but no effect was seen at the other levels tested. In suspension adjusted with D/L lactic acid to pH levels between 4.5 and 6.5 and incubated at 23˚C for 7 days, a 0.5 log decrease in infectivity was observed only at pH4.7 (an increase of approx. 1 log was seen at pH5.7). Wolff et al. (2020a,b) concluded that the salt conditions and pH levels used during curing or fermentation of meats would not be sufficient to eliminate all risk of contamination with infectious HEV.

The latter studies described here illustrate several issues with comparability, mainly;

1. Viral source and starting input.
2. Degree of processing/addition of treatments which may impact HEV detection/infectivity.
3. Method of detection, for example, detection of nucleic acid vs infectivity by IFA.
4. Relevance to cooking of food in comparison to environment and storage.

This was reflected in the varied results observed for reduction of viral quantities or infectivity in the publications. Nonetheless, a trend of higher log reductions after longer incubation periods is evident, as would be expected.

5.4 Discussion

There have been several studies which have examined aspects of the thermal inactivation of hepatitis E virus (HEV). A key question in the food industry is whether the inactivation documented is relevant and can be utilised to predict whether cooking processes are adequate to reduce/eliminate potential HEV contamination and reduce risk to consumers. To address this question, a literature search was performed and a critical review carried out focused on information from studies on the effect of thermal treatment on Hepatitis E virus. Only those studies which produced quantitative data were included in the review (Table 2). Data was compiled by temperature tested, from 4˚C to 95˚C. The compiled data shows that temperatures of 65˚C and above are necessary to achieve a substantial (i.e. >2.0 log) reduction in HEV infectivity or measured parameter in a short time (for example 5 min or less), albeit the data is limited. However, the relevance to food remains to be established in some cases, and experimental differences between studies, including detection/quantification methods, complicate the data. This temperature may however be insufficient to completely eliminate infectivity within cooking procedures.

Of the 9 studies selected for inclusion in the model, the main differences observed were in the source of HEV utilised in the study, viral starting input, processing and the method of detection. Sources described were faeces (Barnaud et al., 2012; Wolff et al., 2020a; Wolff et al., 2020b; Yunoki et al., 2016; Yunoki et al., 2008), liver (Farcet et al., 2016; Schielke et al., 2011; Randazzo et al., 2018) and cell culture adapted virus (Johne et al., 2016). The only dataset providing any information for a time series to contribute to a predictive model was that described by Johne et al. (2016).

Barnaud et al. (2012) observed HEV shedding in pigs intravenously inoculated with naturally HEV -contaminated liver suspensions treated at 68˚C and 71˚C for 5 min, indicating that infectivity remained intact. Only at 71˚C for 20 min was HEV infectivity eliminated in this study, and they reported that at 10 min some virus infectivity remained. However, due to an experimental weakness (the pigs inoculated with liver treated at 71˚C 10 min were housed in the same pen as pigs inoculated with liver treated at 62˚C 10 min, and thus it cannot be certain whether cross-contamination did

not occur), this latter result is ambiguous, and the true elimination time at 71˚C would have lain somewhere between 5 and 20 min (Cook and van der Poel, 2015). At temperatures of 75˚C and above, Johne et al. (2016) observed complete or almost complete elimination of infectivity, as assayed by cell culture, after 1 min. Using higher starting HEV numbers than Johne et al. (2016), Randazzo et al. (2018) and Scheilke et al. (2011) observed lower reductions using capsid integrity assays at this time / temperature combination, but the limitations of such assays have to be borne in mind. Previous work had indicated that capsid integrity assays did not make a difference to the detected log reduction after thermal treatment when compared to untreated virus (Scobie et al, unpublished data).

As indicated, not all of the studies are created equal in terms of methodology, and it is difficult to compare the data in order to consider a predictive model on thermal inactivation. At 56°C and 60˚C, we see the greatest disparity in log reduction. The high amount of virus input used by Schielke et al. (2011) may contribute to the low log reduction seen over time at 56˚C; however, this does not follow at 60˚C (Appendix 2). It has been proposed that these temperatures are insufficient to inactivate HEV effectively, and the inability to truly compare each data set makes it almost impossible to determine the virus behaviour. When we go to the higher temperatures, the data is more comparable. It is clear that the available data in the literature at the higher end of temperature (70-95˚C) and those relevant to cooking are much less than the data available at the lower temperatures indicating a gap in the information and the ability to assess the real impact of thermal inactivation on HEV.

Comparison of the sensitivity of either RTPCR or infectivity assay is not possible from the literature and in some cases, log reduction is seen in a very short time (Johne et al., 2016; Schielke et al., 2011). Here it would appear that the use of cell culture adapted virus in comparison to virus isolated from liver would suggest that the virus derived from liver is more stable, albeit number of data points are low. This is also reflected where the reduction in viral quantity/infectivity is less in liver derived HEV (Johne et al., 2016; Randazzo et al., 2018; Schielke et al., 2011).

An additional complication in stability and infectivity also lies with the presence or absence of a membrane associated quasi envelope around the viral capsid (Qi et al., 2015; Yin et al., 2016; Nagashima et al., 2017). What this means is that the virus is

present in the bloodstream as an ‘enveloped virus’ whereas in other locations the virus is non-enveloped (Feng., 2020). In general, the impact of quasi envelopment in the viral life cycle and pathogenesis is largely unknown. Evidence to date would suggest that quasi enveloped HEV (eHEV) is important for cell to cell spread, whereas standard HEV virions are optimised for environmental transmission; both are infectious (Feng., 2020; Riviera-Serrano et al., 2019). Quasi enveloped viruses are also known for being able to evade antibody-mediated immune responses; eHEV has been shown more resistant to neutralising antibodies.

Given the above, we would suggest that HEV in food exists as a mixture of viral states. So, what does this mean? Products containing pork from mixed sources such as charcuterie or pork derived muscle/tissue can be assumed to contain blood and/or serum. This means that both HEV and eHEV would be present and therefore it is relevant to assess both types of the virus. As mentioned earlier, the relevance of using a cell culture adapted virus is difficult to predict, although it could be argued that due to eHEV that is found in both serum and in cell culture, this source is relevant to model. The virus in faeces has no lipid membrane and therefore could be considered representative of HEV. (Qi et al., 2015; Yin et al., 2016; Nagashima et al., 2017). Yunoki et al., (2016) observed that properties varied between the source and treatment of HEV which could affect this virus envelope. These factors add to the complicated outcomes seen in the data and need to be considered in further assessment. Some may consider that the virus represented in faeces is also not relevant to food, however, given there is no full analysis of the status of the HEV virus present in food, all sources should be considered to ensure the range of virus that could be present is fully assessed. In addition, if the analysis is to extend to other foods such as shellfish or berries, then faecal contamination via environment, irrigation etc., will be relevant.

Distilling the above information, it would appear that the time / temperature combination which would be best to use in short-time cooking lies between 65˚C and 75˚C but the data are inconsistent. Further studies, preferentially using assays directly evaluating detection or infectivity, such as cell culture or ICC-PCR, are necessary to determine the precise elimination conditions.

It was anticipated that there may be gaps in the literature due to the varying sources used to obtain HEV. Indeed, the data by Johne et al., the only current limited predictive model provided, is derived from cell culture adapted virus (R. Johne; personal communication) and did not provide a full dataset with respect to the analysis of HEV and eHEV, or sufficient data points at the relevant time/ temperature parameters required. As there was no comparison of virus from different sources. for our purposes, we considered the effect of the source virus in the data to take forward to the model.

Overall, the outcomes were:

I. From the limited literature data available, it is not possible to confirm an optimal time/temperature combination to assess the thermal response of HEV
II. Data in the literature are insufficient to create a consistent and robust model to assess the thermal stability of HEV over time at temperatures relevant to food
III. Further data using a consistent method of detection and varied viral sources for input over a relevant time/temperature setting are required.

6. Experimental assessment of thermal stability of HEV

Based on the findings in section 5, the second objective of this work was to produce additional experimental thermal stability data for the hepatitis E virus. Section 5 identified clear knowledge gaps and it was concluded that data for cooking-relevant temperatures was limited in the literature and therefore it was not possible to develop a robustly predictive model based on this alone. In order to create sufficient information for a predictive model, we designed the experiments to have a minimum of 5 data points for time at each temperature assayed (Bertrand et al; 2012). Experiments were carried out in duplicate and assayed by PCR in triplicate. Overall, a total of 204 data points were generated. We also utilised 3 sources of the virus which were considered to contain both HEV (faeces) and eHEV (serum/cell culture adapted virus) in order to determine the role this may play in thermal stability and if we observed varying responses after thermal treatment. The effect of the matrix was not assessed.

6.1 Materials and Methods

In brief, heat blocks were prepared at each temperature and temperature probes containing 500µl of H_2O were placed in each block to record temperature over the duration of the experiment. Experimental tubes were set up containing RNase- and DNAse- free water. Given that current general food safety advice is to cook food to an internal temperature of 70°C for a minimum of 2 minutes and the lack of information provided in section 5.3, thermal treatments applied were 70, 75, 80, 85, 90, 95 °C with room temperature (RT 22-25°C) and 100°C as negative and positive thermal inactivation controls, respectively. A time series for each temperature was carried out over 10 or 20 minutes with samples taken at 2 or 5 minute intervals respectively. Only temperatures relevant to food and cooking were addressed as per guidance from FSA.Once the relevant temperature was reached, WHO serum-derived HEV nucleic acid standard (6329/10), cell culture-derived virus (kindly provided by R Johne, Bundesinstitut für Risikobewertung, Germany) or porcine virus (faecal source, kindly provided by B Choudary, APHA, UK) was added to each tube to achieve a final concentration of 5000, 10,000, 15,000 or 20,000 IU/ml dependent on source availability. Liver-derived HEV was unavailable to us for comparison. All viruses were genotype 3. Virus was quantified using qRTPCR against the known HEV WHO standard 6329/10 (primary standard) which was prepared according to suppliers' instructions. In brief, the lyophilized sample was resuspended in 500 µl sterile nuclease free water in order to obtain 250,000 IU/ml. 140µl of the virus stock was extracted using the Qiagen QIAamp viral RNA mini extraction kit (Qiagen, Surrey, UK). The eluted viral HEV RNA is currently quantified as 1.6 x 106 IU/ml (working standard solution). This was used to quantify the virus in each source which was then diluted to achieve the relevant viral quantities for input.

For the analysis, duplicate 1ml samples of PBS containing HEV were heated to the relevant temperature. 140µl aliquots were removed from the tubes at specific time intervals and immediately placed on ice to halt the thermal treatment. vRNA was extracted from the sample using the Qiagen mini vRNA kit (Qiagen, Surrey, UK), eluted in 80µl and stored at -80°C. The hypothesis is that, if we achieve effective degradation of the viral particles at a particular temperature/time combination then we would expect a negative result in the analysis. If the virus is able to withstand the thermal treatment without damage to the viral particle, then a positive result will be

obtained on molecular analysis. If the virus is damaged, and a positive result is seen using the standard isolation, it is possible that this is due to the persistence of viral nucleic acid derived from damaged particles.

Additional controls were included, and quantification carried out using the viral RNA standard. The HEV WHO standard 6329/10 (primary standard) was prepared according to suppliers' instructions. The eluted viral HEV RNA is currently quantified as 1.6 x 10^6 IU/ml (working standard solution). It is important to note that the WHO standard has an assigned unitage of 250,000 IU/ml (Baylis et al, 2011). The assigned value of 250.000IU however does not give a clear indication of how many genome copies of hepatitis E virus is present in the standard. It is possible to extrapolate and estimate the genome copy number based on the validation trials conducted by the team that developed the Ceeram Tools ® (BioMerieux, France) detection kit, based on which the 250.000IU corresponds to 125.000 genome copies (hepatitisE@ceeramTools™ . validation report . sl : Ceeram S.A.S , 2013. RV-HEV-AN-V3-130610). Dilutions of the viral RNA were performed to provide a linear range from 250 to 250,000 copies and stored in aliquots at -80ºC. All the quantifications were performed in duplicate with the HEV WHO international standard and PCR detection initially using the commercial validated assays Ceeram Live technology HEV qPCR kit or qRT-PCR for HEV as described previously (Jothikumar et al,, 2006). The Ct values were determined and log reduction calculated utilising the standard curve. Log viral copy per ml of input was plotted against time for each temperature. The limit of detection for the Ceeram assay was 250 IU per reaction and for the Jothikumar assay (2016), 100 IU per reaction.

Data was analysed for significance using ANOVA then a pairwise Wilcox test with Bonferoni adjustments.

The Positive and negative controls used were as provided by Ceeram assay components HEV CTRL+ and CTRL-. The absence of residual DNA was confirmed by a real-time PCR without reverse transcription, which showed a negative PCR result. In addition, a water only control was also utilized. Each sample was run on the PCR in duplicate. Reagent controls were water only added to the master mix and as it was vRNA extracted, the internal positive control to ensure integrity of the PCR assay an internal positive control (IPC) PCR was present in all reactions.

The PCR assays were considered valid when the following were met:

1. Fluorescence signals above background were not detected in the reagent only controls.
2. The R2 value of the standard line over four points was ≥0.98. Slope value between -3.0 and -3.6.
3. No signal was detected in any of the negative control samples.
4. IPC provides a positive signal in each reaction.

6.2 Results

Assays were carried out in duplicate at each time-temperature combination and analysed in duplicate by PCR. Each experiment was carried out a minimum of two times. The control at 100˚C followed the expected pattern of reduction but interestingly it appeared to follow a two phase pattern of reduction that has been seen previously for Hepatitis A virus (Bertrand et al, 2012); that is, the virus was stable for around 5-6 minutes then degraded over the remaining time monitored (Figure 1). Our method used does not eliminate contaminating RNA so it is possible that fragments remain detectable giving a lower reduction value. The room temperature control demonstrated stability over the period of analysis (Figure 2). Temperature was monitored at each minute of incubation over the time course. Temperature was maintained over time course with a standard deviation of ≤± 0.05 - 0.3ºC. Readings were taken in solution volume as per experiment.

The initial analysis of HEV inactivation over time (ten minutes) was carried out using the WHO serum virus. Overall, as observed, virus detection fluctuated over the time period reflecting the varying rates of degradation at temperatures analysed (figures 3, 4, 5 and 6). Overall, a significant reduction was seen at 70 and 75° C; p=0.036 (Figure 3b) and 0.032 (Figure 4b) respectively.

Figure 1: Thermal inactivation at 100˚C of HEV derived from human serum.

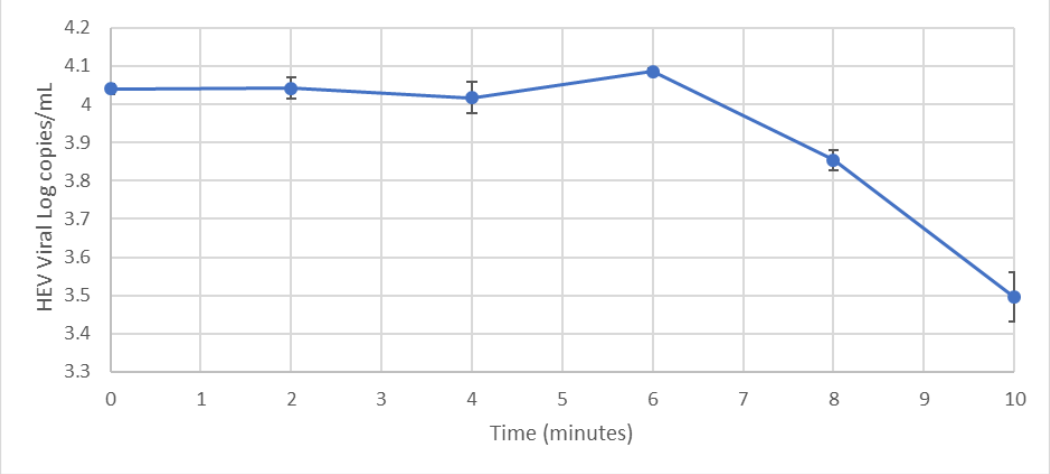


Figure 2: Thermal inactivation at room temperature (RT 22-25°C) of HEV derived from human serum.

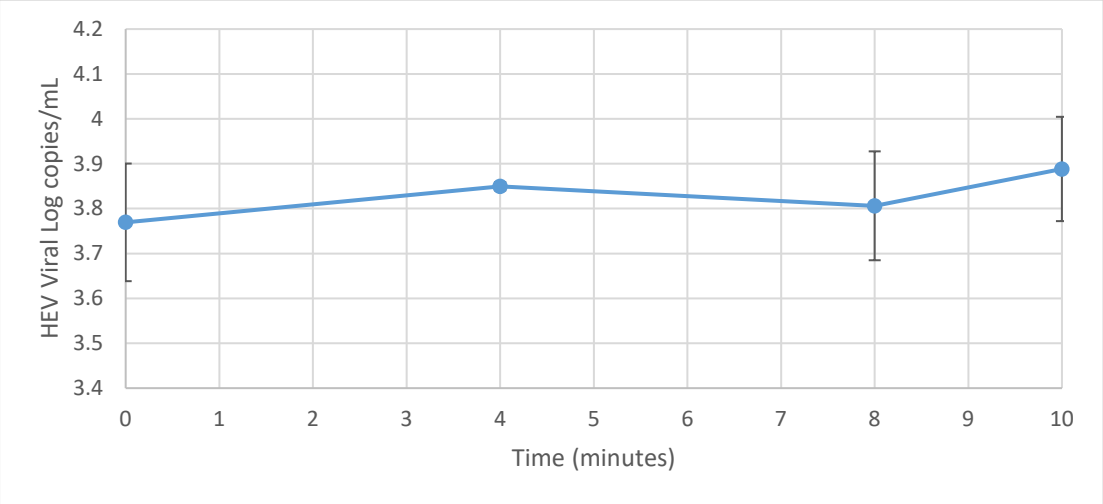


Figure 3a: Thermal inactivation at 70˚C, over a 10minute time period of HEV derived from human serum.

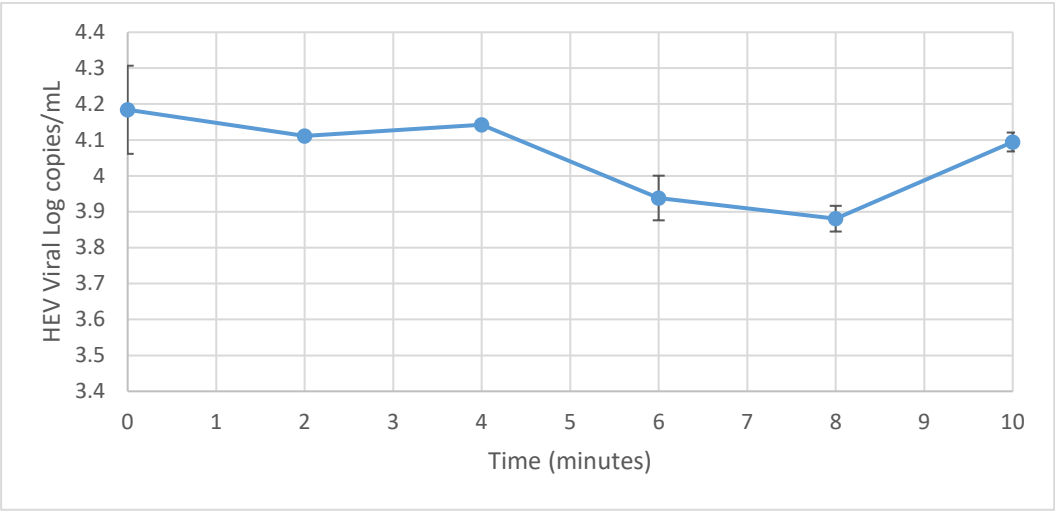


Figure 3b: Statistical analysis of viral quantity reduction at 70°C, over 10 minutes with HEV derived from human serum.

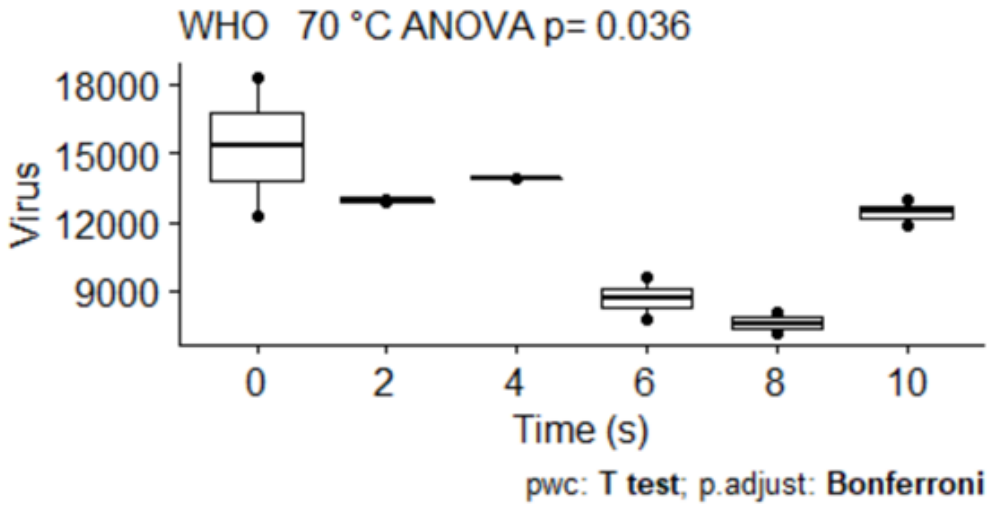


Figure 4: Thermal inactivation at 75˚C, over a 10minute time period of HEV derived from human serum.

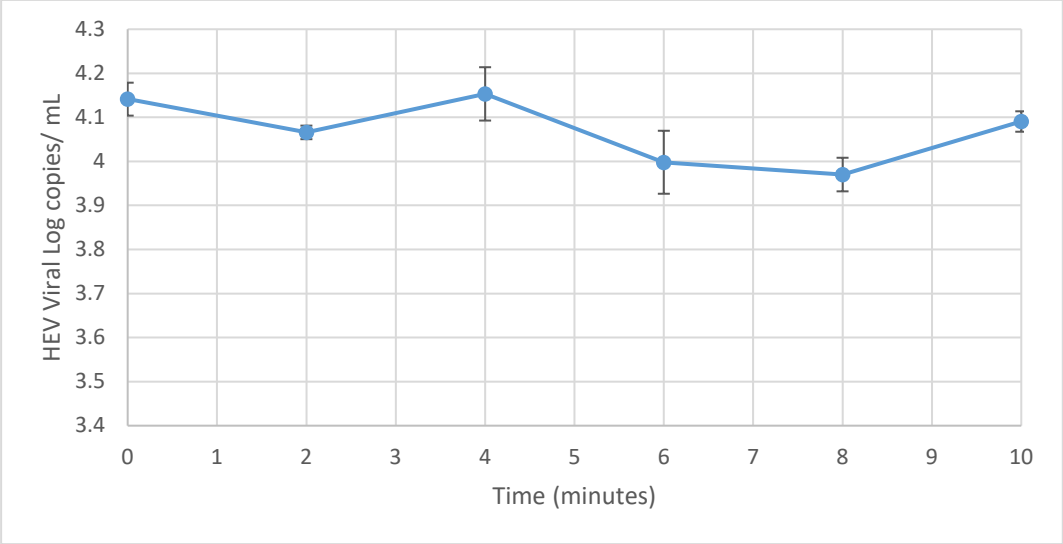


Figure 4b: Statistical analysis of viral quantity reduction at 75°C, over 10 minutes with HEV derived from human serum.

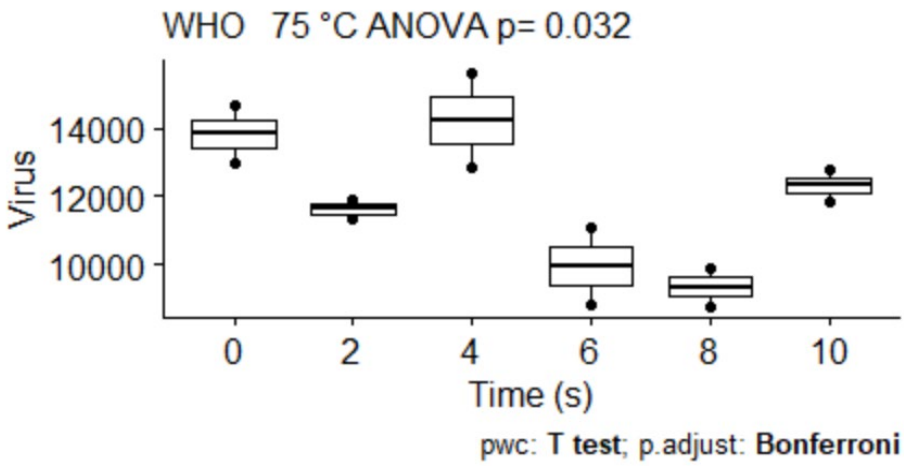


Figure 5a: Thermal inactivation at 80˚C, over a 20minute time period of HEV derived from human serum.

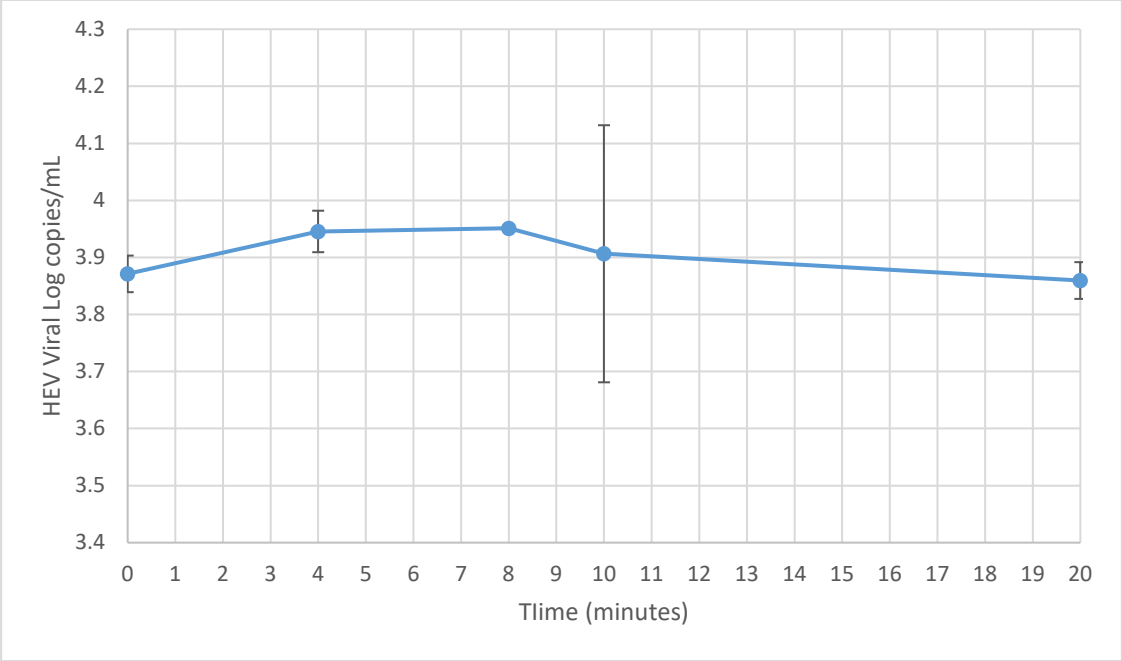


Figure 5b: Statistical analysis of viral quantity reduction at 80°C, over 20 minutes with HEV derived from human serum.

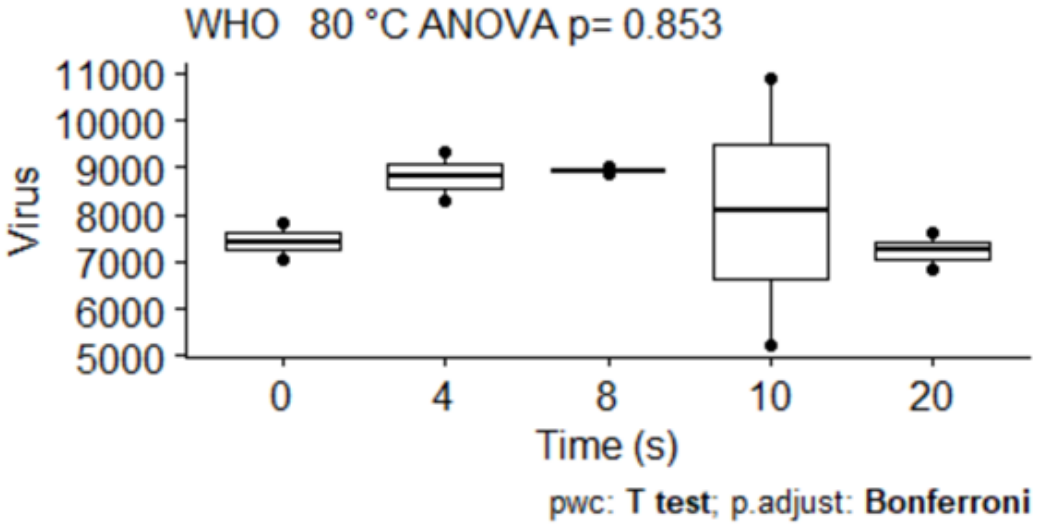


Figure 6a: Thermal inactivation at 85˚C, over a 20minute time period of HEV derived from human serum.

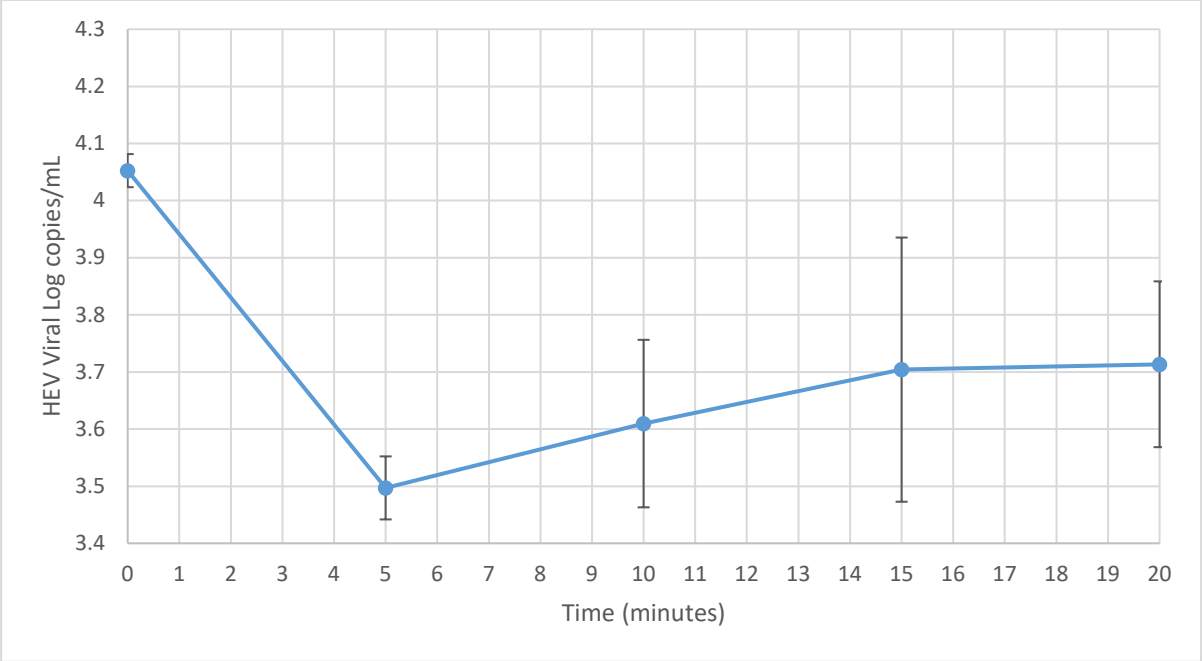


Figure 6b: Statistical analysis of viral quantity reduction at 85°C, over 20 minutes with HEV derived from human serum.

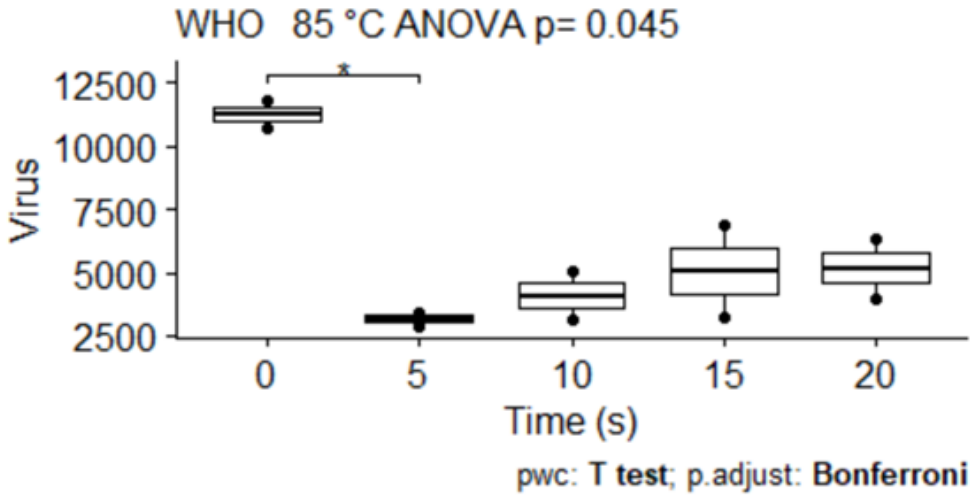


Figure 7a: Thermal inactivation at 95˚C, over a 20minute time period of HEV derived from human serum.

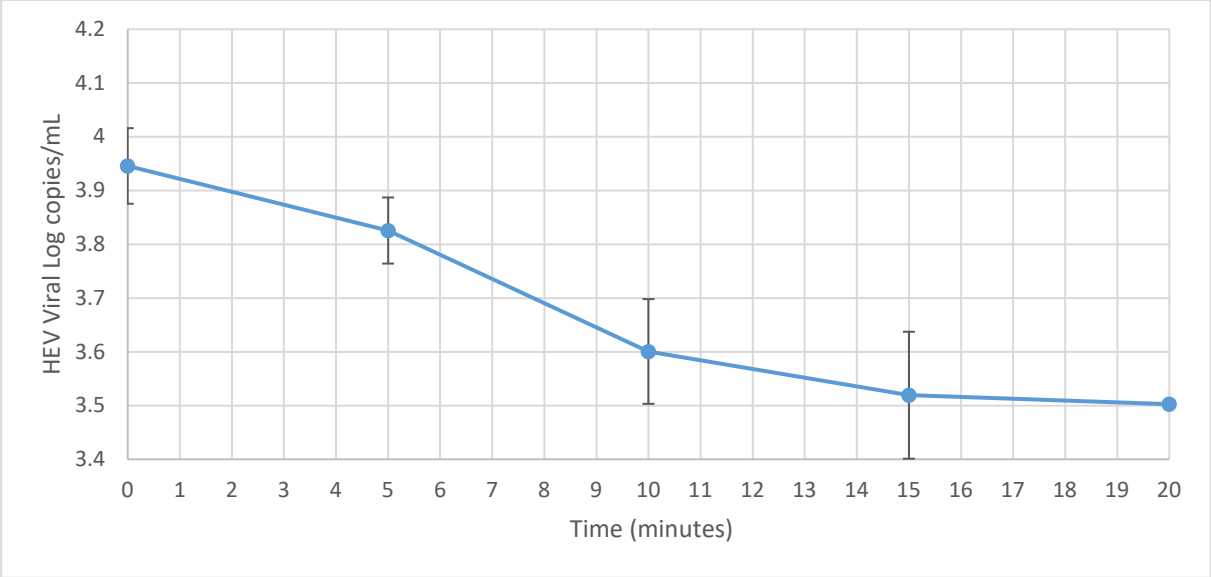
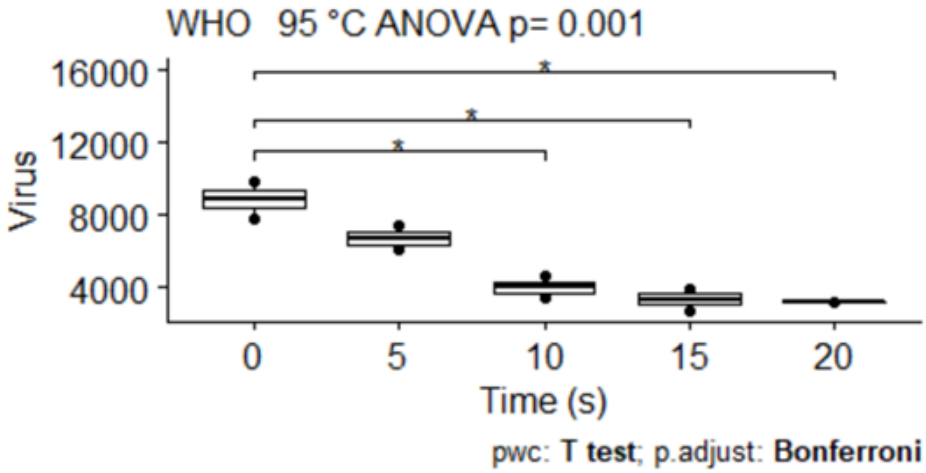


Figure 7b: Statistical analysis of viral quantity reduction at 95°C, over 20 minutes with HEV derived from human serum, p=0.001.



No effect on HEV derived from human serum was seen at 80°C, p=0.853 (Figure 5b).

At 85˚C, an initial drop in quantity of virus was observed within the first 5 minutes (p=0.045) but this did not change over the remainder of the time period (Figure 6b). At 95˚C, a significant reduction over all time points was seen (Figure 7b).

The same analysis was performed in duplicate with the cell culture derived virus (Figures 8-12) and the porcine faecally derived virus (Figures 13-17).

Figure 8: Thermal inactivation at 70˚C, over 20minutes of HEV derived from cell culture supernatant

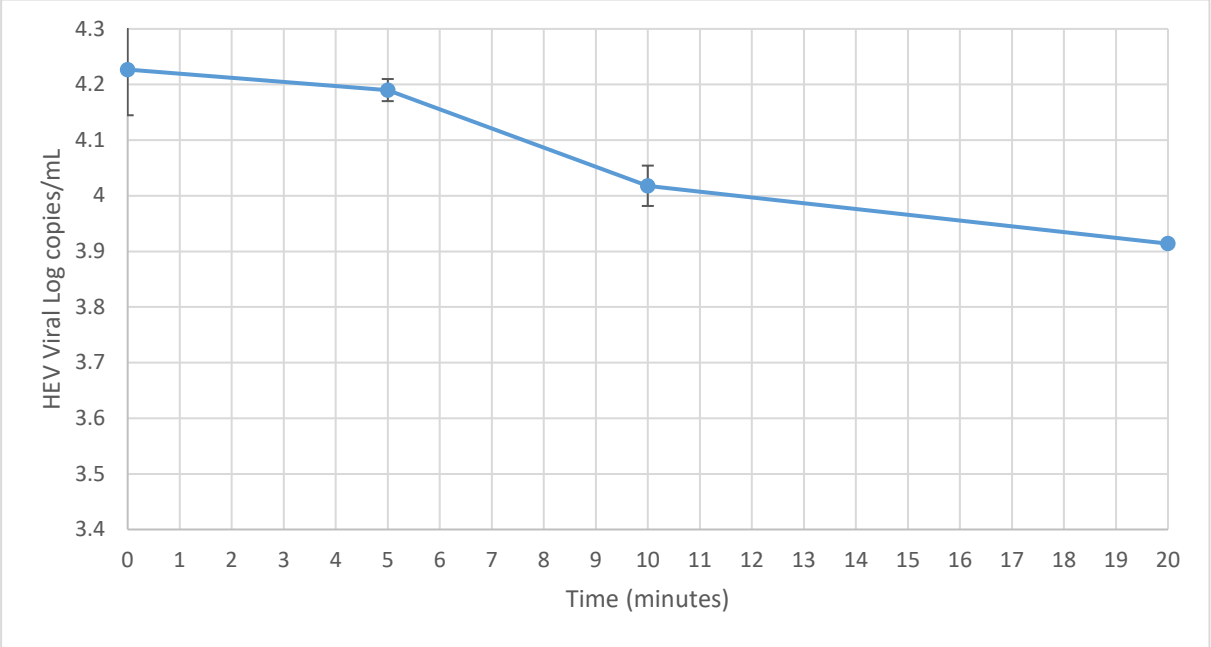


Figure 9: Thermal inactivation at 75˚C, over 20minutes of HEV derived from cell culture supernatant

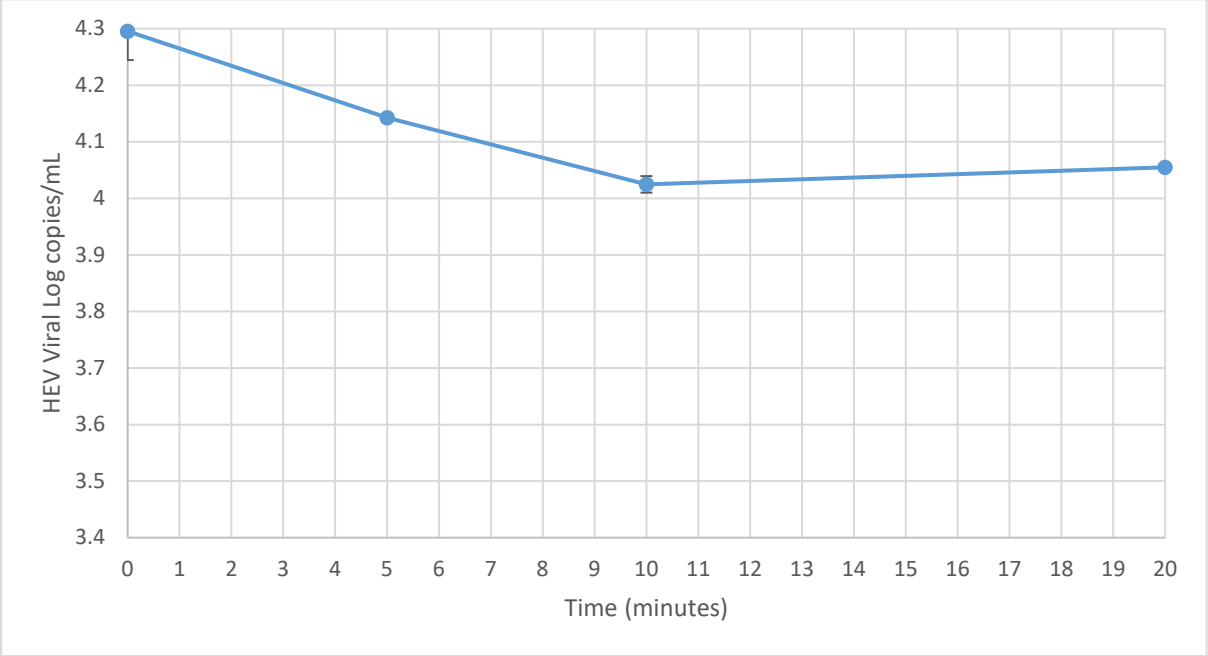


Figure 10: Thermal inactivation at 80˚C, over 20minutes of HEV derived from cell culture supernatant

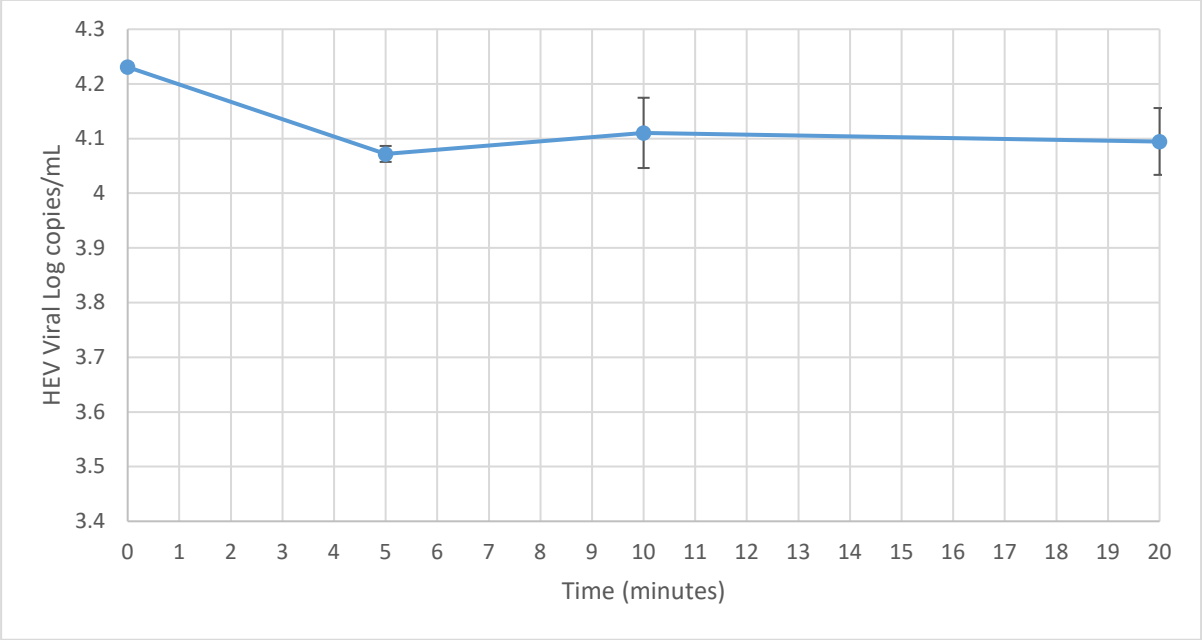


Figure 11: Thermal inactivation at 85˚C, over 20minutes of HEV derived from cell culture supernatant

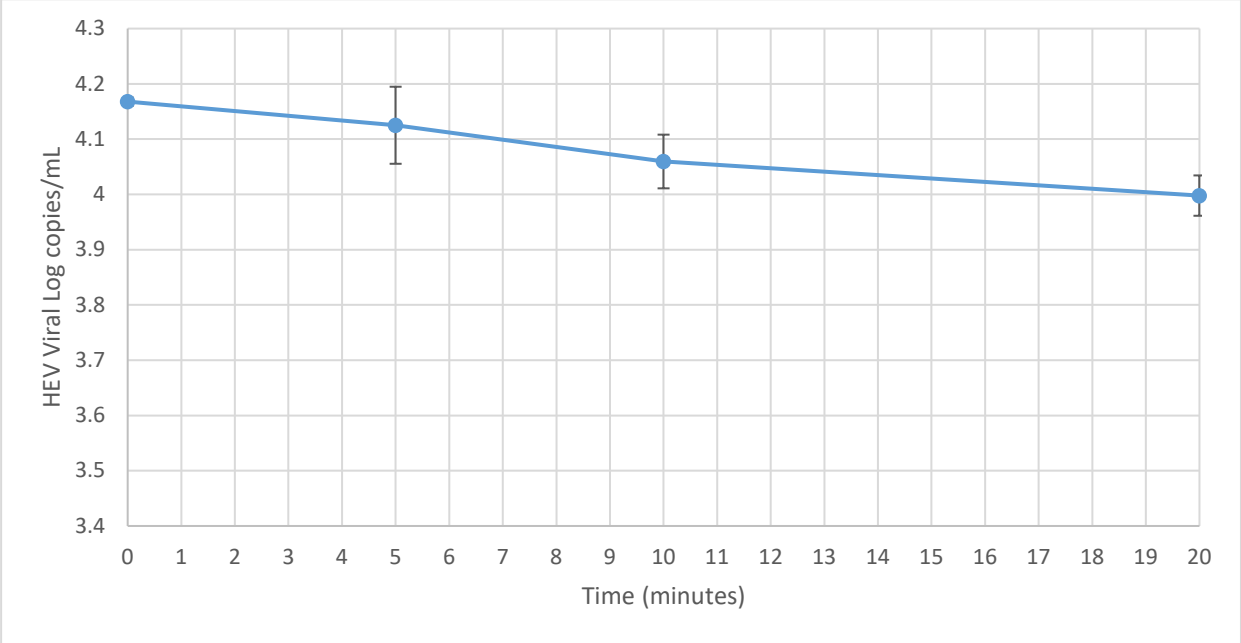
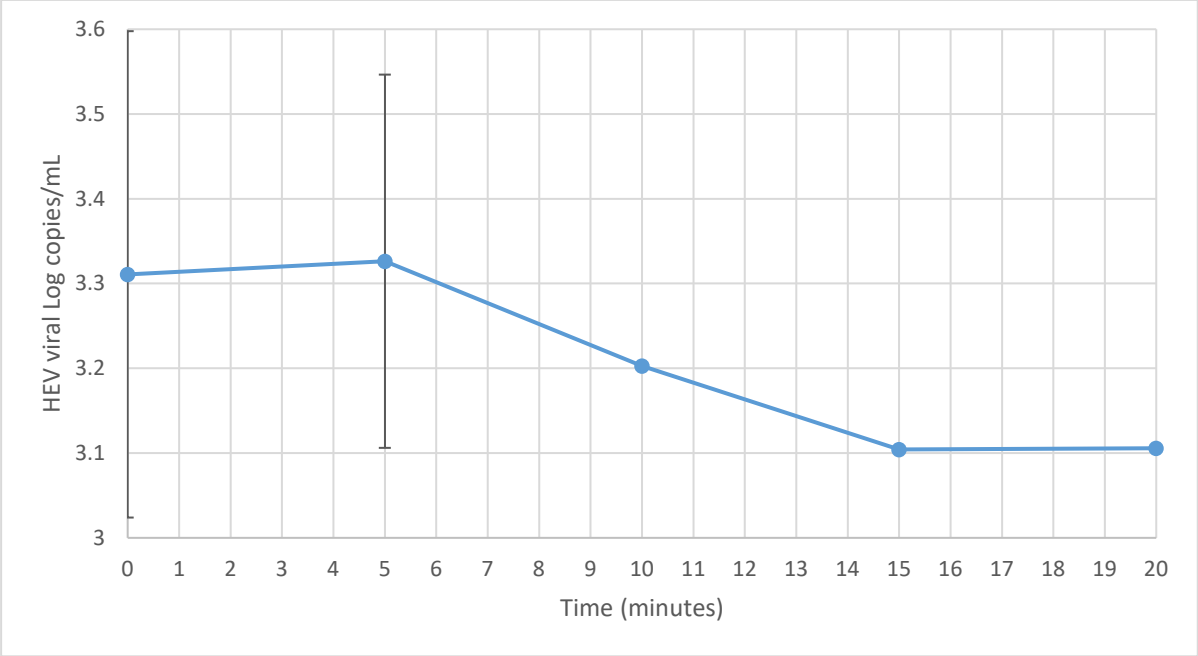


Figure 12: Thermal inactivation at 95˚C, over 20minutes of HEV derived from cell culture supernatant



Again, thermal inactivation at the temperatures expected to be achieved in the cooking process (70 and 75˚C), showed reductions with p values of 0.036 and 0.032 respectively. At 80 and 85˚C no significant reduction in the quantity of virus as detected by PCR was observed (figures 8-11). Unlike that seen for the WHO serum derived virus, the cell culture adapted virus showed no significant reduction at 95˚C (figure 12).

Figure 13: Thermal inactivation at 70˚C, over 20minutes of HEV derived from porcine faeces. p=0.544.

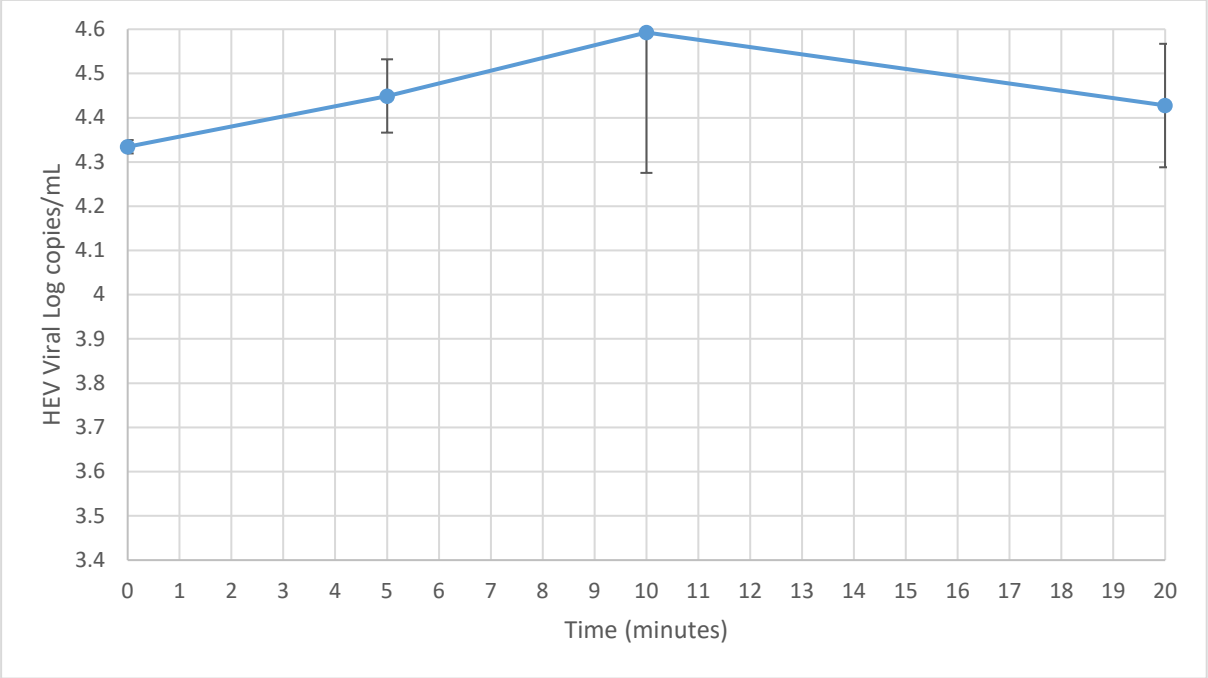


Figure 14: Thermal inactivation at 75ºC over 20minutes of HEV derived from porcine faeces. p=0.232.

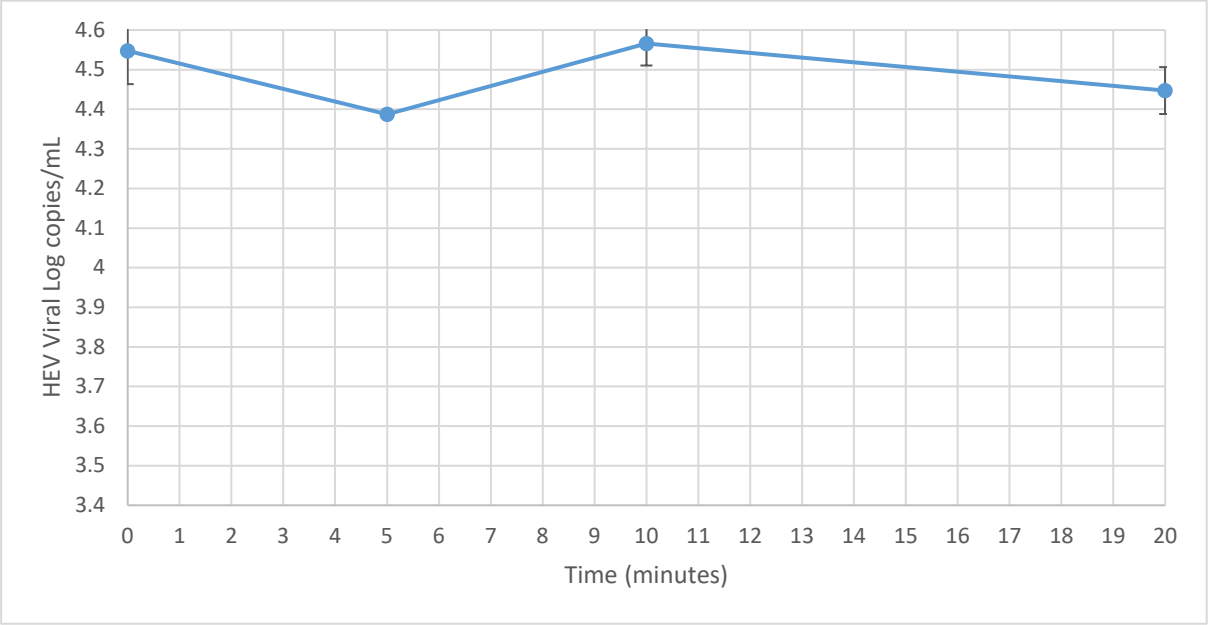


Figure 15: Thermal inactivation at 80˚C, over 20minutes of HEV derived from porcine faeces. p=0.682.

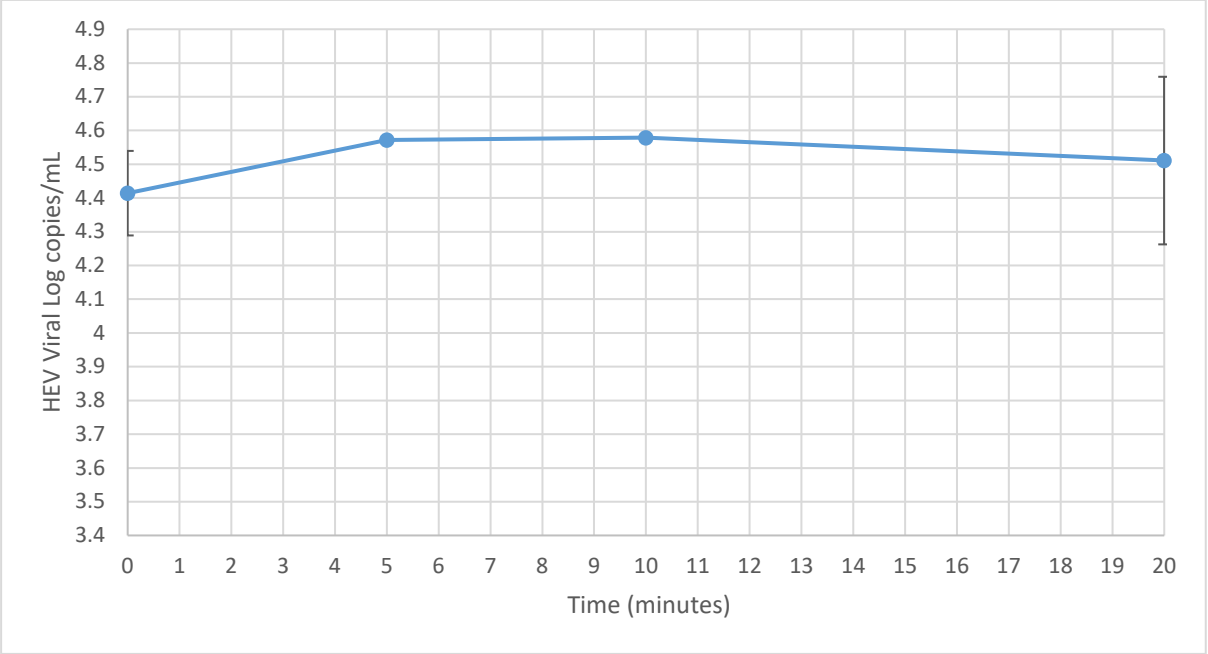


Figure 16: Thermal inactivation at 85˚C, over 20minutes of HEV derived from porcine faeces. p=0.338.

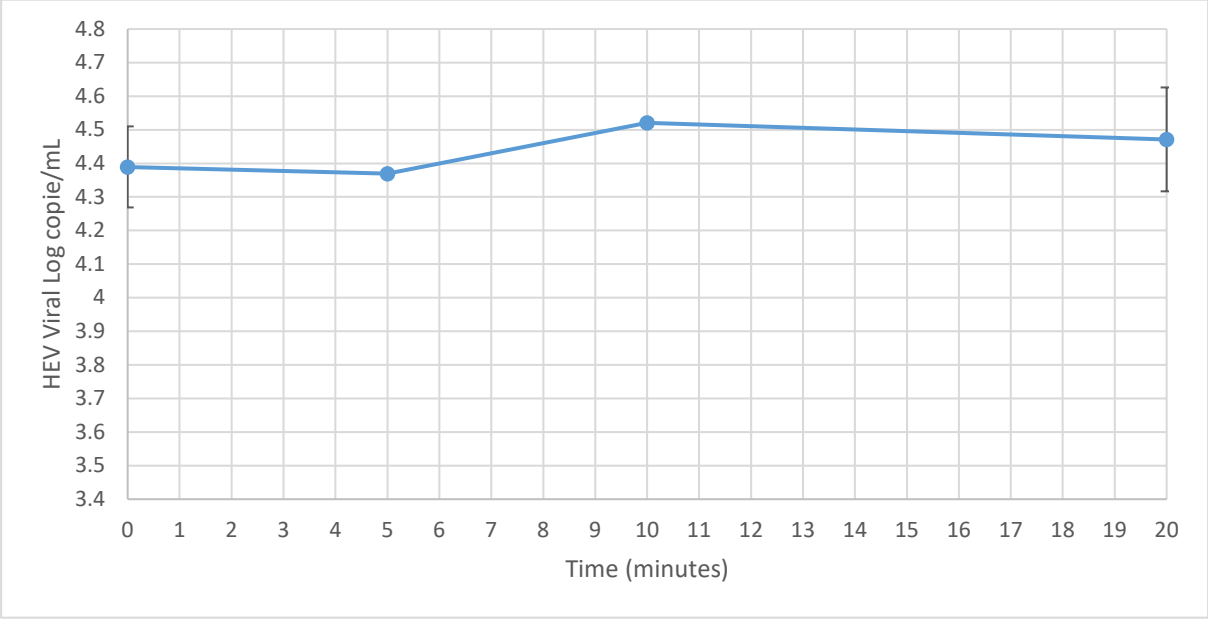
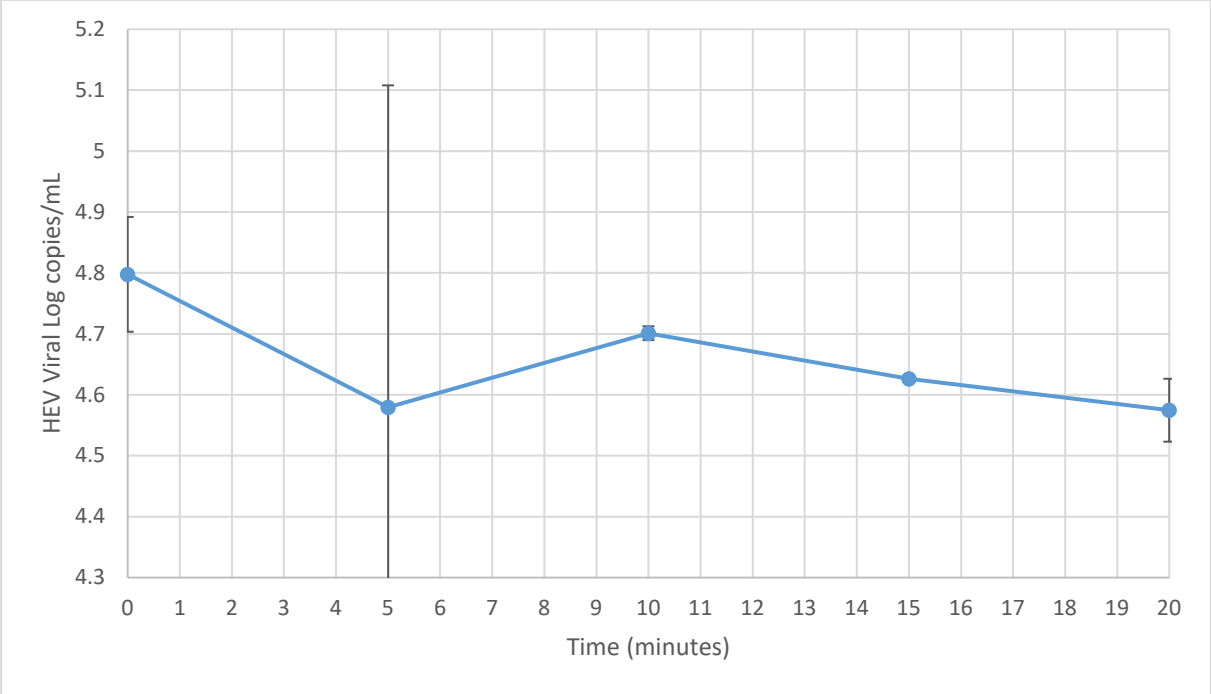
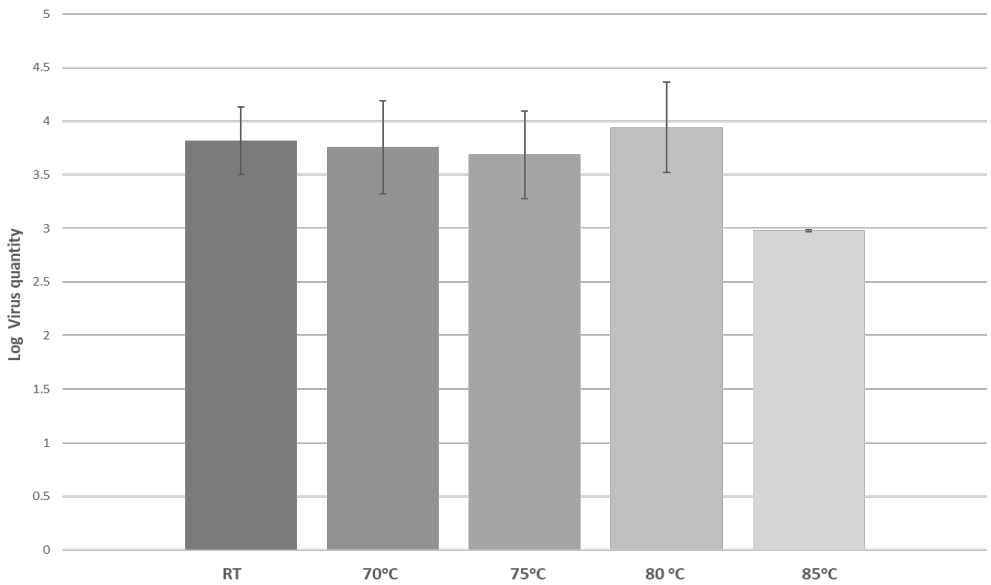


Figure 17: Thermal inactivation at 95˚C, over 20minutes of HEV derived from porcine faeces. p=0.692.



For the faecal porcine virus, no significant change was observed at 70, 75, 80, 85˚C or 95˚C (Figures 13-17).

Figure 18: Effect of short term heating on HEV stability. Viral source was serum and the period of time assayed was 2 minutes. Columns represent means of three independent tests and log of viral RNA detected by PCR. RT=room temperature equivalent to no thermal treatment.

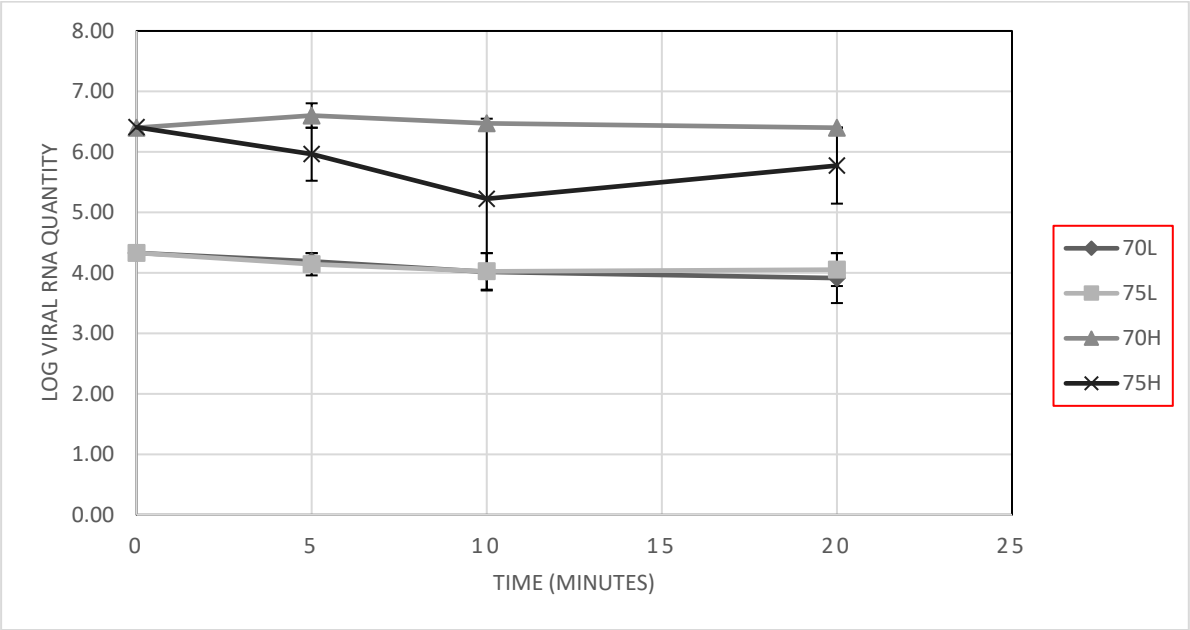


In addition to generating the data to feed into the model, based on the literature we also looked at the reduction in HEV by RT-PCR detection over a short time period and the effect that starting input may have on the log reduction observed.

Analysis of the effect of short term heating over a period of 2 minutes did not reveal any significant change in the thermal response of serum-derived HEV until a temperature of 85˚C was achieved (p=0.019) (Figure 18). Virus input for replicates ranged from log 4.13-4.17.

From the data obtained, we were also able to assess if viral input influenced the response to thermal treatment for the cell culture adapted HEV. High virus input was represented by log 6.4 and low input was equivalent to log 4.33. When we examined the effect of high and low input virus, we saw no significant variation in the slope or log reduction at 70ºC (average log reduction = 0.285) or 75˚C (average log reduction = 0.253) at low virus input (Figure 19). There was no significant difference between the average log reduction at both temperatures (p>0.05). At high virus input, we saw a change in average log reduction at 70˚C (average log reduction = 0.095) but a higher one at 75˚C (average log reduction = 0.611). The difference between the log reductions at the higher viral input were found to be significant between the two temperatures (p=0.044) (Figure 19).

Figure 19: Comparison of high and low viral input for cell culture derived HEV.



Cell culture virus appeared more sensitive to thermal treatment > serum derived HEV > faecal derived HEV.

6.3 Discussion

Much fewer data are available in the literature at the higher end of temperature (70-95˚C) and those relevant to cooking than at lower temperatures, indicating a gap in the evidence needed to assess the impact of thermal inactivation on HEV at temperatures relevant to domestic cooking behaviours. This work sought to complete the gap by providing a time series at cooking-relevant temperatures using three different sources of HEV. In addition, we expected the 3 sources of the virus to contain both HEV (faeces) and eHEV (serum/cell culture adapted virus) in order to determine the role this may play in thermal stability and represent virus present in liver and products containing blood/serum respectively.

Overall, regardless of viral source input or initial copy number used, there was no significant difference observed in the inactivation/degradation of HEV at each temperature as detected by PCR.

The limitations of the data are as follows:

1. Reduction in thermal stability was not able to be correlated directly with infectivity due to lack of suitable assay.
2. No complex matrix background was introduced.
3. The presence of residual RNA particles being detected at the temperatures of 70, 75, 80, 85 and 95˚C is not accounted for, i.e., we did not use the capsid integrity assay.

With respect to point 1; it is not possible currently to assess this and it was not the focus of the aims. Currently, there is no agreed extraction method for HEV in a matrix and the focus of this work was to develop a model that could be adapted at a later date to include such data. Regarding point 3, it is not possible to completely remove degraded RNA using capsid integrity methods; indeed, Walker et al (2019) indicate that this method would over estimate infectivity. This needs to be considered when assessing viable and no-viable viral presence. As described in section 5.3, to date,

no studies have been reported which have demonstrated a relationship between HEV capsid integrity and infectivity. For the purposes of our study, it could be argued that the use of capsid integrity does not fully reflect the range of viral states present in food, i.e., raw or partially cooked food may contain a mix of intact and damaged viral particles due to the nature of the matrix suspension. This data may reflect the realistic detection of nucleic acid over time as opposed to infectivity analysis which is yet to be done.

Schielke et al. (2011) assessed the thermal response of a liver suspension at 70, 75, 80, 85, 90 and 95˚C for one minute. In our study, we used a minimum time of 2 minutes to reflect the recommendations of the FSA. This is the most comparable data to ours albeit the source and use of the capsid integrity assay introduce variables. The exposure of HEV to 70°C for one minute led to an average log reduction of protected RNA of 0.48. At 75°C the reduction rate slightly increased (-0.72 $\log_{10}$). Incubation at 80°C and 85°C for one minute resulted in an average 2.47-$\log_{10}$ and in a 2.58-$\log_{10}$ reduction, respectively, whereas the incubation at 90°C for one minute led to a 3.58-$\log_{10}$ reduction. Finally, the exposure of HEV to 95°C for one minute resulted in a log reduction of 3.67. The range of $\log_{10}$ reduction as Schielke et al (2011) describe isn't considered to be significant until a temperature of 90˚C is achieved. We would argue that anything below 0.5$\log_{10}$ is a significant reduction (Walker et al; 2019).

In our study, for faecally derived virus, which could be considered similar to liver, we observed lower log reductions when compared to the study above. This reduced reduction = compared to 90˚C, over one minute (Schielke et al., 2011), could be potentially explained by i) the extended time of two minutes at each temperature; and ii) our analysis used HEV derived from serum, faeces and cell culture in comparison to liver derived HEV. It had been previously indicated in the literature that liver derived HEV was more stable than cell culture adapted virus and the reduction in viral quantity/infectivity was observed to be less in liver derived HEV (Johne et al., 2016; Randazzo et al., 2018; Schielke et al., 2011). For the other viral sources, the overall times were longer so no further comparisons can be drawn.

Based on the literature review and the data presented, if we focus on the relevant cooking temperatures starting at 70, 75 and 80˚C; we do observe some differences

between our experimental data and that described previously. We did not observe any significant reduction in thermal stability at these temperatures, however, Barnaud et al. (2012) showed that at 71˚C for 20 min, HEV infectivity was eliminated, but reported that at 10 min at this temperature, some virus infectivity remained. Again, the measure of outcome here is different; we have detected viral RNA not infectivity. However, the limit of detection for the cell culture system used by Barnaud et al, (2012) was 10^4–10^5 genome copies, and therefore, some viable virus may have remained in the minced pork but not have been detectable using the cell culture system.

At temperatures of 75˚C and above, Johne et al. (2016) observed complete or almost complete elimination of infectivity of cell culture derived virus as assayed by cell culture, after 1 min. However, some infectious virus could still be detected at 75˚C, but not at 80˚C. Without full assessment of all viral sources by infectivity assay vs RNA detection we cannot provide a comparison but our data is consistent in showing that HEV RNA is thermostable up to 85˚C.

Another caveat of our data is that it could be argued that the viral input contributed to the lack of reduction seen at 70, 75, 80 and 85˚C, however, we do see reductions at 95˚C. An average viral input of 15,000 or 20,000 copies was used for serum derived virus due to availability (log 4.0-4.17) but for the porcine faecal virus and cell culture derived virus, higher inputs were also assessed. In figures 1-17, this did not appear to affect the log reductions observed across the different viral sources or the percentage reduction (Tables 3-5). However, individual analysis of cell culture derived virus did provide some evidence that higher viral input gave a lower log reduction at 70˚C but this did not follow at 75˚C. Indeed, discrepancies in the literature have also been observed. The high amount of virus input used by Schielke et al. (2011) may have contributed to the low log reduction seen over time at 56˚C; however, this did not follow at 60˚C. Using higher starting HEV numbers at temperatures of 75˚C and above, Randazzo et al. (2018) and Scheilke et al. (2011) also observed lower reductions in viral quantity using capsid integrity assays at this time / temperature combination. When we consider higher temperatures of 95˚C that have been described in the literature for inactivation of HEV, we see that a proportion of capsids remain potentially intact and HEV RNA detectable therefore it is not impossible to observe lower reductions in detectable RNA as seen in our study.

(Schielke et al., 2011; Randazzo et al., 2011). This needs to be addressed further. In terms of food, it is anticipated that virus will be present at low quantities in some foods but higher in others ($1x10^2$ to $2.3x10^6$) as described for products containing raw pork liver and $7x10^4$ copies/g in liver pate (Pavio et al., 2014; Boxman et al., 2019). The oral infectious dose for humans remains unknown.

Our data does indeed support the observation that HEV is more thermally resistant than expected and that cooking at 70-75˚C may not be sufficient to fully eliminate viral RNA. Overall, our data suggests that viral particles and subsequent nucleic acid is detectable over a 20minute time period up to 95˚C, however, if that virus remains infectious is a question still to be answered. Utilising RT-PCR rather than a culture assay does create a caveat that although we can detect the virus nucleic acid, it may indeed be rendered non-infectious. Until, we can replicate this in an infectivity assay then we can only predict the required thermal treatment and time to reduce the detection of virus.

Further research is clearly required to investigate HEV inactivation within food matrices in a consistent manner as we have done here. This may require a more efficient cell culturing method and an assessment of different foods, cooking methods and HEV strains. Importantly, it will be very useful to correlate the PCR detection with infectivity as this has not yet been done. However, taking the results of studies conducted in non-food and food (pork product) samples together, a conservative measure would be to cook pork products for longer than 20 min at temperatures higher than 72°C (Treagus et al., 2021) however, this may affect sensory quality.

Overall, the outcomes were:

1. HEV RNA from either faecal, serum or cell culture sources is stable and detectable by RT-PCR up to 85˚C over a period of 20 minutes. The relationship between these results and whether the virus is still infectious requires further study.
2. Viral quantity appears not to affect the rate of log reduction.
3. The origin of the virus can influence the reduction in viral detection and should be considered as independent in the model.
4. This data provides a consistent time series over relevant temperatures to carry over to the model.

7. Construction of the predictive model

7.1 Introduction

Mathematical models to predict the thermal inactivation of food-borne pathogens assist in developing adequate thermal processes. The generation of precise thermal process data and the establishment of proper thermal processes for inactivating HEV in food is important both for consumers and for industry.

A number of different models were reviewed, including a simple log-linear model, before the Weibull predictive-microbiology model was selected. It was found that the simpler models didn't fit the data well, whilst more complicated models (Bozkurt et al.; 2014) proved too complicated to achieve reliable/consistent convergence for the available data. A Weibull predictive microbiology model was selected as a suitable means to describe the thermal death of Hepatitis E virus (HEV) particles in foodstuffs (Smith R. , 1987), and fitted to available data using a Bayesian approach. The model equation takes the form:

$$N_t = N_0 \, exp\left[-(A\,t\,)^{\beta}\right]$$

where, N_t is the number of surviving virus particles left after a sample has been exposed to a particular temperature T (Celsius), for time t (minutes), and N_0 is the number of particles initially, before any heating has taken place. A is a constant frequency parameter, whilst β is a constant shape parameter, and both are different for each temperature T. These parameters are fitted using existing data from the literature described in section 5.0, alongside the new data from experiments carried out at Glasgow Caledonian University (GCU) and described in section 6.0, both of which present the log reduction in HEV over time, for various times and temperature combinations.

The model has been coded into a user-friendly ‘Shiny’ app that runs within the RStudio software, and a separate user manual provided to guide the user through the operation of this app. The RStudio runs upon the open source R-Project software language for statistical programming.

In summary, a model has been fitted which estimates parameters, for each temperature (T) and to allow the virus remaining after time (t) to be predicted. Experimental data gathered during this project is presented over 10 or 20 minutes. The general hypothesis is that the lower the temperature the longer the time required to inactivate the virus. The literature data was complex and highly variable, and it was difficult to predict a trend due to the different sources and different detection methods used.

In what follows; background information is presented, to support our choice of predictive microbiology model, and outline the benefits that it provides over others.

Having selected an appropriate model, an exploratory look into the literature data is presented. The aim being to gain an insight into the data's relevance to cooking foodstuffs, alongside its usefulness for informing the predictive model. This highlights the sparseness of the available literature data (relevant to cooking foodstuffs). However, despite the obvious data gaps, it was still possible to fit the Weibull model for a singular temperature and virus source combination.

Motivated by the obvious gaps within the literature review data, we next explore the new data generated from the experimental work carried out as described in 6.0. First the data is presented on its own, then the Weibull model is fitted to the data for various temperature and virus source combinations.

The model was then successfully validated against further experimental trials carried out at GCU. Finally, we present a discussion of the findings, along with suggestions for future work.

When combined with equation (1) or (2) below, the median estimates presented in Tables 3-19 for model parameters A and β allow for the reduction in HEV particles to be predicted at any given time. These parameter estimates are temperature dependent, and thus predict the reduction for the temperature that each table corresponds to. Likewise, they are intended to be unique to the viral source of the HEV that they have been estimated from (i.e., cell-culture, faeces, or serum).

Plots of the corresponding fitted model curves, for each temperature and virus source can be found in Figures 30-45.

Naturally, as both the quantity and quality of available data increases, these estimates will further increase in accuracy.

7.2 Materials and Methods for the predictive microbiology model

7.2.1 Review of potential models

Initial models proposed included a simple log-linear model of the form:

$$\log \mathrm{N_t} = \beta_0 + \beta_1 \mathrm{T} + \beta_2 \mathrm{t}$$

where N_t is the number of remaining virus particles at time t, and temperature T. The three $\beta_{1,2,3}$ parameters were then to be fitted to the data using non-linear least squares or bootstrapping techniques. However, it soon became obvious, due to the non-linear nature of the literature data, that the log-linear model was unsuitable.

It was subsequently agreed that a better approach would be to adopt a predictive microbiology model which would be based on biological dynamics rather than only a description of the data. The first of these predictive microbiology models was selected from (Bozkurt, 2014), and took the form:

$$N_t = N_0 \left[\exp\left\{\left(-Aexp\left(\frac{-E}{8.314T}\right)t\right)\right]^{\beta}\right\},$$

where N_t was the number of remaining virus particles at time t, for temperature T (Kelvin). N_0 was the initial particle count, whilst parameters A, E and β corresponded to a constant frequency factor, the virus activation energy, and a constant shape parameter, respectively.

Attempts were made to fit this three-parameter model to the data, using Bayesian inference techniques, however, the estimated parameter values proved too dependent upon their prior estimates. Parameters A and E in particular, would only converge when the other effectively became fixed. This led to alternative approaches being sought, and subsequently this three-parameter approach was dropped, in favour of a more established, two-parameter Weibull predictive microbiology model.

7.2.2 Mathematical model

Variables such as genotype and source of virus were evaluated as to how they would impact the chosen predictive microbiology model. This was done based on indications in the literature which suggested that a one size fits all option is not possible. As a result, it was decided to fit the model to each of the virus sources detailed in section 6.0 separately – the main HEV sources available relevant to foodstuff described as serum, porcine faecal and cell-culture adapted virus. Unfortunately, infected liver was unavailable for assessment.

In what follows, a two-parameter Weibull mathematical model (Smith R. , 1987) is introduced, which takes the form $N_t = N_0\, exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right]$, where α and β are the scale and shape parameters, respectively (Cunha, 1998). The Weibull mathematical model is successfully used to predict the thermal death times for Hepatitis E virus (HEV) in foodstuffs. Commonly used in survival analysis to predict the time to death of biological organisms, and within engineering applications to predict time to failure of components parts (Smith R. L., 1991), the Weibull model has also recently been used to describe the thermal inactivation of Hepatitis A virus (HAV) in blue mussel (Bozkurt, 2014).

The two-parameter Weibull model equation takes the form:

(1) $N_t = N_0\, exp\left[-(A\, t\,)^{\beta}\right]$

where, N_t is the number of surviving virus particles left after a sample has been exposed to a particular temperature T (Celsius), for time t (minutes). N_0 is the number of virus particles initially, before any thermal treatment has occurred. Here, A is a constant frequency parameter and β is a constant shape parameter. These will be different for each temperature.

Using rules of logs:

$-\big||\text{Log Reduction}|\big| = [log_{10}[N_0] - log_{10}[N_t]]$

$= \log_{10}[\,N_t\,] - \log_{10}[N_0] = \log_{10}\left[\frac{N_t}{N_0}\right]$

and rearranging equation (**1**), the negative of the log reduction, in the number of virus particles at time t is given by the equation, (Equation 2):

$$-\left|\left|\text{Log Reduction}\right|\right| = log_{10}\left[\frac{N_t}{N_0}\right] = log_{10}\left(exp\left[-(A\,t)^{\beta}\right]\right)$$

$$= \frac{1}{2.303} ln\left(exp\left[-(A\,t)^{\beta}\right]\right)$$

$$= \frac{-1}{2.303}(A\,t)^{\beta}$$

2

For a given temperature, the model parameters A and β are then estimated using Bayesian inference, within the open-source R software (R Project, 2021). This is achieved using the JAGS (Just another Gibbs Sampler, mcmc-jags.sourceforge.net) package, which uses Markov chain Monte Carlo simulation (MCMC) to estimate the parameters. Doing so allows us to account for some of the uncertainty and noise found within the literature/GCU trial data. The resulting model has been coded into a user-friendly Shiny app that runs within the RStudio software.

For the Bayesian MCMC simulations, the log change data (negative log reduction) is assumed to be normally distributed, with mean given by equation (**2**), and variance described by a gamma distribution; Gamma (1,4) with shape parameter 1, and rate parameter 4. The values of the gamma distribution were selected by preliminary analysis, and deemed to be a suitable prior given the available data

Priors for the two parameters A and β are assigned uniform prior distributions, covering the ranges [0, $2\mu_{A_0}$] and [0, $2\mu_{\beta_0}$], respectively. Where μ_{A_0} and μ_{β_0} are sensible initial guesses as to the true value of the parameters A and β.
The JAGS simulation uses a burn in period of 1,000 MCMC iterations (these are early samples which are subsequently discarded), followed by 5,000 further MCMC iterations, across three separate chains (to allow different starting values).

The model then returns estimated values of the parameters A and β via a summary table. This contains the mean parameter estimate, alongside the standard deviation, 2.5% quantile, median and 97.5% quantile.
Additional statistics labelled “Rhat” and “n.eff” are also returned. Here, Rhat is the Gelman-Rubin convergence diagnostic and compares the estimated variance between-chains and variance within-chains used by the Bayesian simulations for

each model parameter. Therefore, if these two variances are similar (i.e. 1.0), it means the chains have converged, whilst, If the difference between chain variances is bigger, it means the chains have not converged. Thus the standard approach is to assume we do not have convergence, if the Gelman-Rubin diagnostic value is greater than 1.05 (noting: here, 1.05 an arbitrary choice, but is generally accepted as standard practice).
“The second parameter “n.eff” represents the effective sample size.

Trace and density plots of the MCMC simulation are also returned. These density plots are essentially smoothed histograms of the estimated values of each parameter (usually termed the “posterior distribution”) and indicate the likelihood of observing the parameter values on the x-axis.

7.3 Results

7.3.1 Data distilled from the literature review

The literature review data provides an initial insight into the survival characteristics of HEV for various time-temperature combinations. To visualize this data and the relationship between time, temperature, and reduction in HEV particles that it describes, Figure 20 plots the log change (negative log reduction) in HEV particles against time. This plot focuses on the time range 0-60 minutes, which contains the data points most relevant to food preparation/cooking times, however timescales of up to 10 weeks are observed within the full literature dataset (Appendix 1). Note: Data plotted in this section is purely literature data, and excludes new data obtained by experiments carried out during this project. In total, 86 data points were evaluated from the literature screen described in section 5.2 out of 95 (Table 2; Appendix 2). After exclusion and validation, a final total of 52 data points were put forward for the final model. Data with < 3 points were unable to be fitted to the model.

To further simplify this initial visualization, the literature data was split according to the source of the HEV it describes, with: figure 21 and figure 22 depicting HEV sourced from porcine liver; figure 23 depicting HEV sourced from soil; figure 24 illustrating HEV sourced from faecal matter; and figure 25 - 26 HEV sourced from cell

culture adapted virus. This emphasizes any characteristic behaviors that are unique to each virus source and illustrates the full range of times found within the literature.

In figures 21-26, data points were plotted as per method of detection either PCR or cell culture.

Figure 20: ALL literature data. Log change plotted against time, 0-60 min range.

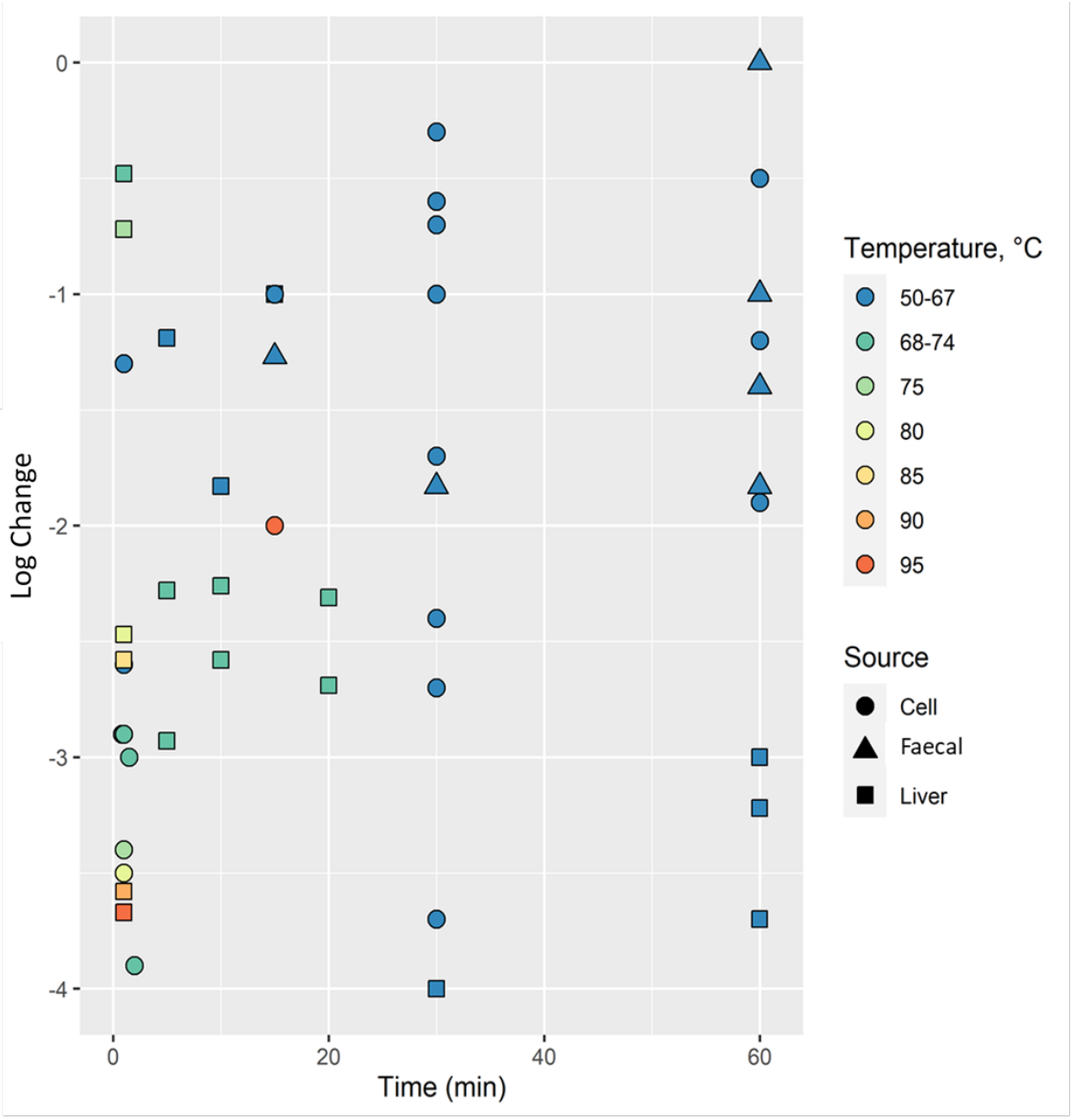


Figure 21 - Literature data, illustrating the log change for HEV sourced from liver.

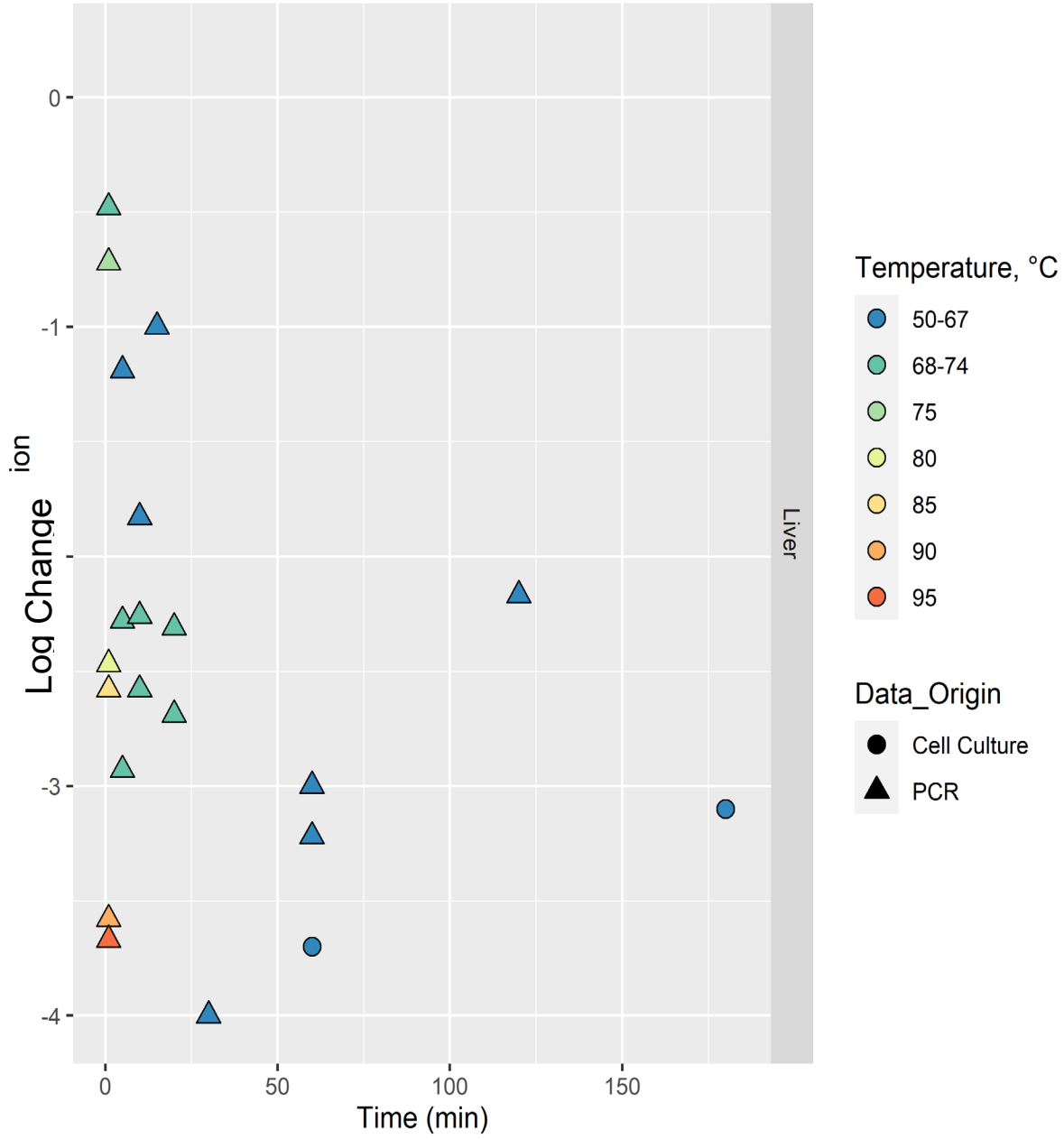


Figure 22 – Literature data, illustrating the log change for HEV sourced from liver (times 0-60mins).

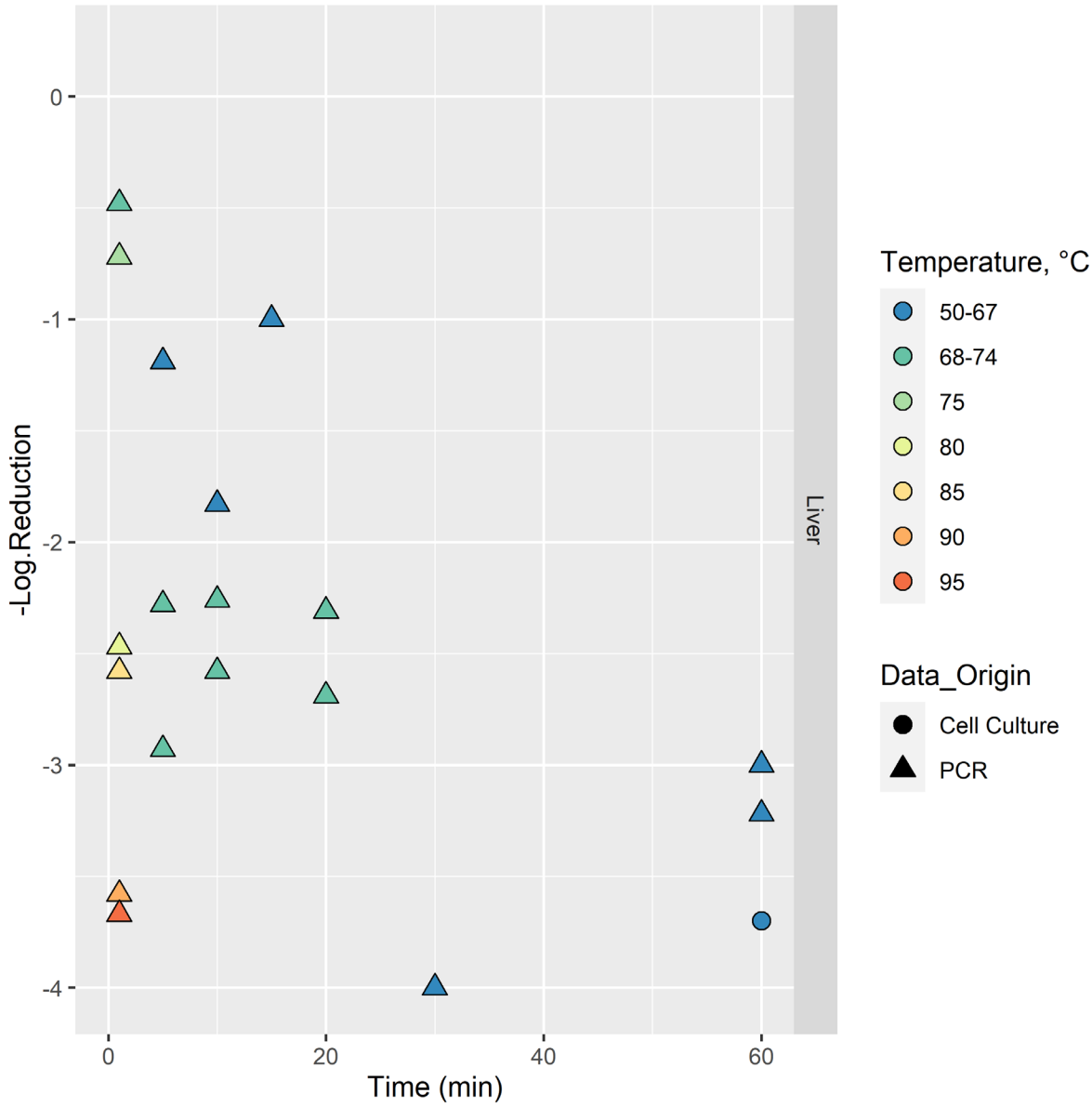


Figure 23 – Literature data, illustrating the log change for HEV when evaluated at 37°C in soil.

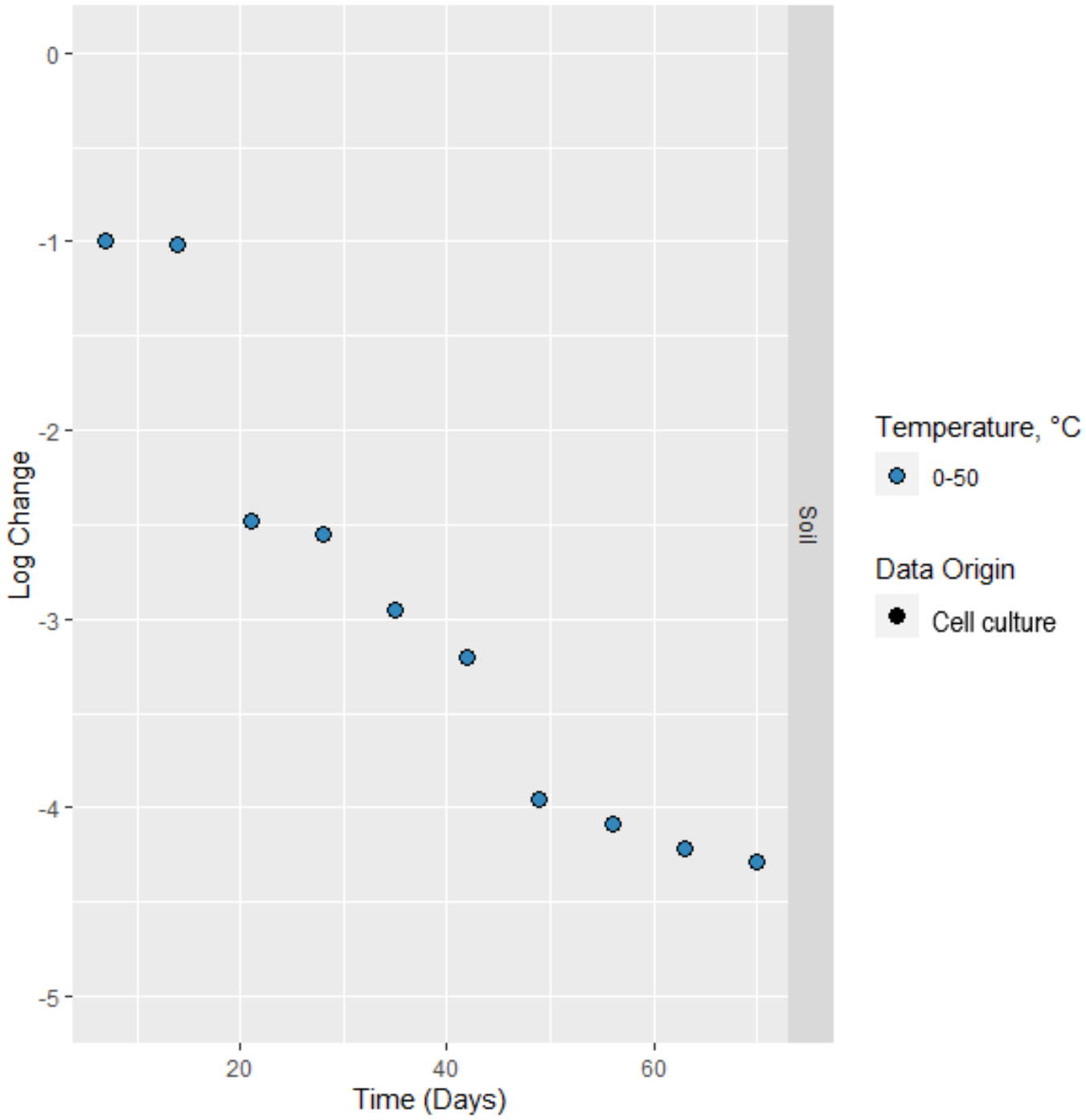


Figure 24 - Literature data, illustrating the log change for HEV sourced from faeces.

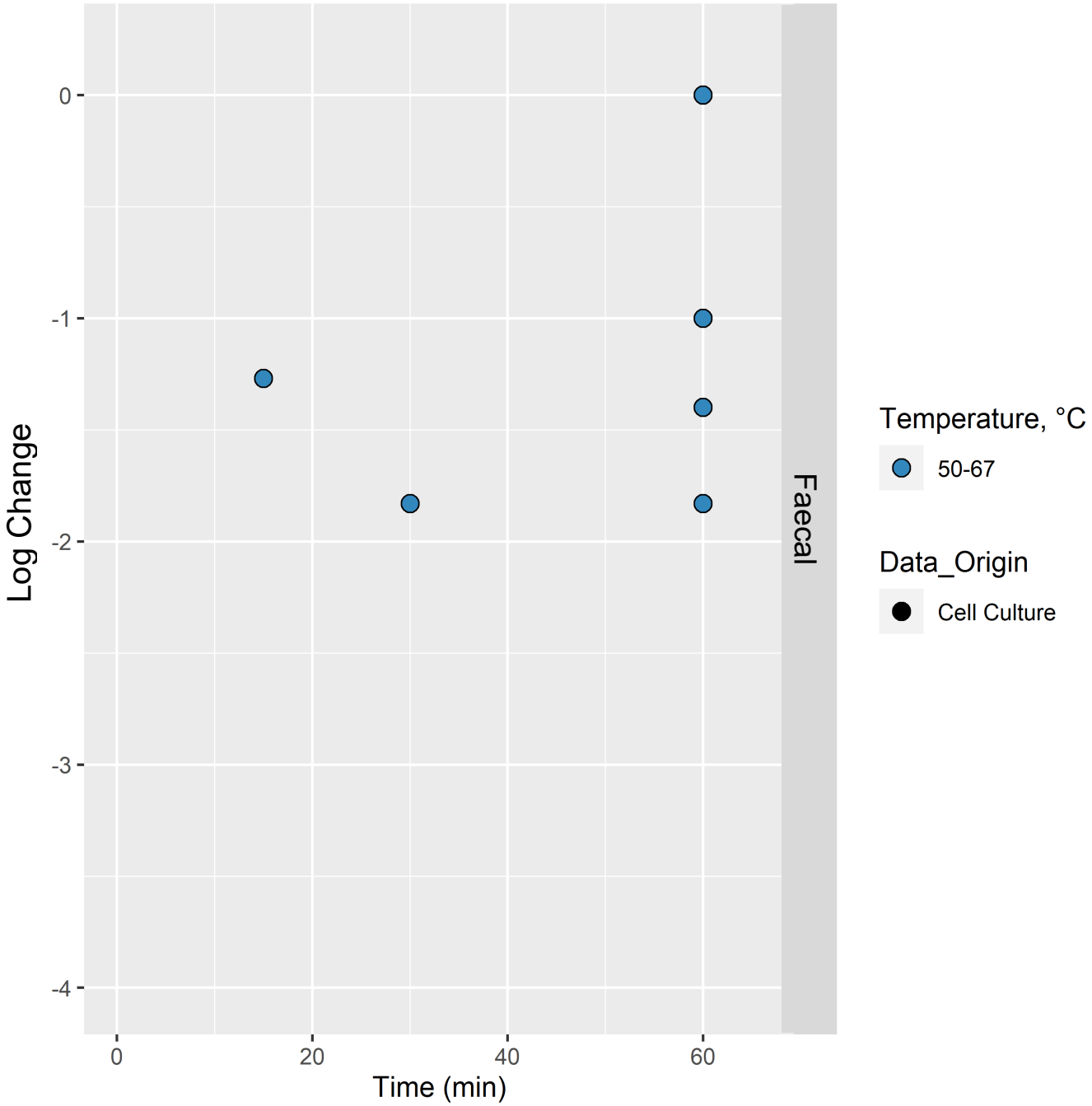


Figure 25 – Literature data, illustrating the log change for HEV sourced from cell culture.

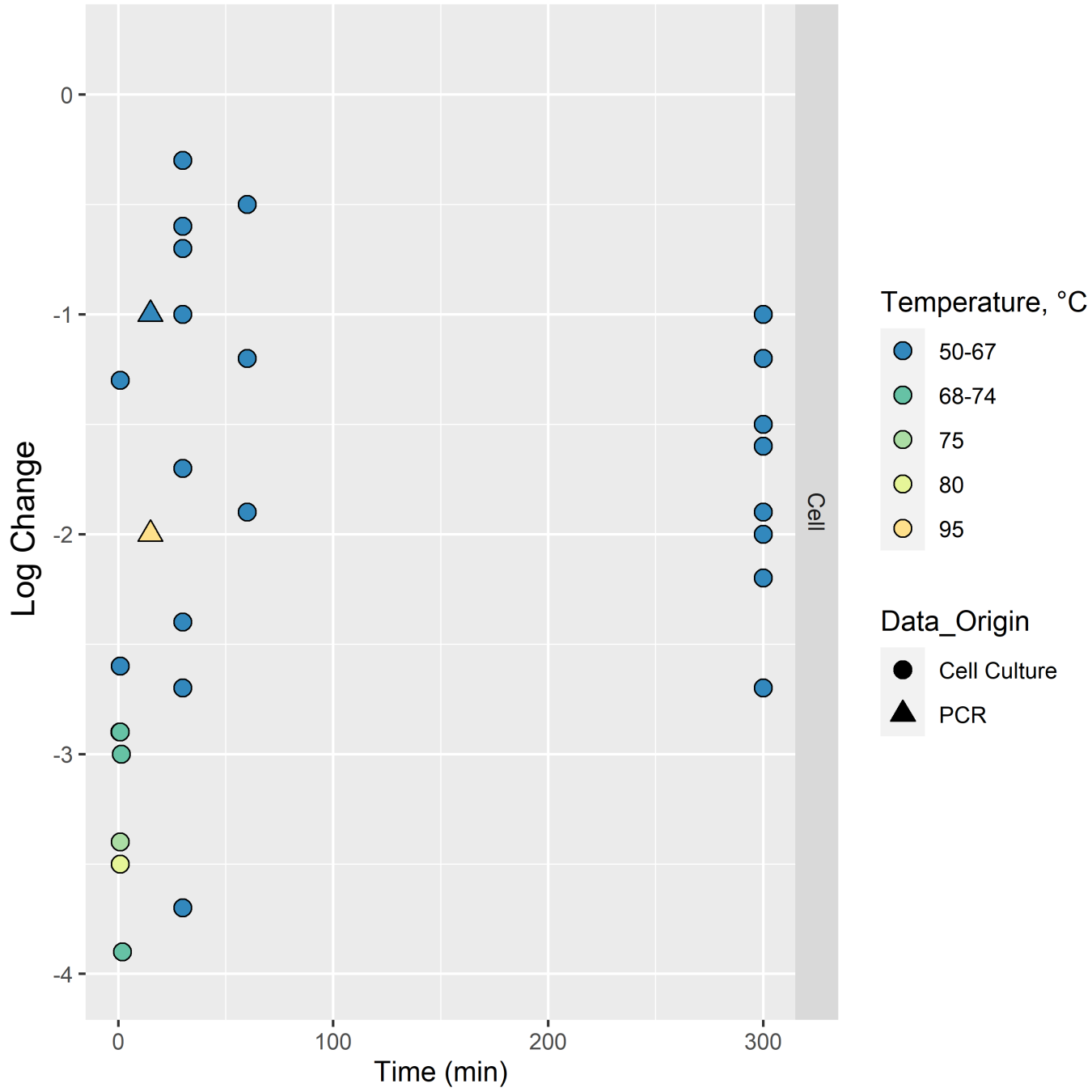
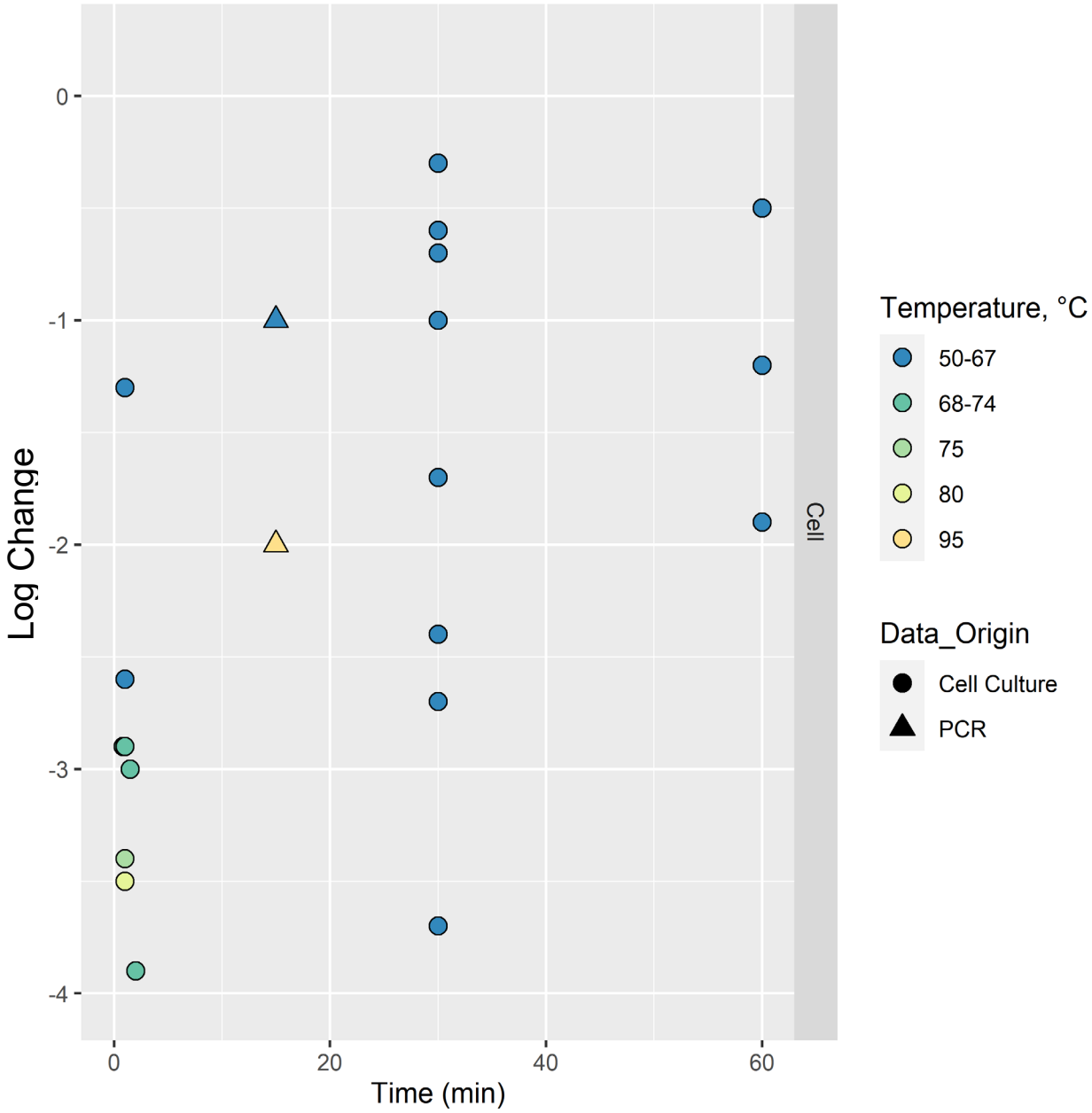


Figure 26 – Literature data, illustrating the log change for HEV sourced from cell culture. Area amplified to focus on data up to a maximum time of 60 minutes.



Analyzing these plots highlighted several issues and data gaps within the existing literature data. In figure 20, it can clearly be seen that for several time-temperature combinations, the observed log reduction covers a very wide range of values. Take for example, the 50-67˚C temperature range at 30 minutes; the literature data suggests the magnitude of the log reduction could lie anywhere between 0 and 4. Similar wide variability can easily be seen at the 60-minute mark, where log reductions are again scattered across the same range, from 0 to 4.

This large variance and noise within the combined dataset (figure 20) motivated separating the literature data by HEV source, as per figures 21-26, to see if this produced more defined trends within the data. However, this was not the case, and there was still significant variation and inconsistency present within the data for each source. Instead, it became more obvious that there were significant data gaps within the literature data, for temperatures and times that were relevant to cooking food. Here, it was deemed appropriate to consider data for temperatures > 70˚C and times < 60mins, as appropriate for cooking food.

Figures 21 and 22 depict the data for HEV sourced from liver. Clearly there is a wider range of relevant temperatures represented by this data (up to 95˚C), and a good range of data points for the 75˚C data. However, the data is sparse for the other temperatures, where there are significant data gaps for times > 1 min, providing clear indication that additional experimental data to fill in these data gaps would be extremely useful.

Figure 23 illustrates the literature data for HEV sourced from soil. Clearly there is a defined pattern here, however the observation times cover a lengthy 10-week period, for temperatures below 50˚C. Subsequently these times and temperatures were deemed irrelevant in the context of cooking food and removed from the dataset used to contribute to the model.

A similar story can be seen in figure 24, for the faecal virus data, as well as figures 25 and 26 for the cell culture derived virus data: for both sources there is a significant lack of data at times greater than around 5mins, for temperatures that are relevant to cooking as discussed in section 5.4. There is also only a very limited number of temperatures represented at those relevant to food. This further motivated additional

experimental work to fill in these data gaps and improve upon the existing literature data set.

In figures 20-26, there is no observable relationship between log reduction and time, whereas it is theorised that, for thermal treatment, reduction in virus particles generally increases as time passes due to degradation process of the external viral capsid or lipid membrane and release of the viral RNA (Treagus et al, 2021). As mentioned in sections 5.4 and 6.3, in the literature, reduction of viral nucleic acid is not correlated with infectivity and the data has non comparable methods of detection to provide clear associations with.

Experimental work was proposed to gather time-series data for temperatures in the 70-100°C range. In addition, the proposed experimental work was to include at least two different HEV sources to compliment the sources found within the literature and be representative of the typical HEV found in pork products.

7.3.2 Fitting model parameters to the literature review data

Initially we fit the model described by equation (**2**) to the literature review data for each of the virus sources. However, for temperatures > 50°C and times within the 0-20 mins range that we have focused upon, due to its relevance to cooking, the literature data is exceptionally sparse, and includes no data whatsoever from HEV serum sources which would be relevant in certain porcine food products such as those which may contain serum fillers or blood.

Figure 27 - Model curve fitted to (very limited) literature data for 71°C, for HEV source: porcine liver by PCR. Solid line indicates the median.

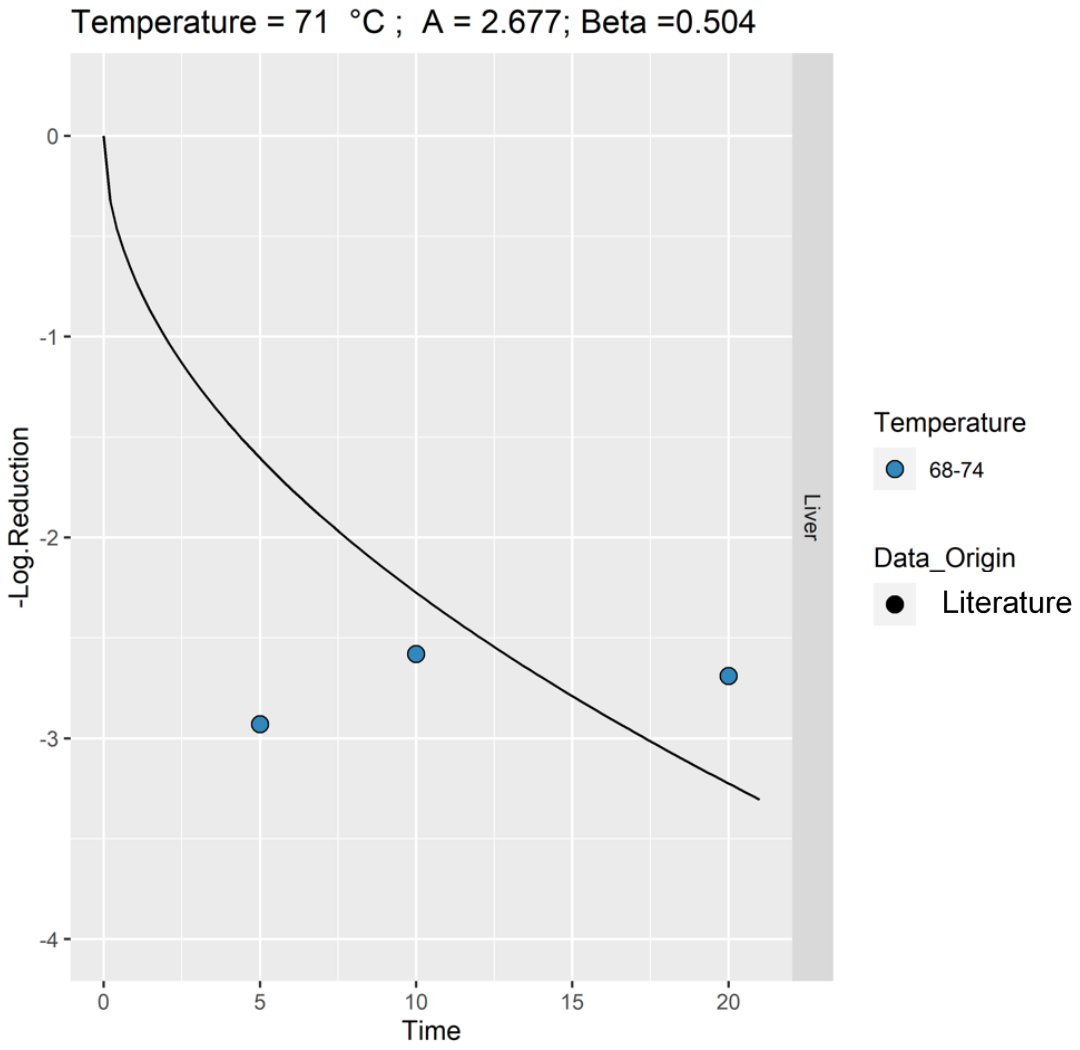


Table 6 – Estimated model parameters. Fitted to literature data for 71°C, for HEV source: Liver.

71c	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	2.554379	0.951431	0.650578	2.67716	3.94234	1	1742
β	0.520126	0.102426	0.365523	0.503758	0.781679	1.01	1455

7.3.3 Fitting the model parameters to the experimental data

In response to the findings of Sections 7.3.1 and 7.3.2, experimental work was carried out to further populate the large data gaps discovered within the literature dataset. The experiments were designed to cover specific cooking temperatures in the 70-100°C range, and time periods up to 20 minutes. In addition, by using a source-directed approach, we believe that the predicted values will be more objective and robust as opposed to an integrated model.

Experiments calculated the log reduction of HEV particles from different sources at 70ºC, 75˚C, 80˚C, 85˚C, 95˚C and 100˚C, although some additional temperatures were also investigated. For each temperature, the remaining HEV particles were measured at times of 0, 2, 4, 6, 8, 10, 15 and 20 minutes, and the corresponding log reduction was calculated for each. Again, some additional data at single alternative time points within this same range were also recorded. Amongst these were: experiments at 65˚C for 10mins; 70˚C for 2 mins; 75˚C for 52 sec and 75˚C for 2mins. HEV sourced from serum, cell-culture and faecal sources was used in the experiments; and the corresponding results can be found in figure 28, figures 29 and 30, respectively. In total, 204 experimental data points were evaluated from the new experimental work described in section 6.0. After validation to the quality criteria described in section 6.1, 180 were used in the final model.

Clearly this new data fills in a lot of the knowledge gaps that were missing from the literature data, for the temperatures (65-100°C) and times (0-20 mins) that were deemed most relevant to the cooking of pork products. Obviously, there is still some noise within the data, however, the model accounts for this inherent variability within

the data by using the Bayesian approach to fit the model parameters, which helps smooth out some of this noise.

To illustrate where the experimental data fills the gaps in the literature data, this new experimental dataset is plotted alongside the literature data in Figure 31; This new data successfully populates the data gaps within the range of temperatures and times relevant to cooking pork. The result is a more complete and consistent dataset, that can be used to fit the parameters of our chosen mathematical model. Note: Some literature data outliers have been removed from this plot.

Figure 28 - Experimental data. HEV sourced from Serum.

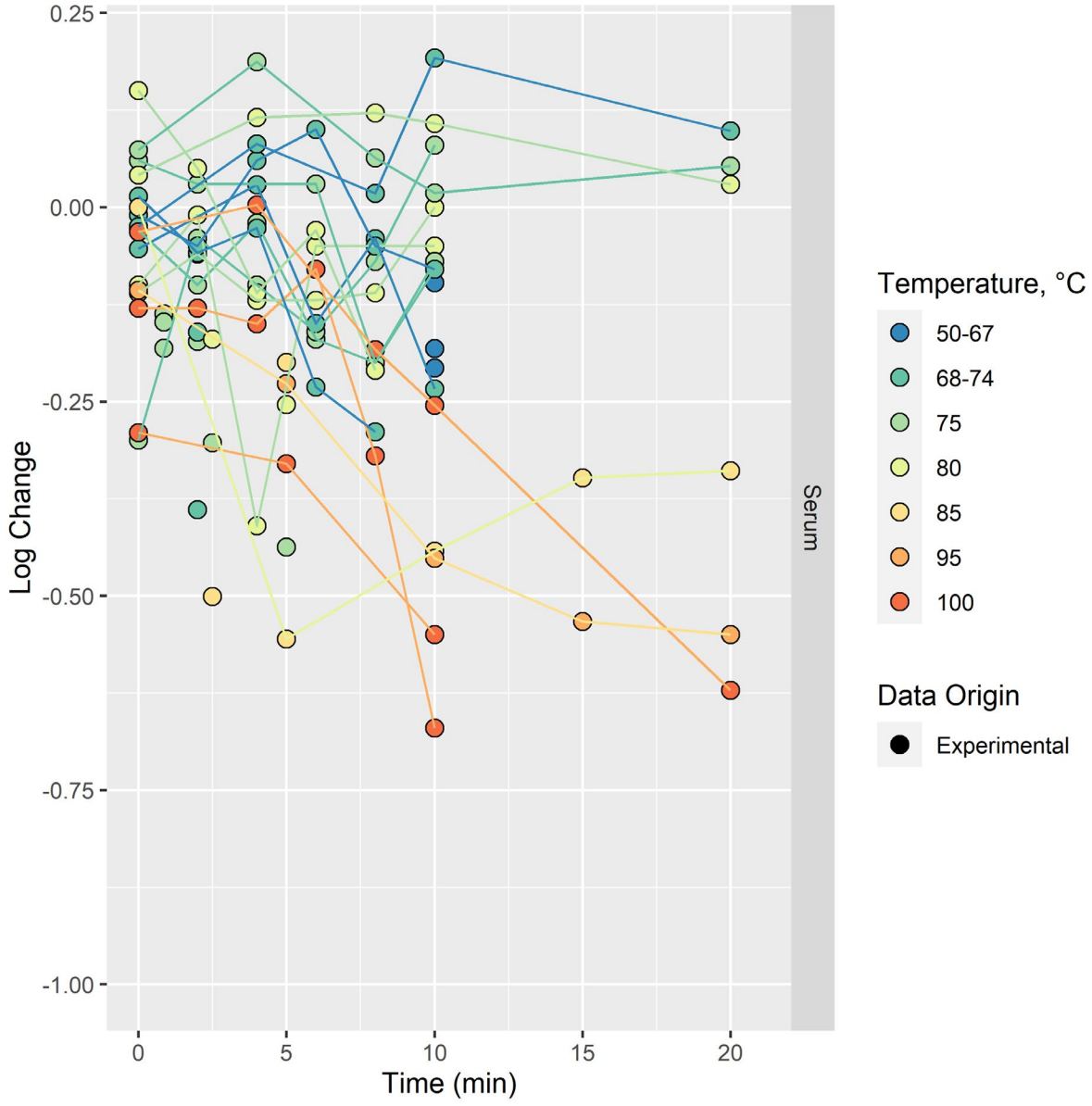


Figure 29- Experimental data. HEV sourced from cell-culture.

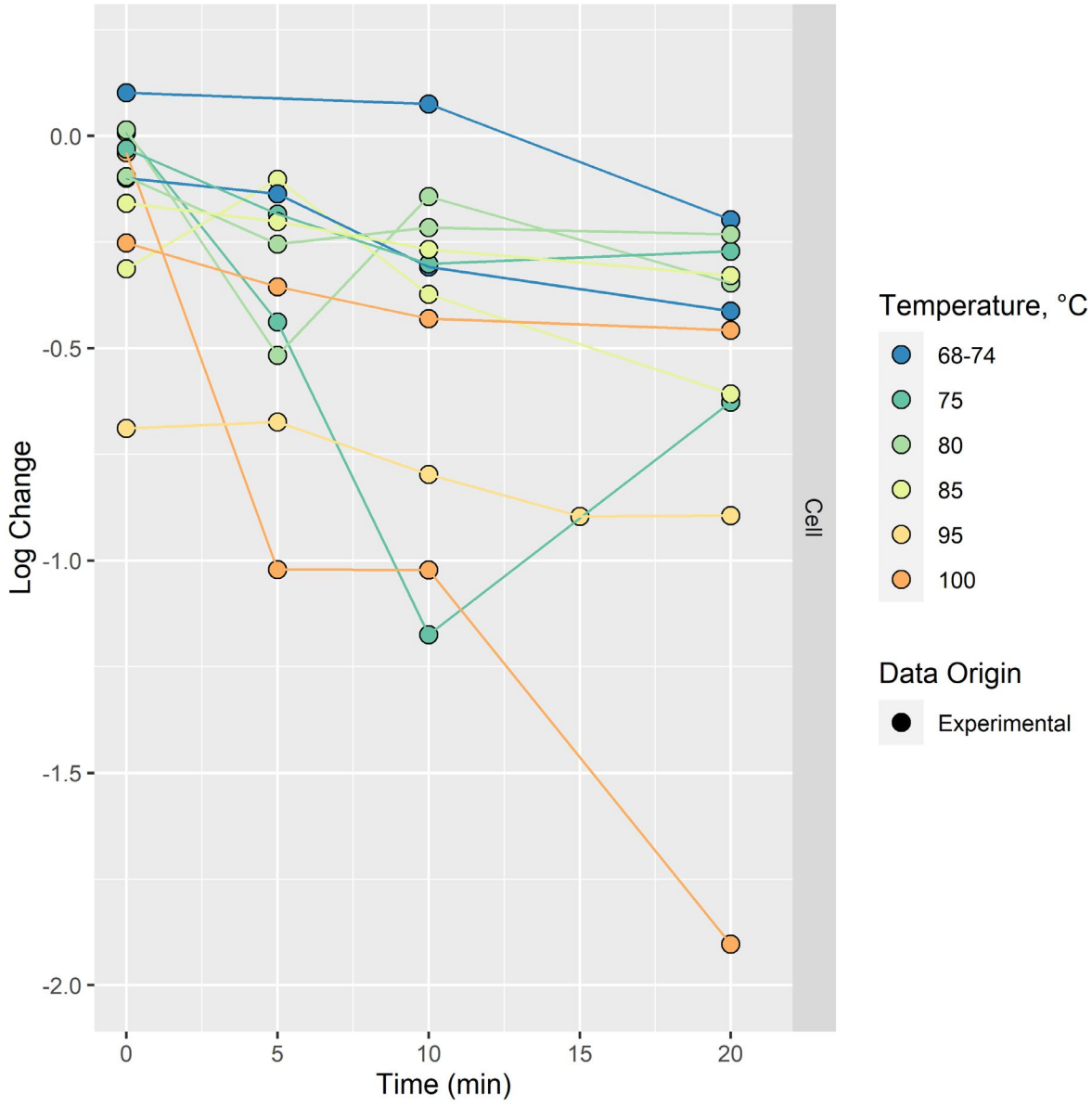


Figure 30 - Experimental data. HEV sourced from faecal sources.

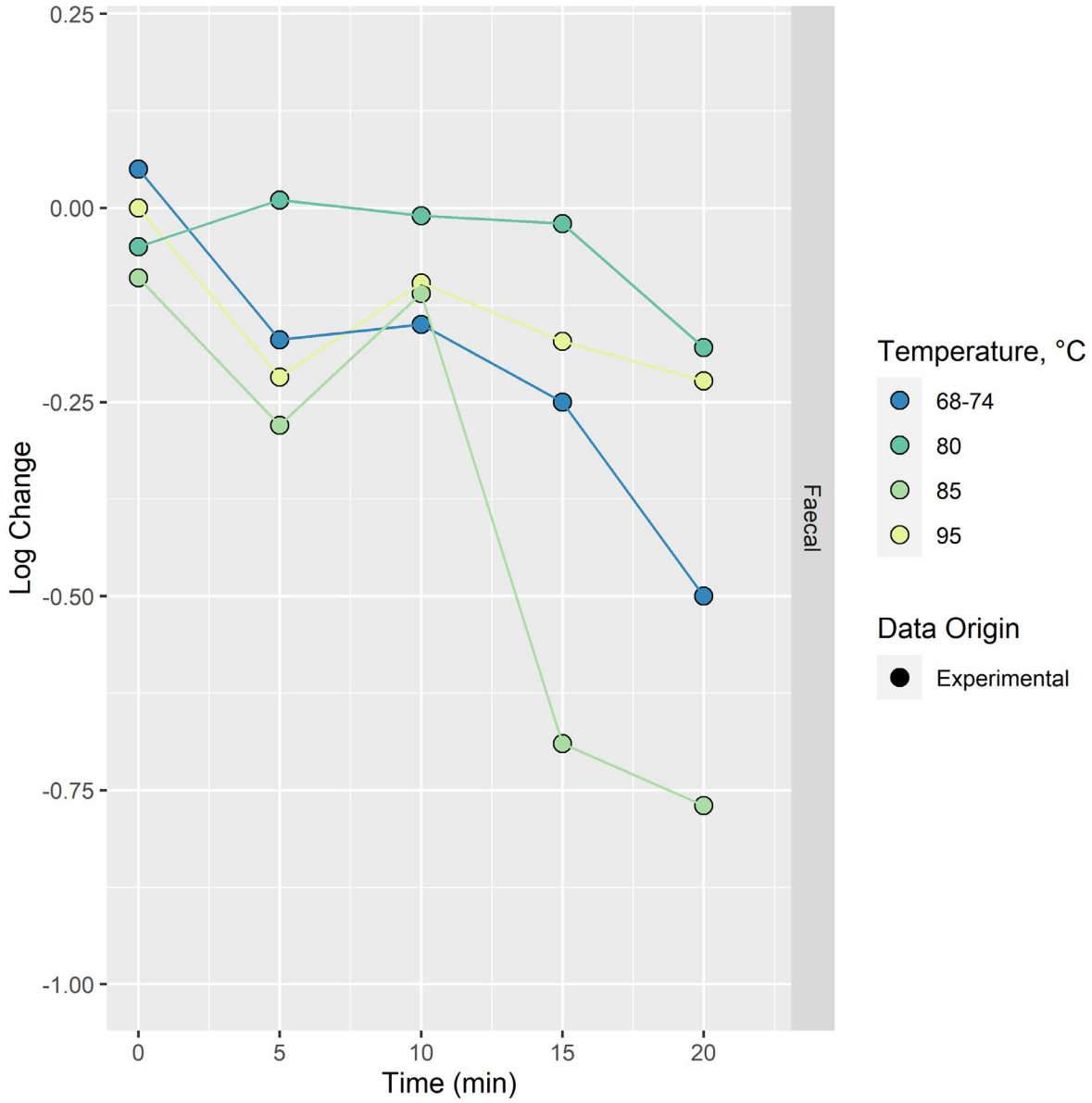
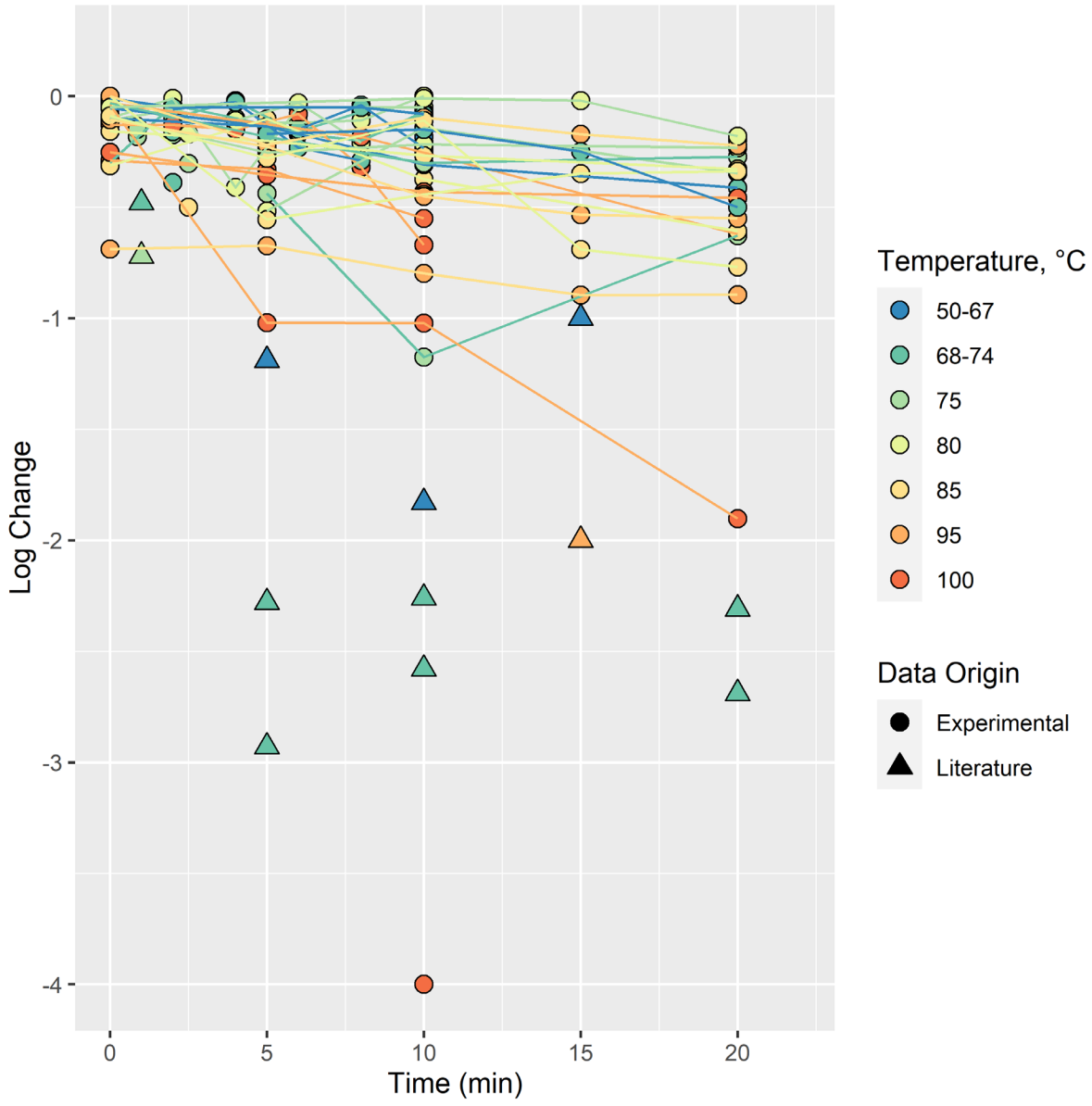


Figure 31 - Combined literature and experimental data; for temperature > 50°C, time 0-20mins; all HEV sources.



Having established that the new experimental data is vital for successful calibration of the model, we now use it to estimate the model parameters for the three different virus sources, for the temperatures within the 70°C - 100°C range, for which experimental data is available.

Parameter estimates, summary statistics and fitted model curves for each temperature and source combination are illustrated in the tables and figures below. In these tables, Q2.5% and Q97.5% are simply the 2.5% and 97.5% percentiles of the estimated parameter distribution, providing a 95% confidence interval of the

mean. Overall, the model achieves good levels of convergence for all of the fitted parameter estimates listed below.

Results from fitting to experimental data utilising HEV serum data as described in section 6.2:

Table 7 - Estimated model parameters. Fitted to experimental data for 70°C. HEV source: Serum.

70°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.044973	0.020672	0.005882	0.046174	0.080933	1	893
β	1.273717	0.634446	0.373874	1.147954	2.753795	1	994

Table 8 – Estimated model parameters. Fitted to experimental data for 75°C. HEV source: Serum.

75°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.0238	0.016885	0.001327	0.020679	0.063027	1	1736
β	0.484873	0.198365	0.187619	0.450826	0.93046	1	1711

Table 9 - Estimated model parameters. Fitted to experimental data for 80°C. HEV source: Serum.

80°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.02146	0.014672	0.001118	0.019087	0.054417	1	1929
β	0.664376	0.254012	0.246158	0.637885	1.158333	1	1782

Table 10 - Estimated model parameters. Fitted to experimental data for 85°C. HEV source: Serum.

85°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.718212	0.595377	0.025201	0.558058	1.913063	1	3577
β	0.077817	0.10557	0.001497	0.043018	0.381675	1.04	1207

Table 11 - Estimated model parameters. Fitted to experimental data for 95°C. HEV source: Serum.

95°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.092038	0.030432	0.030718	0.08891	0.158334	1	7660
β	0.462078	0.242718	0.045304	0.447386	0.982434	1	5781

Table 12 - Estimated model parameters. Fitted to experimental data for 100°C. HEV source: Serum.

100°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.086905	0.026139	0.034786	0.085847	0.149406	1	5877
β	0.709309	0.323659	0.143631	0.688355	1.413292	1	5197

Figure 32 - Model curve fitted to experimental data for 70°C. HEV source: Serum.

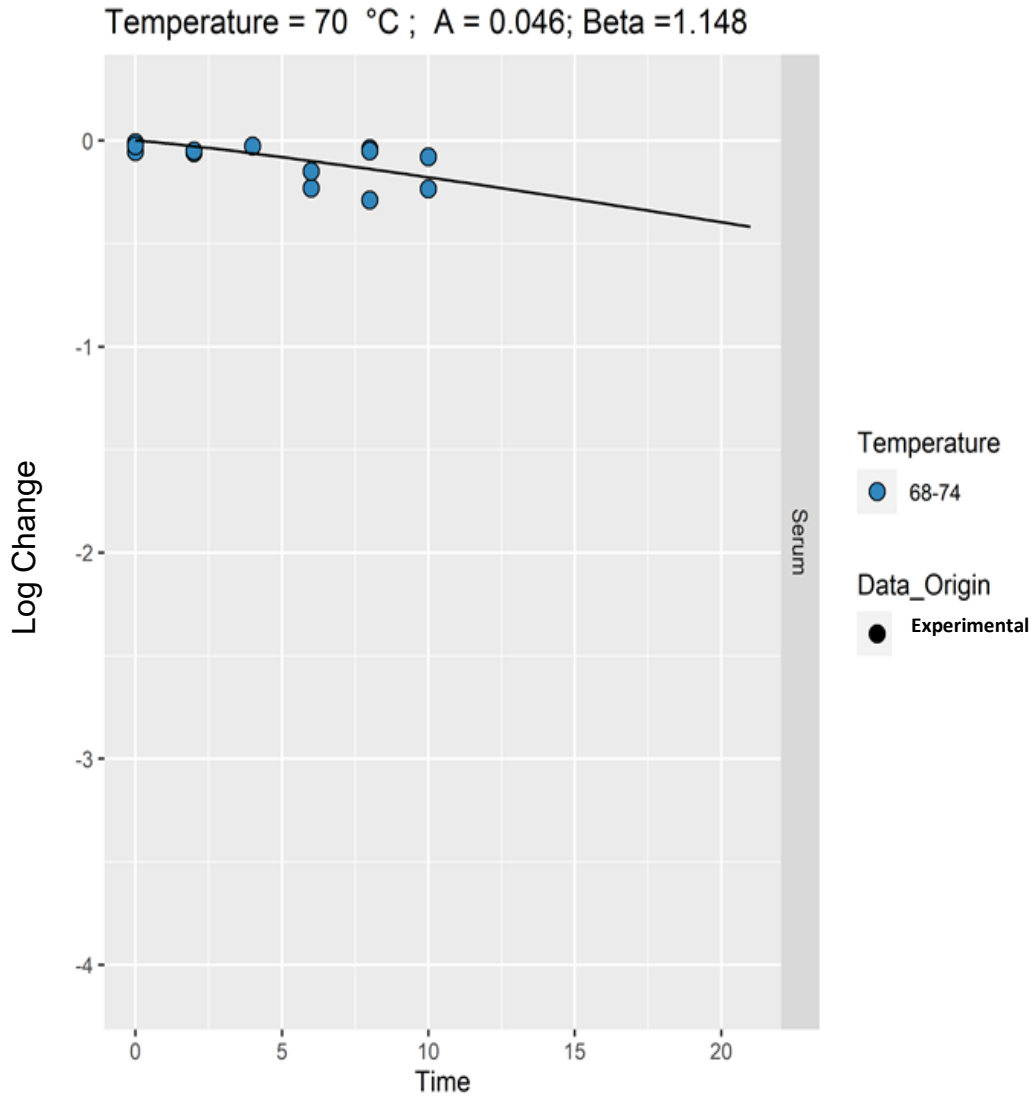


Figure 33 - Model curve fitted to experimental data for 75°C. HEV source: Serum.

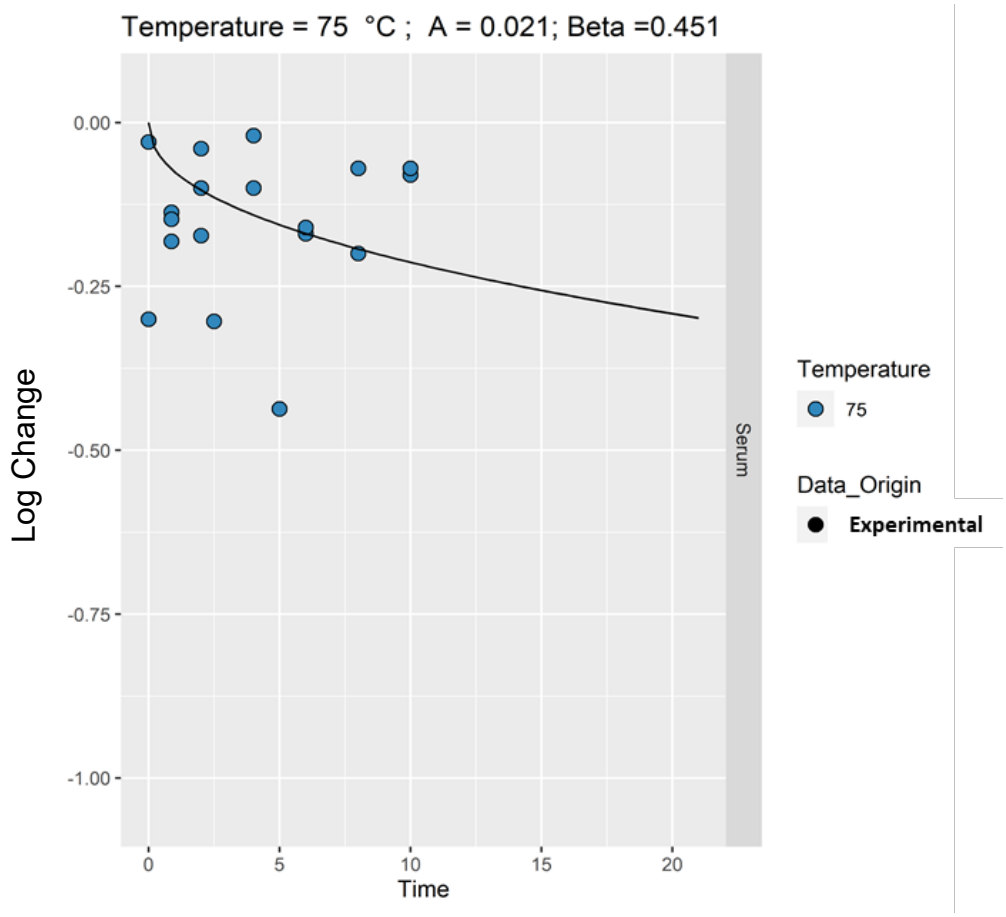


Figure 34 - Model curve fitted to experimental data for 80°C. HEV source: Serum.

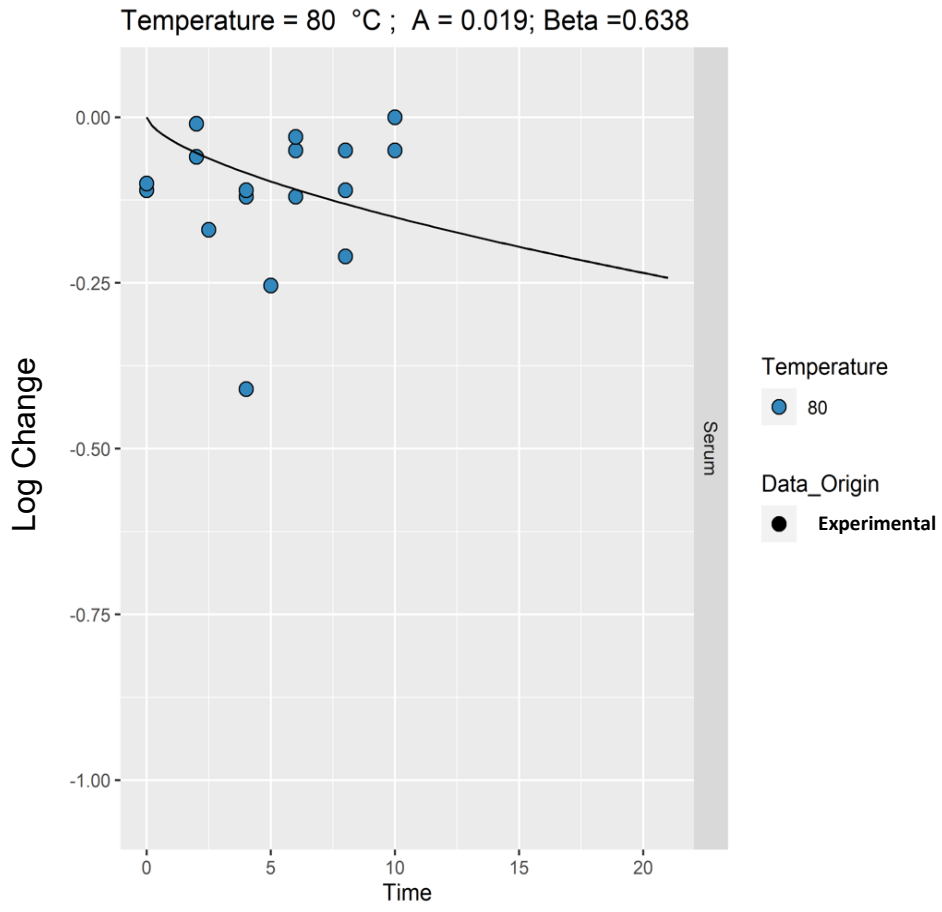


Figure 35 - Model curve fitted to experimental data for 85°C. HEV source: Serum.

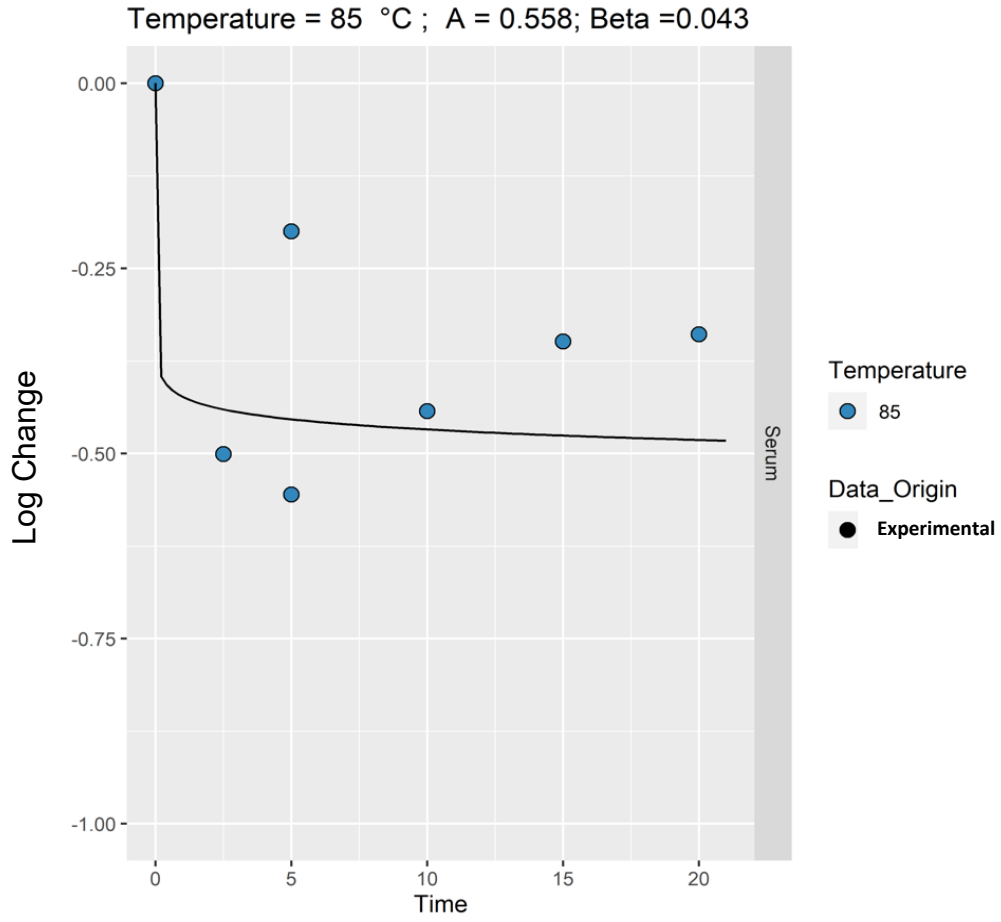


Figure 36 - Model curve fitted to experimental data for 95°C. HEV source: Serum.

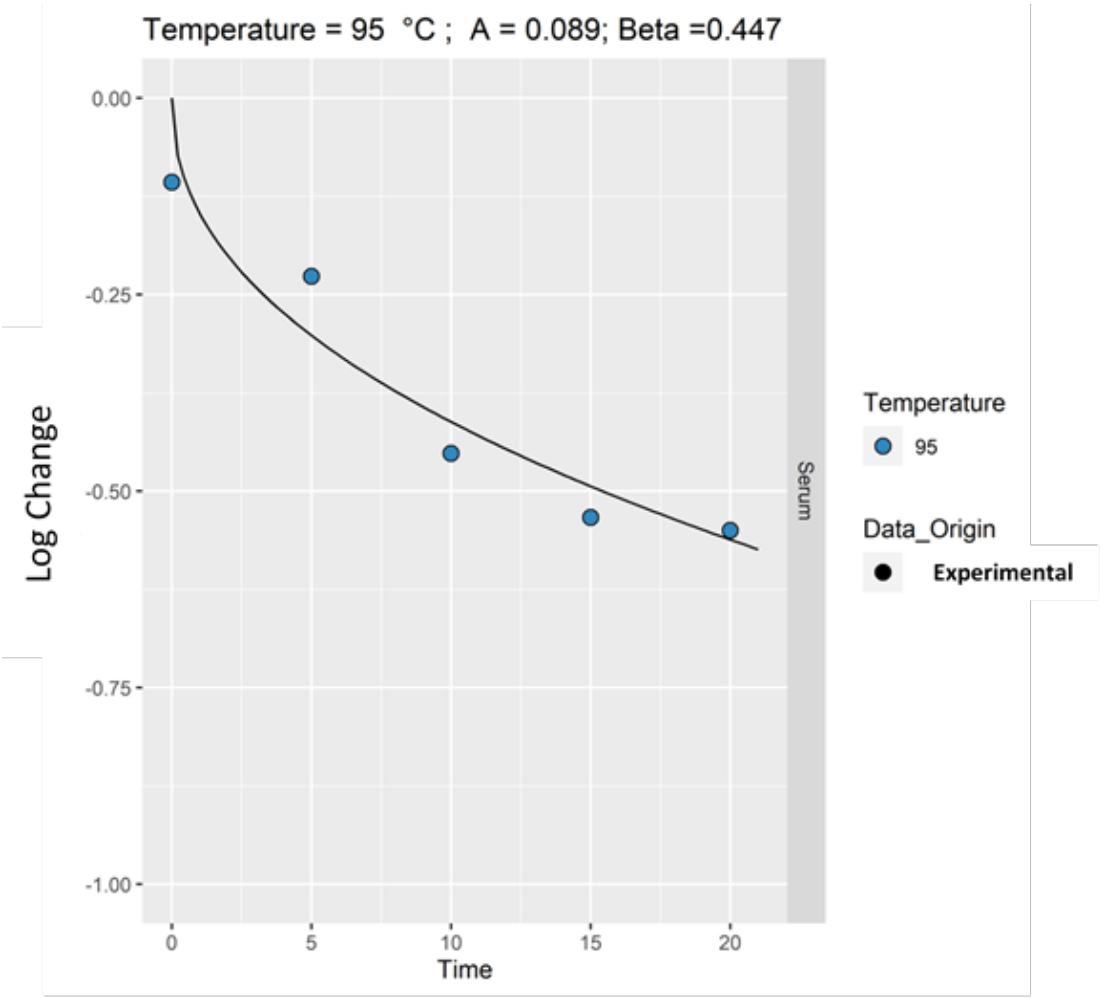
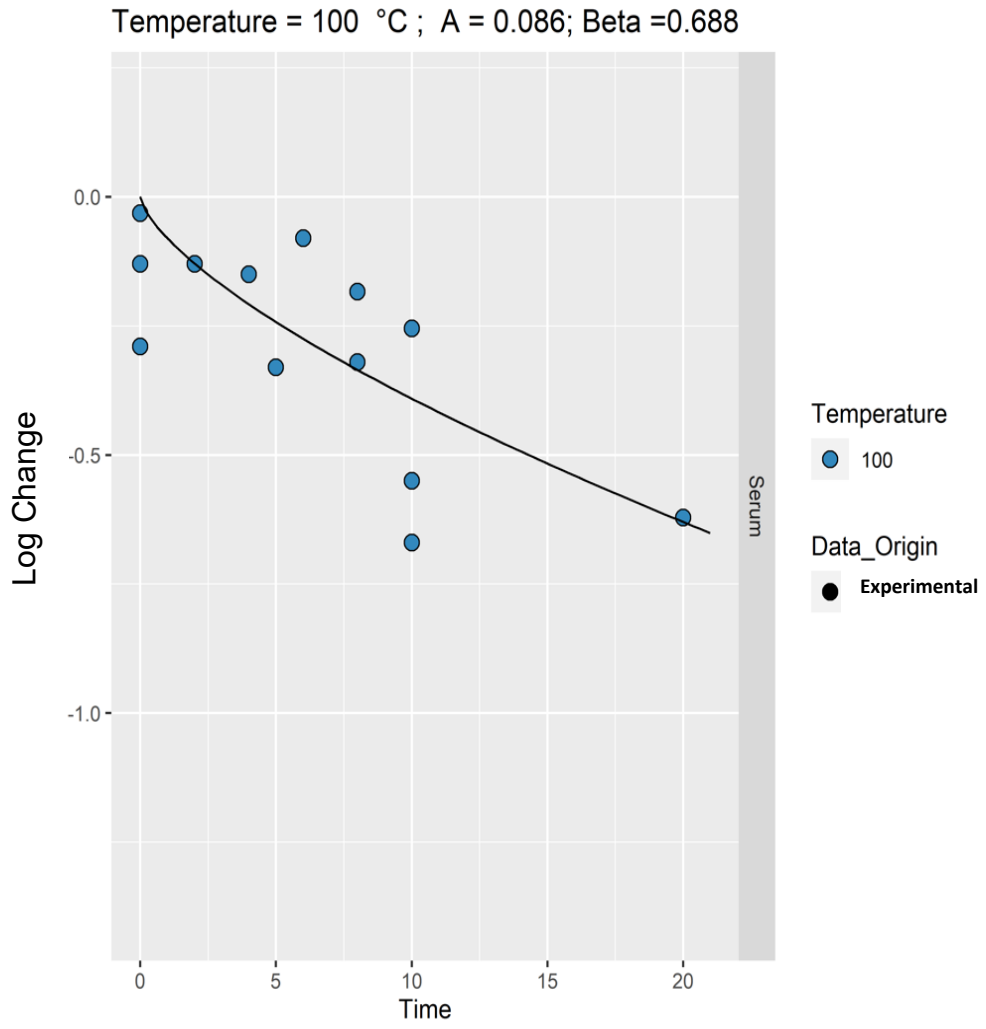


Figure 37- Model curve fitted to experimental data at 100°C. HEV source: Serum



Results from fitting to experimental cell-culture data generated in section 6.2:

Table 13 - Estimated model parameters. Fitted to experimental data for 70°C. HEV source: Cell-culture.

70°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.035411	0.015395	0.005251	0.036693	0.065184	1	4705
β	1.454019	0.834748	0.22363	1.295673	3.151922	1	5155

Table 14 - Estimated model parameters. Fitted to experimental data for 75°C. HEV source: Cell-culture.

75°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.048181	0.020026	0.006063	0.051435	0.075787	1	6373
β	0.338435	0.2645	0.010481	0.272763	0.924623	1	4981

Table 15 - Estimated model parameters. Fitted to experimental data for 80°C. HEV source: Cell-culture.

80°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.031348	0.019002	0.002107	0.029373	0.071163	1	5279
β	0.348094	0.2317	0.034467	0.293224	0.899877	1	4975

Table 16 - Estimated model parameters. Fitted to experimental data for 85°C. HEV source: Cell-culture.

85°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.060609	0.029441	0.01266	0.056582	0.146376	1	4654
β	0.819095	0.597142	0.062123	0.676034	2.457638	1	3669

Table 17 - Estimated model parameters. Fitted to experimental data for 95°C. HEV source: Cell-culture.

95°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	1.414697	0.880737	0.149381	1.32261	3.029105	1	3767
β	0.24044	0.132759	0.030879	0.221662	0.583705	1	3556

Table 18 - Estimated model parameters. Fitted to experimental data for 100°C. HEV source: Cell-culture.

100°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.358549	0.162203	0.096927	0.347876	0.645476	1	3543
β	0.494049	0.228957	0.086148	0.464968	1.038622	1	3541

Figure 38- Model fitted to experimental data for 70°C. HEV source: Cell-culture.

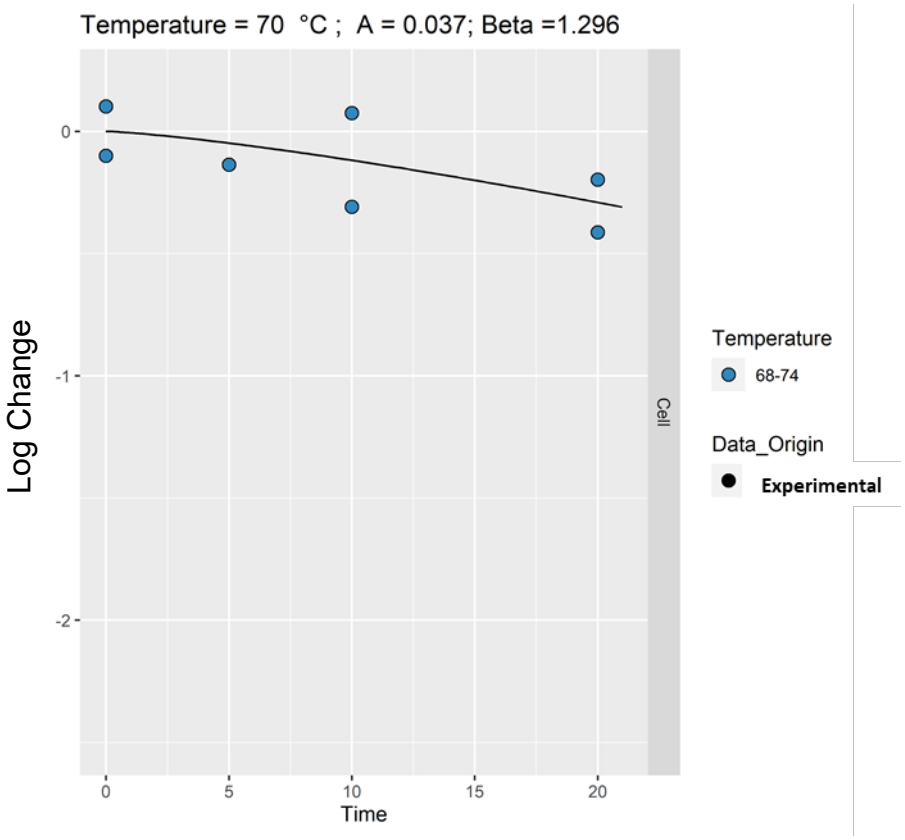


Figure 39- Model fitted to experimental data for 75°C. HEV source: Cell-culture.

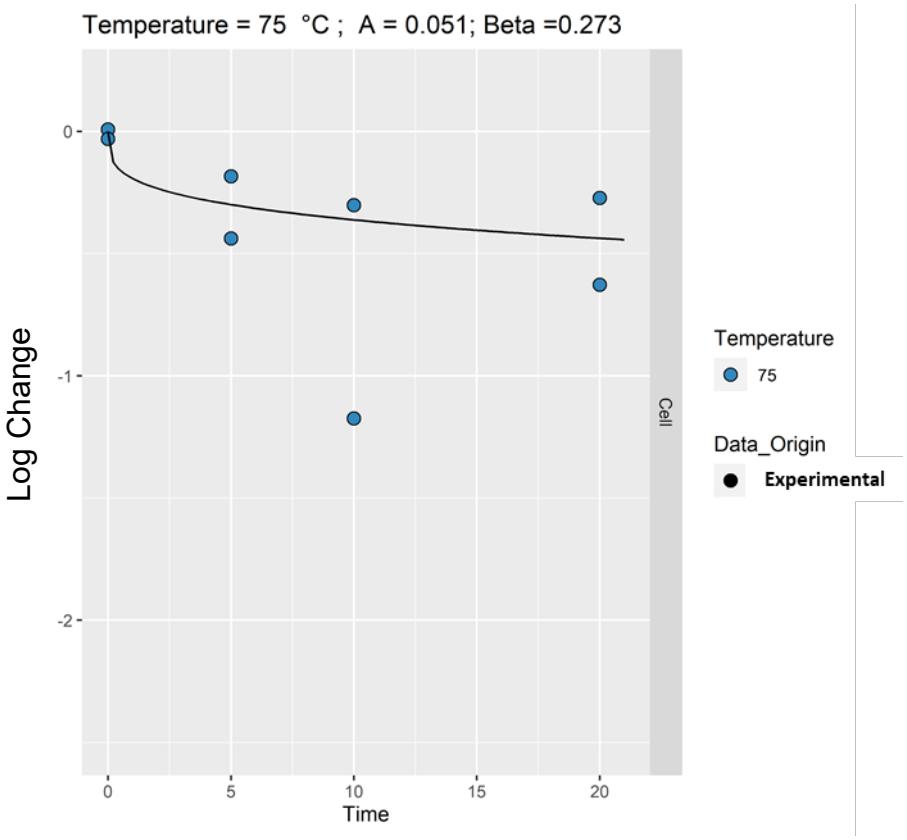


Figure 40- Model fitted to experimental data for 80°C. HEV source: Cell-culture.

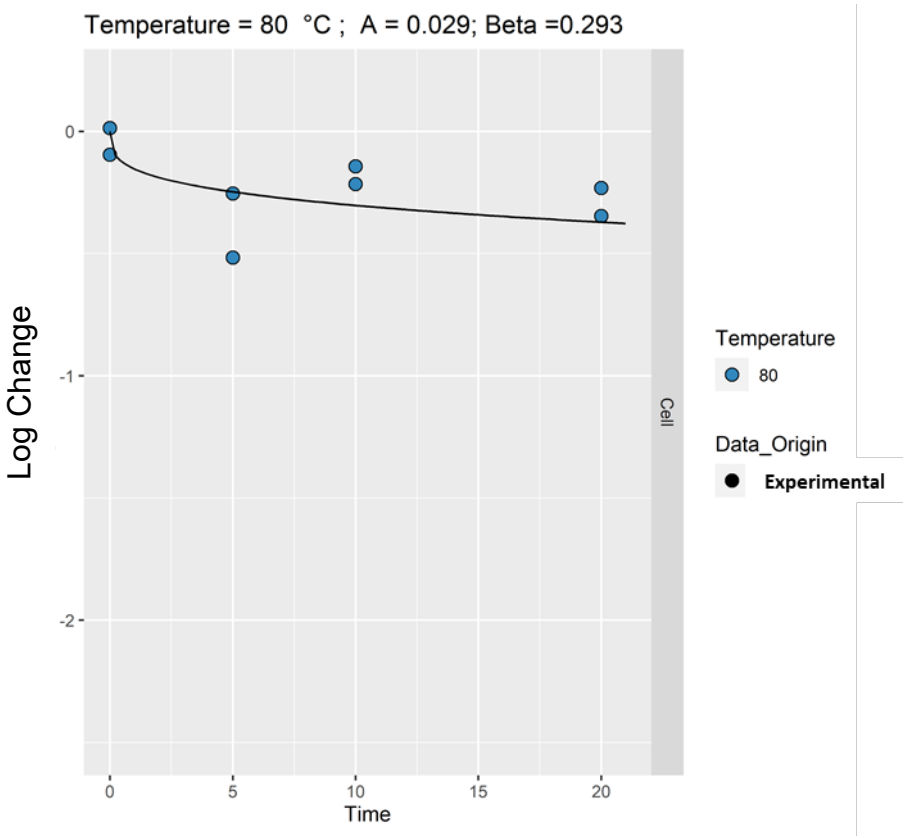


Figure 41- Model fitted to experimental data for 85°C. HEV source: Cell-culture.

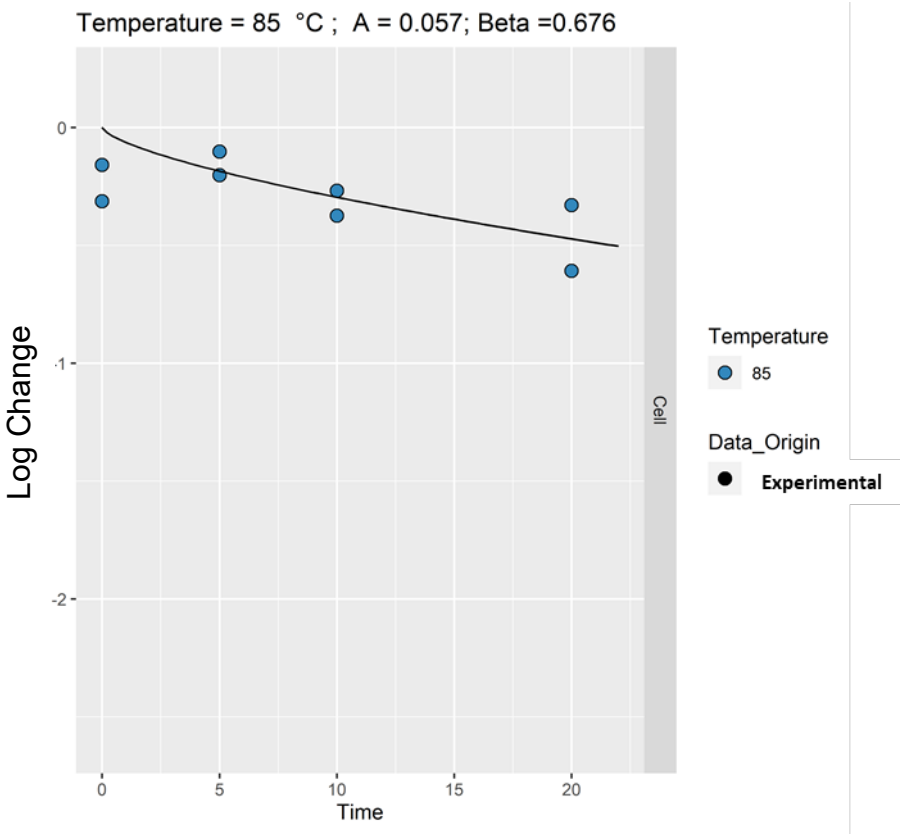


Figure 42- Model fitted to experimental data for 95°C. HEV source: Cell-culture.

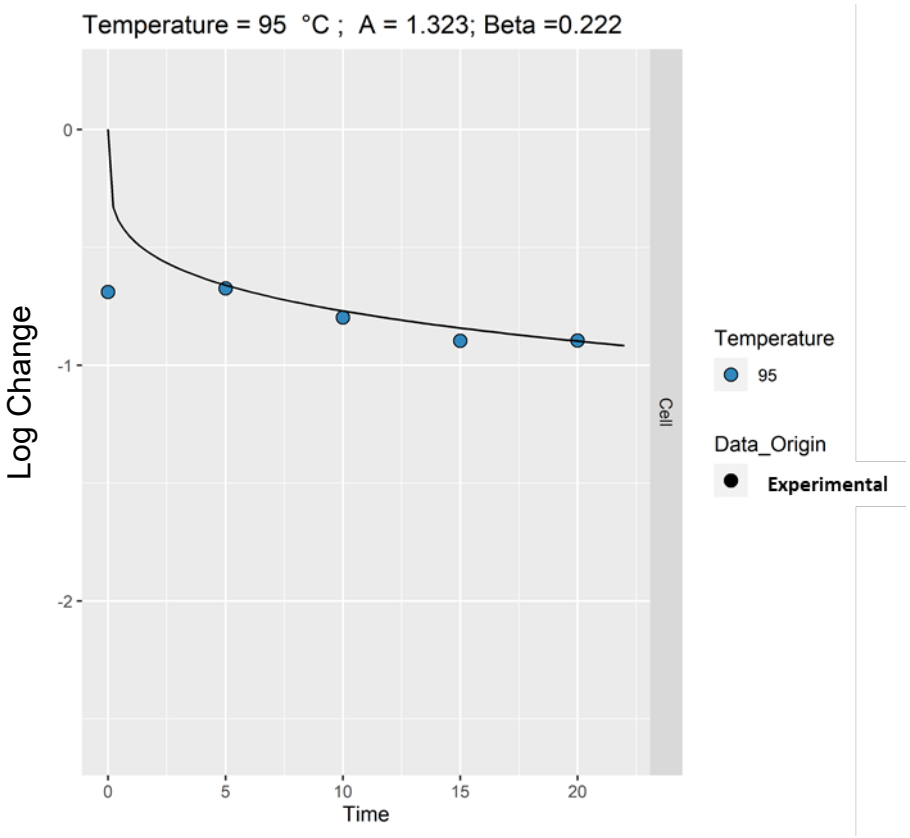
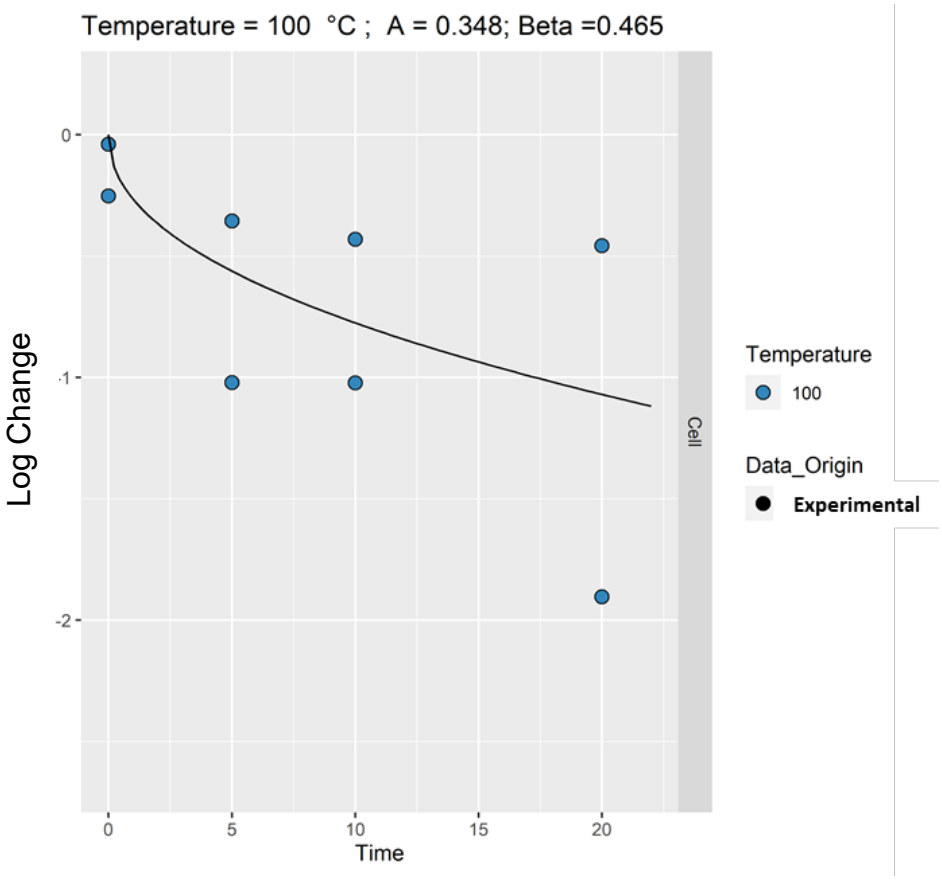


Figure 43- Model fitted to experimental data for 100°C. HEV source: Cell culture



Results from fitting to experimental faecal data in section 6.2:

Table 19 - Estimated model parameters. Fitted to experimental data for 74°C. HEV source: Faecal.

74°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.047741	0.014623	0.013085	0.049668	0.076183	1	5006
β	1.136805	0.555061	0.17837	1.105563	2.198776	1	4370

Table 20 - Estimated model parameters. Fitted to experimental data for 82°C. HEV source: Faecal.

82°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.026957	0.010875	0.002892	0.029407	0.042956	1	2696
β	2.352786	0.77259	0.762402	2.462573	3.451433	1	2677

Table 21 - Estimated model parameters. Fitted to experimental data for 87°C. HEV source: Faecal.

87°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.099655	0.041467	0.027646	0.090634	0.198348	1	5607
β	0.763529	0.424783	0.057487	0.73604	1.539626	1	3695

Table 22 - Estimated model parameters. Fitted to experimental data for 95°C. HEV source: Faecal.

95°C	Mean	Std.dev	Q 2.5%	Median	Q 97.5%	Rhat	n.eff
A	0.020657	0.012351	0.00147	0.019559	0.047692	1	2406
β	0.698544	0.328004	0.184668	0.653746	1.344113	1	2867

Figure 44 - Model curve fitted to experimental data for 74°C. HEV source: Faecal.

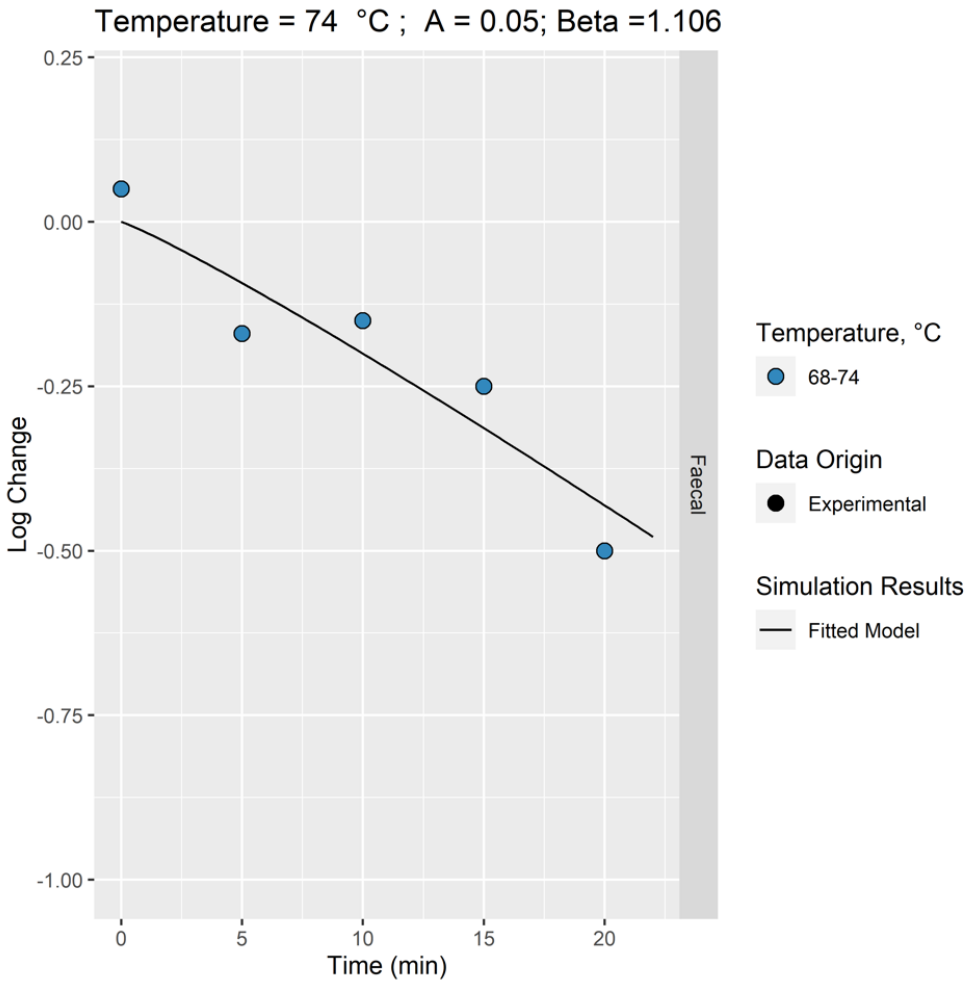


Figure 45 - Model curve fitted to experimental data for 82°C. HEV source: Faecal.

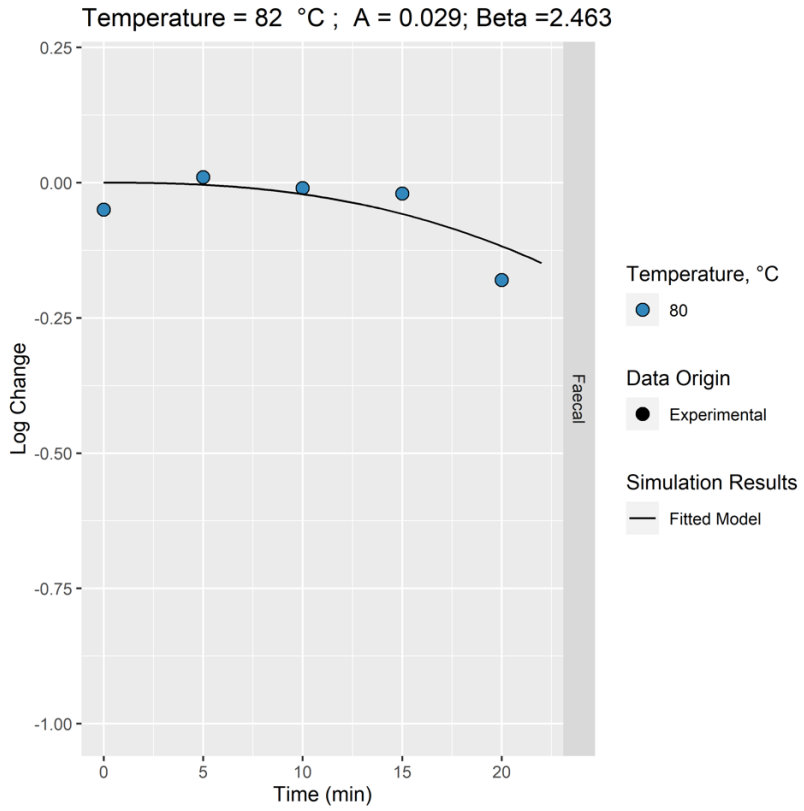


Figure 46 - Model curve fitted to experimental data for 87°C. HEV source: Faecal.

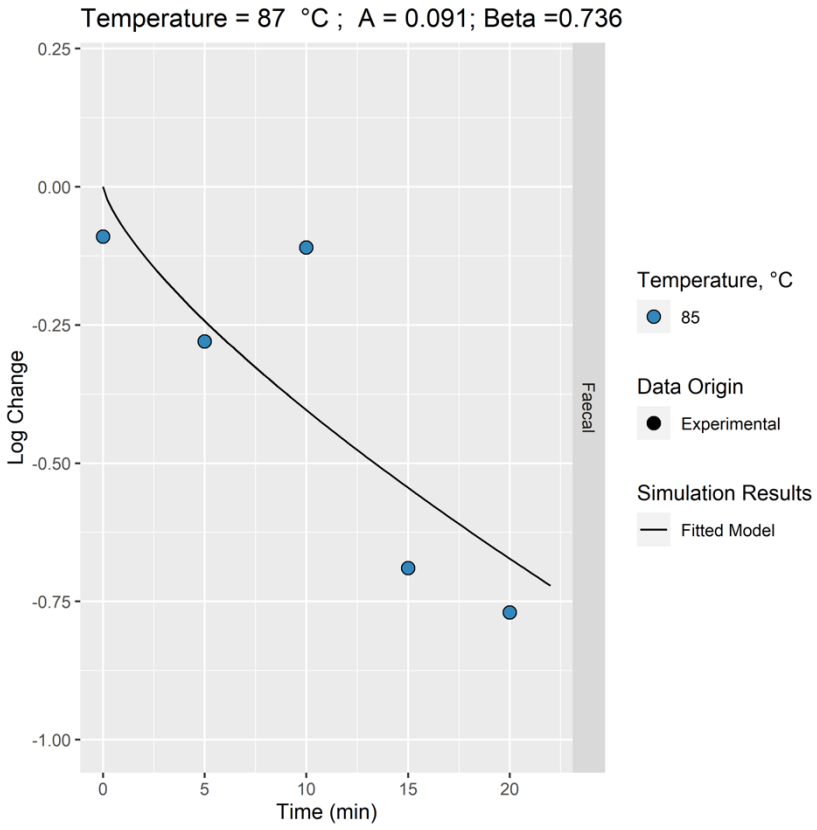
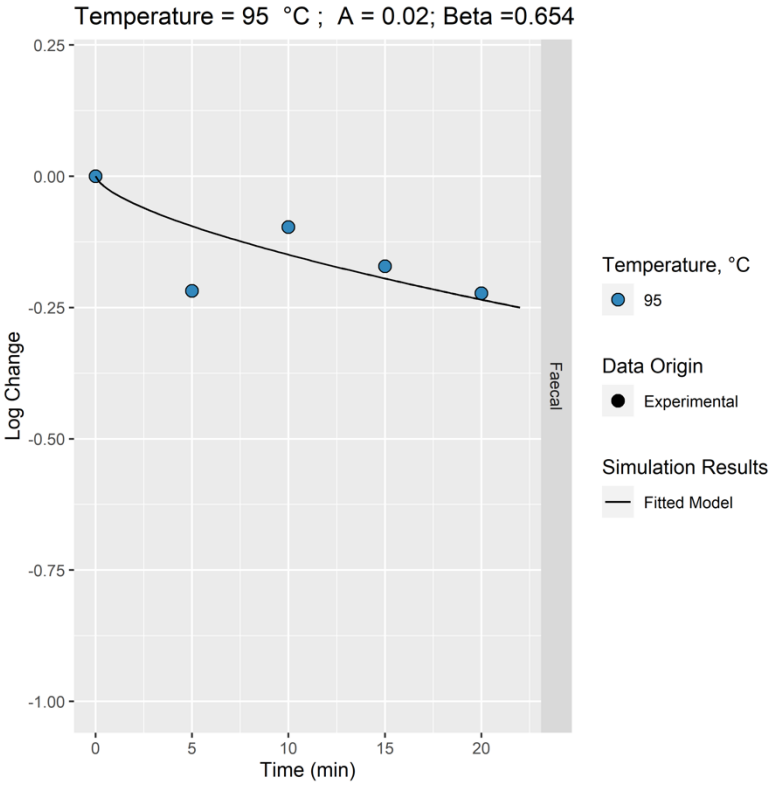


Figure 47 - Model curve fitted to experimental data for 95°C. HEV source: Faecal.



7.3.4 Discussion

Predictive microbiology models are essential for risk assessments when concentrations of human pathogens in food cannot be measured and must be predicted, for example at the time of consumption after distribution of food to consumers (Stavropoulou and Bezirtzoglou, 2019). Likewise, web resources are available to view data deposited from research establishments and the literature and provide software tools for modelling, for example, ComBase, and SafeCONSUME. However, most are for bacteria and there is no such dataset is available for a predictive model to estimate the thermal response of HEV. The model described in section 7.3 was created to predict the relevant log reduction in HEV viral quantity at specific temperatures associated with cooking of food.

Combined with equation (1) or (2), the estimates presented in Tables 7-22 for model parameters A and β, allow for the reduction in HEV particles to be predicted at any given time. These parameter estimates are temperature dependent, and thus predict

the reduction for the temperature that each table corresponds to. Likewise, they are intended to be unique to the viral source of the HEV that they have been estimated from (i.e., cell-culture, faeces, or serum). Plots of the corresponding fitted model curves, for each temperature and virus source can be found in figures 32-47. Equation 2 does not account for input value. Naturally, as both the quantity and quality of available data increases, these estimates will further increase in accuracy.

The “Rhat” value in tables 7 -22 is a convergence diagnostic statistic, that aims to quantify the convergence of the MCMC simulation that generated the fitted model results. If we have Rhat close to 1, this indicates the model has converged, whilst Rhat greater than 1.05 indicates we do not have good convergence. Clearly, for all parameter estimates in tables 7 -22, the model shows great convergence, with Rhat values close to, or equal to 1 in every single case.

The “n.eff” value is the effective sample size, that is kept from the posterior distribution of the MCMC simulation used to generate the parameter estimates. As a default rule, for this particular model, any n.eff value greater than 30 is good (This corresponds to 10 times the number of MCMC chains used by the model simulations). This should typically correspond to stability of the convergence (Gelman, 2013). Again, we clearly have good effective sample sizes for all the results in tables 7 -22. Note: The model uses three “Markov chains”, in its simulations. These three chains simultaneously estimate the two model parameters, at each time step. So “convergence” refers to all three chains converging upon approximately the same value.

Looking at the results for each individual source: Parameter estimates obtained using the experimental data for serum-derived virus, along with plots of the log-change curves these describe, are shown in tables 7-12 and figures 32-37, respectively. It can be seen from the figures, that the fitted model produces a very good fit to the available data.
The corresponding parameter estimates for cell-culture-derived virus, based upon the new experimental data, can be found in tables 13 – 18 and figures 38 – 43. Again, the parameter estimates are reasonably consistent in value, show excellent convergence (Rhat equal to 1, and n.eff much larger than 30), and the fitted log-

change curves show excellent agreement with the experimental data in the plots. The obvious exception is the parameter estimates for 95°C shown in table 17, where the A parameter estimate is much larger than that for the neighbouring temperatures (90°C and 100°C, in tables 13 and 15, respectively). Again, this can easily be explained by looking at the experimental data points used to fit these parameters, shown in figure 42. Clearly, there is a minor inconsistency, as the measured log change at time t=0 is close to 0.75, whereas it would be expected to be nearer 0. So clearly this has skewed the parameter estimates in this case.

Finally, the parameter estimates for faecal-derived virus, can be found within tables 19 – 22, and figures 44 – 47. Once again, these all produce consistent values for parameters A and β , with excellent convergence and visually the fitted model curves can clearly be seen to fit the data very well.

The model was verified by the FSA.

Future steps could include evolving the model equation further, in order to incorporate temperature into the model equation itself. This would allow ALL data points, no matter what the temperature they correspond to, to be used to estimate model parameters for a particular source. Obtaining more experimental data, that can be added to the log-reduction dataset, will also be extremely beneficial, in further increasing the quality of the model predictions. It should also be noted that if the predicted time for cooking is greater than 20 minutes then more data will be required to predict accurately.

Outcomes of the analysis.

1. It has been successfully demonstrated that the Weibull model is both a good fit to the available experimental data, and thus a suitable model for describing the thermal reduction of HEV within foodstuffs. This is clear from the convergence results shown.

2. The model/application can easily be adapted to new data as this becomes available. This is a major advantage of developing the model into a user-upgradable application. As such, is anticipated that the model predictions will further increase in accuracy, as the dataset it uses develops and expands to include data from new studies.

3. The model benefits from being flexible. Theoretically, users could replace the data (contained in the apps .csv fie) with data for the thermal reduction of some other virus, and in theory, the model should be able to provide a reasonable fit, with little to no modification.
4. The model can predict the time temperature combination to reduce the virus quantity by the log reduction required. To calculate which time/temperature combination will provide a log reduction of 2, then you can rearrange equation 2 to calculate the time (t) required to give you a certain log reduction: t = { [2.303 x | Log reduction |] ^ (1/beta) } x (1/A). So if you want the predicted time, for Log reduction = 2; Simply set the Log reduction to 2, add the median estimated and parameters A and Beta, from the tables, and that will give you the time predicted, to achieve that log-reduction, i.e., t = { [2.303 x 2] ^ (1/beta) } x (1/A).
5. The model has its limitations. The estimates it provides are only as good as the available data that they are based upon. Thus, the more data available, the better the model predictions will become. Similarly, if the quality of the data input into the model is poor, the model predictions will mirror this.

7.4 Validation of the model

No model predictions can be used with confidence unless they are validated by data from independent laboratories which we were unable to achieve at this time due to the SARS-CoV2 pandemic affecting laboratory access.

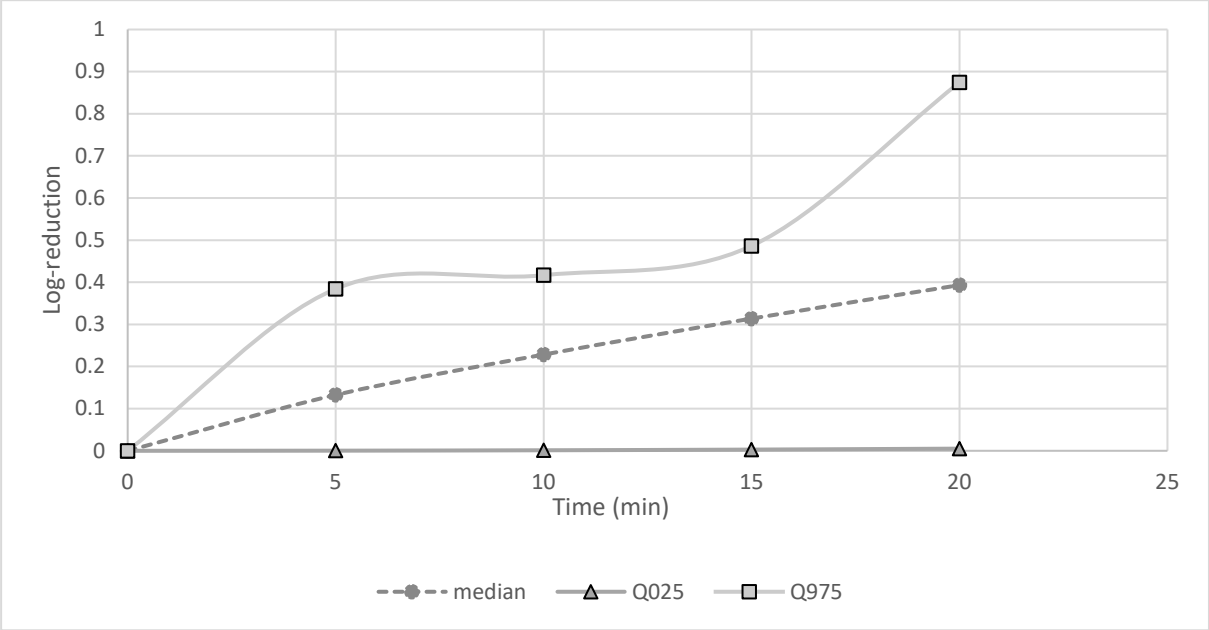
7.4.1 Materials and Methods

In order to test the model, anonymized data was generated in-house. Test conditions (consisting of various temperature and virus source combinations) were provided to UoS, and both the predicted model parameters and corresponding predicted log reductions were returned for validation purposes. Experimental analysis was carried out as described in section 6.1 to replicate the chosen test conditions and the experimental results compared to the predicted values. For the test conditions, random temperatures were selected within the model range; these were 74, 82 and 87˚C. Predictions were requested for cell culture derived virus and faecal porcine virus over a 20minute period at 5 minute intervals. As the dataset used to fit the mathematical model does not explicitly contain data points at any of these three selected temperatures, parameters for these test temperatures were estimated by averaging the parameter estimates of the two closest neighbouring temperatures, present within the existing model dataset. For example, for 74˚C, parameter estimates for 70 ˚C and 75 ˚C were averaged in order to approximate parameter values for the 74 ˚C test conditions (Table 23). Likewise, to estimate parameters for 82 ˚C, the existing parameter estimates for 80 ˚C and 85 ˚C were averaged, whilst, for 87˚C the existing estimates for 85 ˚C and 100 ˚C were averaged. It is expected that the observed experimental results should fall within the range of the estimated 95% confidence interval, which ranges from the Q2.5 to Q975 as shown in figure 48.

Table 23: Parameter estimates for 74˚C test conditions with cell-culture derived HEV virus. Calculated by averaging the parameter estimates of the two closest neighbouring temperatures (70 ˚C and 75 ˚C) available within the model dataset.

Temperature	**-**	**Parameters (CELL) Median**	**Parameters (CELL): Q2.5**	**Parameters (CELL): Q975**
T= 70°C	A	0.036693	0.005251	0.065184
T= 70°C	β	1.295673	0.22363	3.151922
Estimated: T= 74°C	A	0.044064	0.005657	0.0704855
Estimated: T= 74°C	β	0.784218	0.1170555	2.0382725
T= 75°C	A	0.051435	0.006063	0.075787
T= 75°C	β	0.272763	0.010481	0.924623

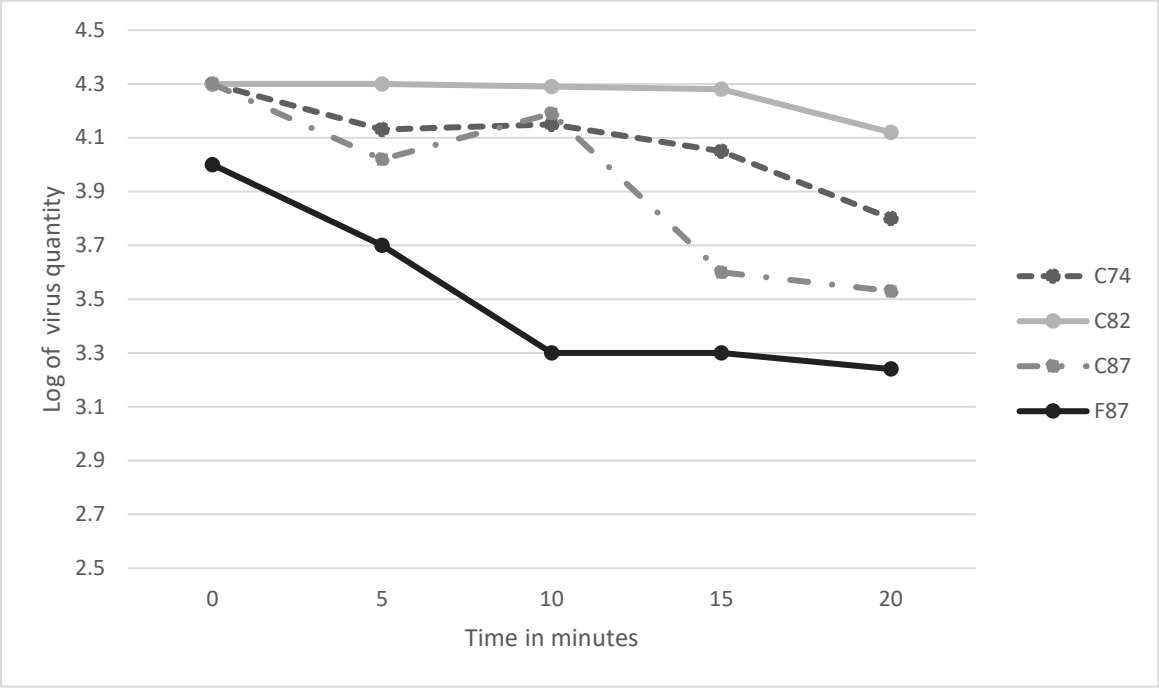
Figure 48: Graphical representation of predicted log reduction at 74˚C for cell culture derived HEV.



7.4.2 Results

Predicted values were provided based on the model described in section 7.3 and equation 2; the actual values for the experimental data points are presented in figure 49.

Figure 49: Log of viral quantity for the validation experimental data at 74, 82 and 87˚C for cell culture derived virus (C) and at 87˚C for faecally derived virus (F87).



Tables 24-26 provide the predicted and actual outcomes for the validation experiments using cell culture derived virus at each temperature proposed with the median, Q2.5 and Q975 values for A and β. Figures 50-52 indicate the parameters for the median, Q2.5 and Q975 alongside the actual values 70 and 75ºC 80 and 85ºC for cell culture derived virus.

The actual data falls within the confidence intervals of the predicted values for all temperature time combinations for the cell culture adapted virus (Tables 24-26; Figures 50-52). We assume that these estimates will be influenced by experimental variation. Indeed, the model cannot currently account for any differences that may be caused by variation in viral input albeit the earlier data would suggest that log reduction does not vary greatly.

Table 24: Predicted and actual values for log change in cell culture derived virus at specific temperature of 74°C using the model described in section 7.3. Log viral input =4.3.

Time (Mins)	Log reduction predicted. (Median)	Log reduction predicted (Lower, Q2.5)	Log reduction predicted (Upper, Q97.5)	Log reduction actual
0	0	0	0	0
5	0.1326	0.000303079	0.384315667	0.162543
10	0.2283	0.001244907	0.416797693	0.149589
15	0.3138	0.002844846	0.486422888	0.247234
20	0.3932	0.005113496	0.874325596	0.49534

Figure 50: Predicted vs actual log reduction in cell culture derived virus at 74°C. 95% Confidence interval: {Q025, Q975].

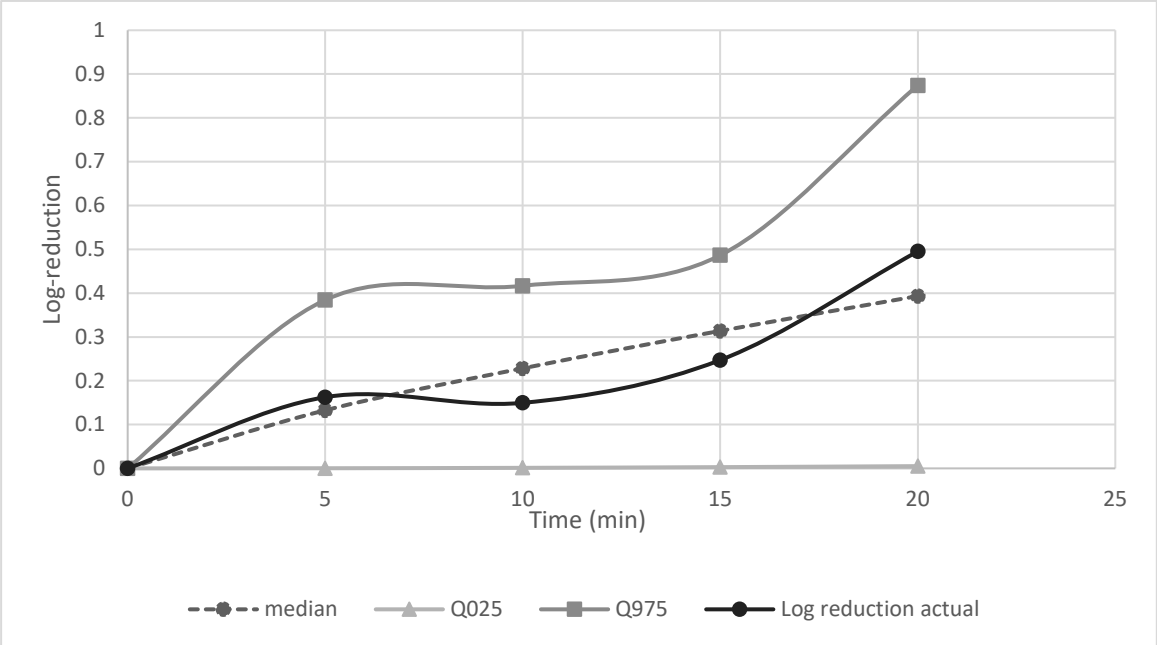


Table 25: Predicted and actual values for log change in cell culture derived virus at specific temperature of 82°C using the model described in section 7.3. Log viral input =4.3.

Time (Mins)	Log reduction predicted. (Median)	Log reduction predicted (Lower, Q2.5)	Log reduction predicted (Upper, Q97.5)	Log reduction actual
0	0	0	0	0
5	0.2060	0.001707798	0.421629443	0.01709
10	0.2884	0.005467551	0.500026112	0.00402
15	0.3510	0.010799593	0.98765941	0.017095
20	0.4035	0.017504481	1.600844126	0.170445

Figure 51: Predicted vs actual log reduction in cell culture derived virus at 82ºC. 95% Confidence interval: {Q025, Q975].

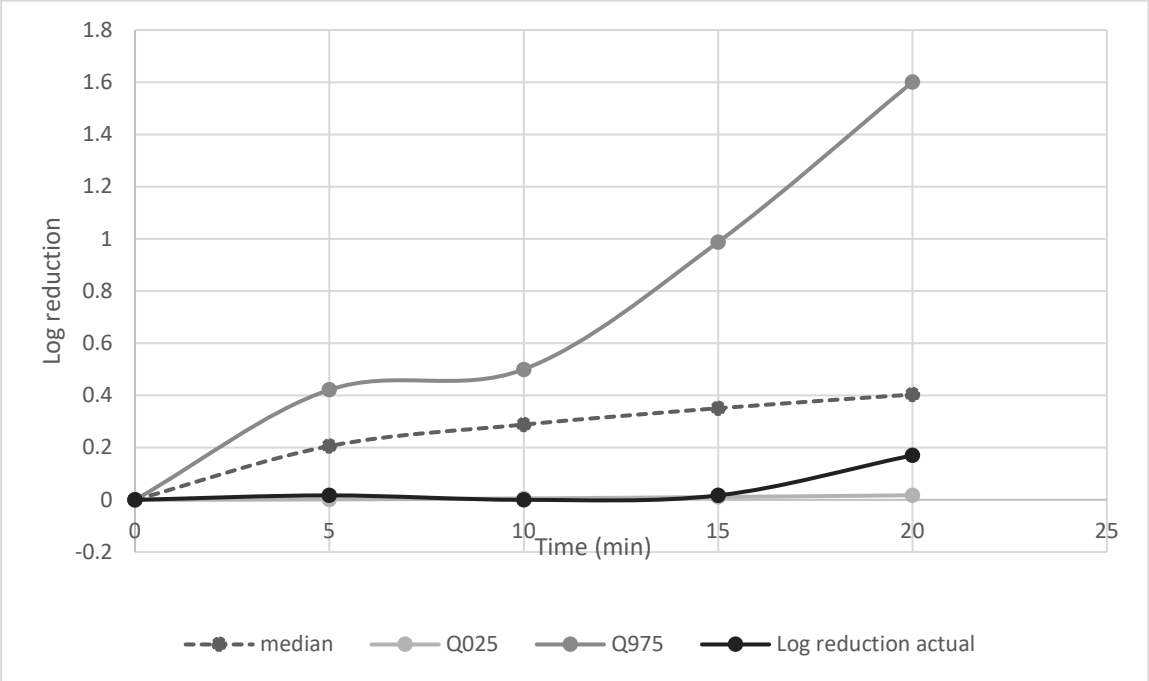


Table 26: Predicted and actual values for log change in cell culture derived virus at specific temperature of 87ºC using the model described in section 7.3. Log viral input =4.3.

Time (Mins)	Log reduction predicted. (Median)	Log reduction predicted (Lower, Q2.5)	Log reduction predicted (Upper, Q97.5)	Log reduction actual
0	0	0	0	0
5	0.4370	0.045157605	1.432760159	0.2755577
10	0.6489	0.151694719	4.812968982	0.108758
15	0.8178	0.308177463	9.777852364	0.690743
20	0.9637	0.437173288	16.16786332	0.76237

Figure 52: Predicted vs actual log reduction in cell culture derived virus at 87ºC. 95% Confidence interval: {Q025, Q975].

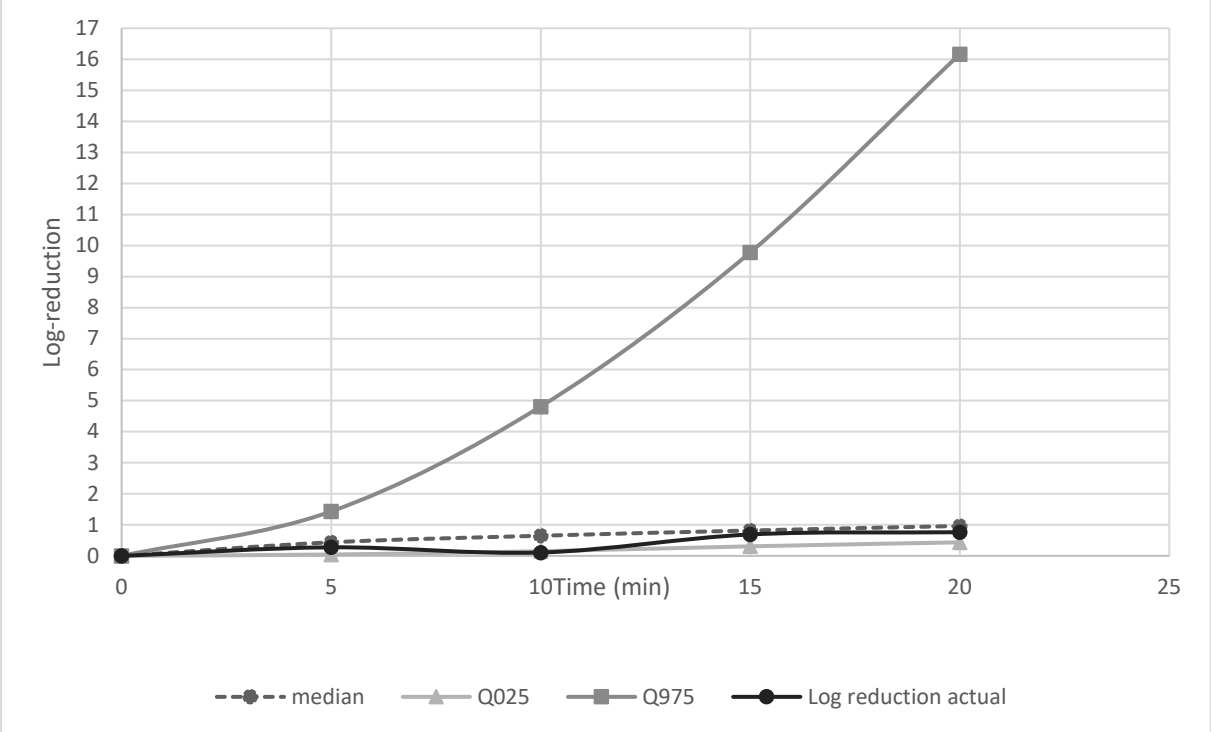
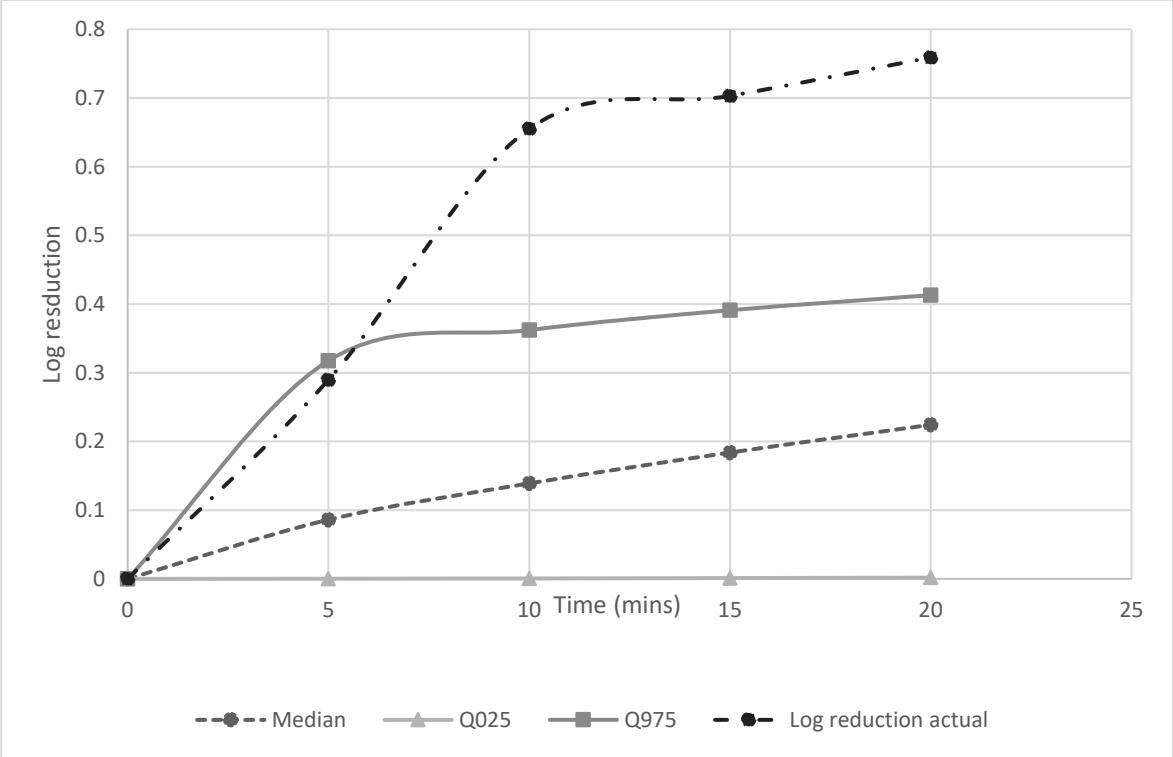


Table 27: Predicted and actual values for log change in faecally derived virus at 87ºC using the model described in section 7.3. Log viral input =4.0

Time (Mins)	Log reduction predicted. (Median)	Log reduction predicted (Lower, Q2.5)	Log reduction predicted (Upper, Q97.5)	Log reduction actual
0	0	0	0	0
5	0.0861	0.000223009	0.317556356	0.289686
10	0.1390	0.000639396	0.362141031	0.655128
15	0.1839	0.001184021	0.391069454	0.70301
20	0.2243	0.001833232	0.412985362	0.758978

Figure 53: Predicted vs actual log reduction in faecally derived virus at 87^{o}C. 95% Confidence interval: {Q025, Q975].



In contrast to the cell culture derived virus, the actual values for the faecally derived virus treated at 87^{o}C, has a much greater log reduction than the predicted median and upper Q975 (Table 27; Figure 53). This would suggest that the level of reduction in viral detection is much greater than the model is predicting. The same did not

follow for 70 and 75°C (Figures 54 and 55) and the actual log reduction fell within the predicted parameters.

Figure 54: Predicted vs actual log reduction in faecally derived virus at 70°C. 95% Confidence interval: {Q025, Q975].

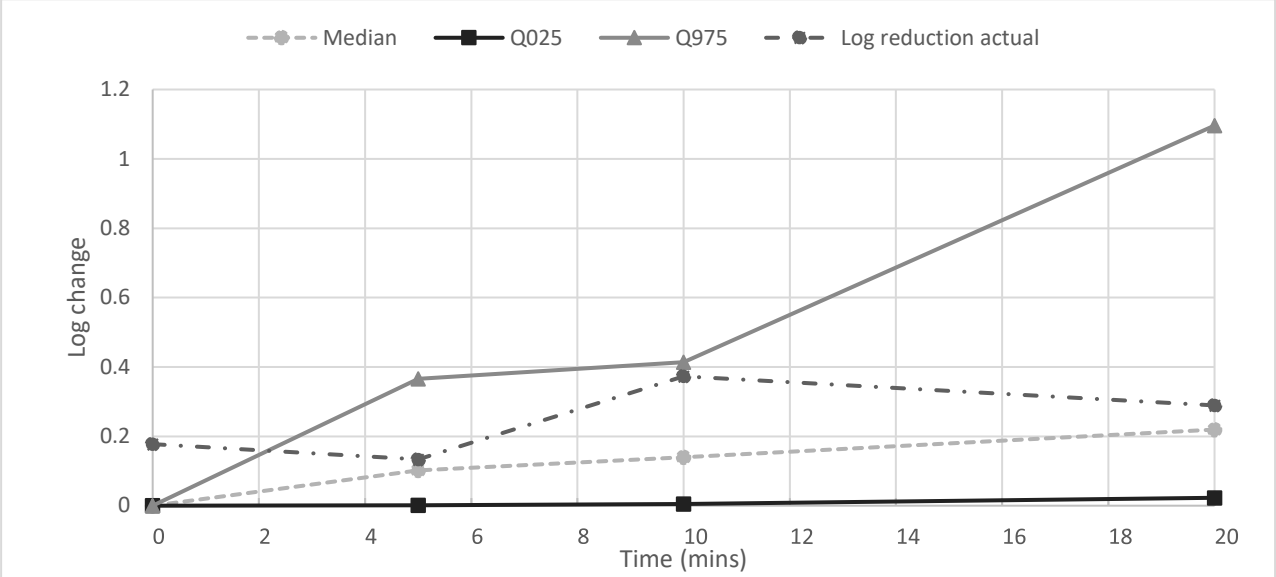
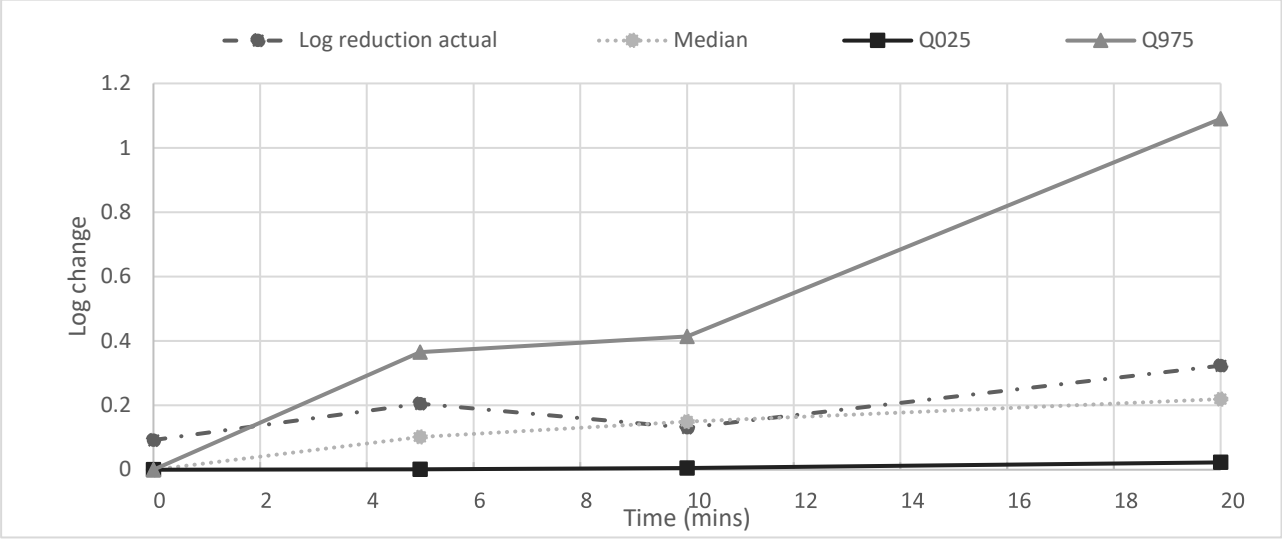


Figure 55: Predicted vs actual log reduction in faecally derived virus at 75°C. 95% Confidence interval: {Q025, Q975].



7.4.3 Discussion

As indicated previously, predictive microbiology models are essential for risk assessments when concentrations of human pathogens in food cannot be measured and must be predicted, for example at the time of consumption after distribution of food to consumers (Stavropoulou and Bezirtzoglou, 2019).
Validation was carried out to ensure that the model was fit-for-purpose and could establish a prediction of thermal reduction which was a reflection of the actual experimental data produced. This is in line with the recommendations of the Aqua Book: guidance on producing quality analysis for government (2015). The intention was to arrange for a third party to carry out the validation experiments but unfortunately, we were unable to secure this due to circumstances in other laboratories as a result of the SARS-CoV2 pandemic.

The expectation was that the outcome should match within the confidence intervals Q025 and Q975 of the model. We did not perceive any uncertainties utilising similar source virus inherent to the model during this quality assurance, however, as noted the model does not account for viral input.

As many more parameters play a role in determining viral inactivation in meat and meat products, the results of predictive microbiology models are often designed to be very conservative (Meat Products Handbook, 2006). The situation in real life is often safer than the results the model suggests. Validation of the results or, in other words, the comparison of calculated with actual results is a major task. Some variables cannot be factored into mathematical models, such as variations in storage temperature and in general, the effects of a maximum of four environmental factors can be incorporated at any one time into most models. Predictive microbiology therefore has its limitations. As we see here, the model is conservative in its estimate for faecally derived virus at the higher temperature of 87˚C and therefore the reality is a greater reduction than estimated. This is the perfect example of the cell-culture derived virus predictions currently being better informed than the faecal virus predictions. This is simply because the current cell-culture predictions/parameter estimates are informed by a much bigger collection of datapoints than we currently have for the faecal virus. As more faecal data becomes available, and is added to the model, it is anticipated that the faecal predictions will become even more accurate.

Thus, the estimates become more realistic and uncertainty is reduced once more data is available to inform the model.

Currently there is no validation data available on any HEV model to compare our outcome to but it is proposed to update the model as part of future work and create predictions prior to comparison with the actual experimental outcome. The next step is to have this model validated by a third party. Indeed, the addition of the thermal stability utilising virus derived from porcine liver (FS307033: Optimising Extraction and Detection of Hep E Virus from Pork Meat and Products) will also strengthen the model and its predictive capacity.

Outcomes of the analysis:

1. The model is fit for purpose and has been validated for cell culture and faecally derived virus.

2. The agreement between the model's predicted log change values and the data from the validation experiments further strengthens the evidence that the Weibull model is a good choice for this application.

3. The model is conservative in its predicted estimates for log reduction in quantity of faecally derived virus.

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Appendix 1: Validated publication database

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Annual report of the Scientific Network on Microbiological Risk Assessment 2016	(EFSA), European Food Safety Authority	2016	EFSA Report	14 pages	3
Rabbit hepatitis E virus infections in humans, France	Abravanel F	2017	Emerging Infectious Diseases	23; 1191-1193	2
The application of food safety interventions in primary production of beef and lamb: A review	Adam K	2010	Int J Food Microbiology	141; S43-S52	3
Survival time of viruses in raw sausage Influence of ripening and storage temperature	Albert T	2012	Fleischwirtschaft	92; 86-90	1
Human and porcine hepatitis E virus strains, United Kingdom	Banks M	2004	Emerging Infectious Diseases	10; 953-955	2
Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food.	Barnaud E	2012	Applied Environmental Microbiology	78; 5153-5159	Accepted
Dry heat treatment (80°C, 72 hours) effectively inactivates a hepatitis E model virus	Belda F	2014	Haemophilia	20; 181	1

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E in pork liver sausage, France	Berto A	2013	Emerging Infectious Diseases	19; 264-266	2
Replication of hepatitis E virus in three-dimensional cell culture	Berto A	2013	J Virol Meths	187; 327-332	2
HEV inactivation assessment using viable virus	Berto A	2011	Iowa State University	Conference Paper	4
Hepatitis E virus : identification and evaluation of the potential for zoonotic transmission in the pork food chain	Berto A	2012	University of Surrey	PhD Thesis	4
Application of ELISA recomWell HEV IgG (human) for detection of virus-specific antibodies in sera of slaughtered rabbits	Bigorai E	2018	Food Anal Meths	12; 3566-3581	2
Analytical Methods for Virus Detection in Water and Food	Bosch A	2011	Food Anal Meths	4; 4-12	1
A novel eukaryotic cell culture model to study antiviral activity of potential probiotic bacteria	Botic T	2007	Int J Food Microbiology	115; 227-234	1
Hepatitis E virus RNA in commercial porcine livers in The Netherlands	Bouwknegt M	2007	J Food Protection	70; 2889-2895	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Comparative risk assessment to prioritize pork products for potential foodborne hepatitis E virus transmission.	Bouwknegt M	2018	J Food Protection	81; 34-35	3
Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA	Boxman ILA	2019	Int J Food Microbiology	296; 58-64	2
Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission	Boxman ILA	2017	Int J Food Microbiology	257; 225-231	3
Evaluation of viral inactivation of pseudorabies virus, encephalomyocarditis virus, bovine viral diarrhea virus and porcine parvovirus in pancreatin of porcine origin	Caruso C	2014	J Virol Meths	208; 79	1
Restricted enzooticity of hepatitis E virus genotypes 1 to 4 in the United States	Chen D	2011	J Cin Micro	49; 4164-4172	2
Control of Viral Contamination of Food and Environment	Cliver DO	2009	Food Environmental Virology	1; 6	3
Virus transmission via food	Cliver DO	1997	Food Tech	51; 71-79	5
Emergence of hepatitis E virus in the pig and human populations of industrialised countries	Conlan J	2015	Food Standards Australia New Zealand (FSANZ)	N/A	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
in the northern hemisphere and why is it not apparent in the southern hemisphere					
A critical review of approaches to assess the infectivity of hepatitis E virus. A report to the United Kingdom Food Standards Agency	Cook N	2016	Food Standards Agency	72 pages	2
Potential approaches to assess the infectivity of hepatitis E virus in pork products: a review.	Cook N	2017	Food Environmental Virology	9; 243-255	2
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	Cook N	2014	Food Standards Agency	98 pages	5
Survival and elimination of hepatitis E Virus: a review	Cook N	2015	Food Environmental Virology	7; 189-194	5
Hepatitis E virus in rabbits, Virginia, USA	Cossaboom CM	2011	Emerging Infectious Diseases	17; 2047-2049	2
Cross-species infection of pigs with a novel rabbit, but not rat, strain of hepatitis E virus isolated in the United States	Cossaboom CM	2012	J Gen Virol	93; 1687-1695	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Serological and Molecular Investigation of Swine Hepatitis E Virus in Pigs Raised in Southern Italy	Costanzo N	2015	J Food Protection	78; 2099-2102	2
The challenge of hepatitis E virus	D'Agostino M	2018	New Food	21; 56-58	4
High levels of Hepatitis E virus in wild boar hunted for domestic consumption in Central Italy.	Dantis P	2018	European J Public Health	28; Supp 4	2
Hepatitis E virus: a nationwide study for risk assessment of foodborne transmission	De Medici, D	2018	European J Public Health	28; Suppl 4	2
High levels of Hepatitis E virus in wild boar hunted for domestic consumption in Central Italy	De Santis P	2018	European J Public Health	28; Suppl 4	2
Acute hepatitis due to hepatitis E virus after raw meat consumption	de Zaraga Mata C	2017	Gastroenterol Hepatol	6; 395-396	3
Hepatitis E virus (HEV)-the future	Denner J	2019	Viruses	11; 251	2
Detection of hepatitis E virus in pork liver sausages	Di Bartolo I	2015	Int J Food Microbiology	193; 29-33	2
Serological survey of hepatitis E virus infection in farmed and pet rabbits in Italy	Di Bartolo I	2016	Archives of Virology	161; 1343-1346	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E virus: a nationwide study for risk assessment of foodborne transmission.	Di Medici D	2018	European J Public Health	28; Supp 4	2
Quantification and genetic diversity of Hepatitis E virus in wild boar (Sus scrofa) hunted for domestic consumption in Central Italy	Di Pasquale S	2019	Food Microbiol	82; 194-201	2
Development of a viability PCR assay for the analysis of Hepatitis E virus in food matrices	Di Pasquale S	2018	European J Public Health	38; Suppl 4	4
Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays	Diez-Valcarce M	2011	Food Anal Meths	4; 437-445	2
Hepatitis E Virus in Wild Boar in Northwest Poland: Sensitivity of Methods of Detection	Dorn-In S	2017	Food Paths Dis	14; 103-108	2
Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses.	EFSA BIOHAZ	2011	EFSA J	9; 2190	4
Thermal stability of hepatitis E virus	Emerson S	2005	J Infect Dis	192; 930-933	Accepted

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Inactivation of viruses and bacteriophages as models for swine hepatitis E virus in food matrices	Emmoth E	2017	Food Environmental Virology	9; 20-34	2
Viruses in food: scientific advice to support risk management activities. Meeting report	FAO / WHO	2008	Microbiological Risk Assessment Series no. 13	Report	2
Hepatitis E virus and the safety of plasma products: investigations into the reduction capacity of manufacturing processes	Farcet MR	2016	Transfusion	56; 383-391	Accepted
Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States	Feagins AR	2008	Int J Food Microbiology	123; 32-37	4
Foodborne Transmission and Molecular Mechanism of Cross-species Infection of Hepatitis E Virus (HEV)	Feagins AR	2010	Virginia Polytechnic Institute and State University	PhD Thesis	4
Transmission of hepatitis E virus by water: An issue still pending in industrialized countries	Fenaux H	2019	Water Res	151; 144-157	5

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
A metagenomic assessment of viral contamination on fresh parsley plants irrigated with fecally tainted river water	Fernandez-Cassi X	2017	Int J Food Microbiology	257; 80-90	1
High load of hepatitis E viral RNA in pork livers but absence in pork muscle at French slaughterhouses	Feurer C	2018	Int J Food Microbiology	264; 25-30	2
Seroprevalence of parvovirus B19, cytomegalovirus, hepatitis A virus and hepatitis E virus antibodies in haemophiliacs treated exclusively with clotting-factor concentrates considered safe against human immunodeficiency and hepatitis C viruses	Flores G	1995	Haemophilia	1; 115-117	1
Wild Boar: A Reservoir of Foodborne Zoonoses	Fredriksson-Ahomaa	2019	Food Paths Dis	16; 153-165	2
Development and evaluation of a RT-LAMP assay for rapid detection of hepatitis E virus from shellfish	Gao SY	2016	Int J Food Microbiology	220; 1-5	2
Minor groove binder modification of widely used TaqMan probe for hepatitis E virus	Garson JA	2012	J Virol Meths	186; 157-160	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
reduces risk of false negative real-time PCR results					
Detection of Hepatitis E Virus in Raw Pork and Pig Viscera As Food in Hebei Province of China	Geng YS	2019	Food Paths Dis	16; 325-330	2
Detection of Hepatitis E Virus RNA in Raw Cured Sausages and Raw Cured Sausages Containing Pig Liver at Retail Stores in Switzerland	Giannini P	2018	J Food Protection	81; 43-45	2
Emerging viruses are inactivated by pasteurization	Groener A	2004	Blood	104; 114B	2
Effective inactivation of a wide range of viruses by pasteurization	Groener A	2018	Transfusion	58; 41	3
Detection of hepatitis E virus genome in pig livers in Antioquia, Colombia	Gutierrez-Vergara C	2015	Genet Mol Res	14; 2890-2899	2
Look to the Future: viruses, parasites and emerging issues in seafood	Hackney CR	1998	Institute of Food Technologists	Conference Paper	2
Summary Report of Joint Scientific Workshop on Foodborne Viruses	Hayward MP	2016	EFSA Report	Report	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Stem cell-derived hepatocytes: A novel model for hepatitis E virus replication	Helsen N	2016	J Hepatol	64; 565-573	2
A Cross-Sectional Study of Hepatitis E Virus Infection in Pigs in Different-Sized Farms in Northern Thailand	Hinjoy S	2013	Foodborne Pathogens and Disease	10; 698-704	2
Animal-borne viruses of relevance to the food industry	ILSI Europe Expert Group on Animal Borne Viruses	2009	ILSI Europe Report Series	64 pages	4
Evaluation of Heating Conditions for Inactivation of Hepatitis E Virus Genotypes 3 and 4	Imagawa T	2018	J Food Protection	81; 957-952	4
Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France	Izopet J	2012	Emerging Infectious Diseases	18; 1274-1281	2
The hepatitis E virus ORF3 protein modulates endocytic trafficking and apoptotic pathways to promote survival	Jameel S	2006	The FEBS Journal	273; poster abstract	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E - a foodborne viral zoonosis in Germany?	Johne R	2008	Archiv Fur Lebensmittelhygiene	59; 44-48	2
An ORF1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture	Johne R	2014	J Viral Hepat	21; 447-456	4
Thermal stability of hepatitis E virus as estimated by a cell culture method.	Johne R	2016	Applied Environmental Microbiology	82; 4225-4331	Accepted
Assessment of F-RNA Coliphage as a Potential Indicator of Enteric Virus Contamination of Hog Carcasses	Jones TH	2012	J Food Protection	75; 1492-1500	1
Survival of Porcine teschovirus as a surrogate virus on pork chops during storage at 2 degrees C	Jones TH	2015	Int J Food Microbiology	194; 21-24	1
F-coliphages, porcine adenovirus and porcine teschovirus as potential indicator viruses of fecal contamination for pork carcass processing	Jones TH	2017	Int J Food Microbiology	241; 237-243	1
Frequency of hepatitis E virus, rotavirus and porcine enteric calicivirus at various stages of	Jones TH	2017	Int J Food Microbiology	259; 29-34	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
pork carcass processing in two pork processing plants					
Effect of handling and storage conditions and stabilizing agent on the recovery of viral RNA from oral fluid of pigs	Jones TH	2014	J Virol Meths	198; 26-31	4
Game Meat Consumption and Foodborne Illness in Japan: A Web-Based Questionnaire Survey	Kadohira M	2019	J Food Protection	82; 1224-1232	1
Hepatitis E Virus in Young Pigs in Finland and Characterization of the Isolated Partial Genomic Sequences of Genotype 3 HEV	Kantala T	2015	Food Paths Dis	12; 253-260	2
Enteric porcine viruses in farmed shellfish in Denmark	Krog JS	2014	Int J Food Microbiology	186; 105-109	2
The detection and characterization of hepatitis E virus in pig livers from retail markets of India	Kulkarni MA	2008	J Med Virol	80; 1387-1390	2
Presence of hepatitis E virus in a naturally infected swine herd from nursery to slaughter	Leblanc D	2007	Int J Food Microbiology	117; 160-166	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E virus load in swine organs and tissues at slaughterhouse determined by real-time RT-PCR	Leblanc D	2010	Int J Food Microbiology	139; 206-209	2
Viruses in food.	Leininger HV	1980	Association of Food and Drug Officials, Quarterly Bulletin	44; 82-85	3
Transmission routes and risk factors for autochthonous hepatitis E virus infection in Europe: a systematic review	Lewis HC	2010	Epidemiol Infect	138 145-166	2
Impact of food unit operations on virus loads in foods	Li D	2017	Quantitative microbiology in food processing: modelling the microbial ecology	263-287	5
Importance of foods in the transmission of pathogenic enteric viruses	Lopez-Sabater EI	1996	Alimentaria	270; 101-115	2
Presence of Mycobacterium avium Subspecies and Hepatitis E Virus in Raw Meat Products	Lorencova A	2014	J Food Protection	77; 335-338	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Reverse Transcription-Real-Time PCR A biochemical Method for Rapid Detection of Hepatitis-E-Viruses	Lorenzen E	2017	Deutsche Lebensmittel-Rundschau	113; 504-507	0
Microbiological Food Safety and a Low-Microbial Diet to Protect Vulnerable People	Lund BM	2014	Food Paths Dis	11; 413-424	1
Foodborne viral diseases	Maifreni M	1993	Annali di Microbiologia ed Enzimologia	43; 181-193	1
Detection of foodborne viruses in ready-to-eat meat products and meat processing plants	Markantonis N	2018	J Food Safety	38; 12436	2
Duplex RT-qPCR for the detection of hepatitis E virus in water, using a process control	Martin-Latil S	2012	Int J Food Microbiology	157; 167-173	2
Method for HEV detection in raw pig liver products and its implementation for naturally contaminated food	Martin-Latil S	2014	Int J Food Microbiology	176; 1-8	2
Evaluation of Extraction Methods for Efficient Detection of Enteric Viruses in Pork Meat Products	Martinez-Martinez M	2011	Food Anal Meths	4; 13-22	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Risk profiles of pork and poultry meat and risk ratings of various pathogen/product combinations	Mataragas M	2008	Int J Food Microbiology	126; 1-12	3
Tracing enteric viruses in the European berry fruit supply chain	Maunula L	2013	Int J Food Microbiology	167; 177-185	1
Meat Juice Multi-Serology - A tool for the continuous improvement of herd health and food safety in the framework of the risk-based meat inspection of slaughter pigs	Meemken D	2011	Archiv Fur Lebensmittelhygiene	62; 192-199	1
Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain	Mesquita JR	2016	Food Microbiol	58; 13-15	2
Thermal processing of live bivalve molluscs for controlling viruses: on the need for a risk-based design	Messens W	2018	Critical Reviews in Food Science and Nutrition	58; 2854-2865	1
Assessment of the risk of foodborne transmission and burden of hepatitis E in Switzerland	Muller A	2017	Int J Food Microbiology	242; 107-115	2
Hepatitis E-Virus in deutschen Wildschweinen	N/A	2010	(BfR), Bundesinstitut fur Risikobewertung	Web Page	0

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Schutz vor viralen Lebensmittelinfektionen	N/A	2013	(BfR), Bundesinstitut für Risikobewertung	Report	0
Current Opinion of the FfR to the Hepatitis E-Virus	N/A	2016	Fleischwirtschaft	96; 96-97	0
Minutes of the meeting of the Scientific Committee - 11 September 2012	N/A	2012	Food Safety Authority of Ireland (FSAI)	Web Page	2
Stability and inactivation of hepatitis E virus in food and the environment (hepatitis E virus)	N/A	2018	Bundesinstitut fur Risikobewertung (BfR)	Web Page	2
Examining the effects of various temperatures on the survival of viruses in fruits and vegetables to enhance understanding of virus contamination in produce and allow better control of infections associated with foodborne viruses	N/A	2019	Illinois Institute of Technology. Institute of Food Safety and Health (IFSH)	Web Page	2
Questions and answers on the transmission of the hepatitis E virus through wild boars and domestic pigs and foods derived from them	N/A	2016	Bundesinstitut fur Risikoberwertung (BfR)	Web Page	3

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Technical report of the Food Standards Agency: Thematic workshop on Hepatitis E virus 26/27 March 2018	N/A	2018	Food Standards Agency	Report	3
Building Capacity to Control Viral Foodborne Disease: A Translational, Multidisciplanary Approach	N/A	2019	Illinois Institute of Technology. Institute of Food Safety and Health (IFSH)	Web Page	3
Hepatitis E virus in food	N/A	2019	Food Standards Australia New Zealand (FSANZ)	Web Page	3
Hepatitis E virus in fresh pig livers	N/A	2010	Centre for Food Safety, Hong Kong	Report	3
Opinion of the French Food Safety Agency regarding the risk to man of infection with the hepatitis E virus (HEV) after ingestion of figatelli (raw sausages containing pork liver)	N/A	2009	Agence Francaise de Securite Sanitaire des Aliments	Web Page	4
Final Report Summary - VITAL (Integrated monitoring and control of foodborne viruses in European food supply chains)	N/A	2011	CORDIS	Web Page	4

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Pathogen Safety Data Sheets: Infectious Substances – Hepatitis E virus	N/A	2011	Government of Canada	Web Page	4
Opinion of the French Agency for Food, Environmental and Occupational Health & Safety concerning the "Request to assess the risks related to contamination of delicatessen meats products derived from raw pork liver with hepatitis E virus (HEV)"	N/A	2013	French Agency for Food, Environmental and Occupational Health & Safety	ANSES Opinion Request No. 2012-SA-0012	4
Hepatitis E Virus and Food; 7. Can cooking kill hepatitis E virus?	N/A	2017	Food Safety Authority of Ireland (FSAI)	Web Page	4
Reflection paper on viral safety of plasma-derived medicinal products with respect to hepatitis E virus. EMA/CHMP/ BWP/723009/2014.	N/A	2015	London: European Medicines Agency; 2015 Jun 25.	Report	5
Molecular Investigation on the Presence of Hepatitis E Virus in Wild Game in Okazaki City, Japan between April 2010 and November 2014	Nakane K	2015	Food Hygiene and Safety Science	56; 252-255	2
Agro-defense: Biosensors for food from healthy crops and animals	Neethirajan S	2018	Trends Food Sci Tech	73; 25-44	1

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E virus, an etiological agent of foodborne and waterborne hepatitis	Netzler NE	2016	Foodborne Viral Pathogens	Book	4
A novel multiplex isothermal amplification method for rapid detection and identification of viruses	Nyan DC	2015	Sci Rep	5; 17925	1
An investigation of the presence of hepatitis E virus in Scottish harvested shellfish	O'Hara Z	2018	Glasgow Caledonian University	PhD Thesis	3
Hepatitis E Virus In Shellfish of Iberian Peninsula: A Risk for Public Health	Oliveira, D	2015	Universidade do Porto	PhD Thesis	3
Stability and inactivation of hepatitis E virus in food and the environment (hepatitis E virus)	Parashar D	2011	Clinical Microbiology and Infection	1; E1-E4	4
Survival of hepatitis A and E viruses in soil samples	Parashar D	2011	Clin Microbiol Infect	17; E1–E4	Accepted
Epidemiology of hepatitis E: a (re) emerging disease?	Pariente A	2015	Presse Med	44; 333-338	0
A molecular survey of farmed and edible snails for the presence of human enteric viruses: Tracking of the possible environmental sources of microbial mollusc contamination	Paszkiewicz W	2016	Food Control	69; 368-372	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Chief Scientific Advisors Science Report: Issue One: foodborne viruses	Poppy G	2015	Food Standards Agency	16 pages	3
Hepatitis E virus in lettuce and water samples: A method-comparison study	Randazzo W	2018	Int J Food Microbiology	277; 34-40	2
Viability RT-qPCR to Distinguish Between HEV and HAV With Intact and Altered Capsids	Randazzo W	2018	Front Microbiol	9; 1973	Accepted
Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen	Ricci A	2017	EFSA J	15; 4886	5
Processing strategies to inactivate enteric viruses in shellfish.	Richards GP	2010	Food Environmental Virology	2; 183-193	3
From hazard analysis to risk control using rapid methods in microbiology: a practical approach for the food industry	Ripolles-Avila C	2020	Comprehensive Reviews in Food Science and Food Safety	2020; 1-31	2
Virus hazards from food, water and other contaminated environments	Rodriguez-Lazaro D	2012	FEMS Micro Revs	36; 786-814	2
Presence of pathogenic enteric viruses in illegally imported meat and meat products to EU by international air travelers	Rodriguez-Lazaro D	2015	Int J Food Microbiology	209; 39-43	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E Virus: A New Foodborne Zoonotic Concern	Rodriguez-Lazaro D	2018	Adv Food Nutr Res	86; 55-70	2
New models of hepatitis E virus replication in human and porcine hepatocyte cell lines	Rogee S	2013	J Gen Virol	94; 549-558	2
Synthese - epidemiology of hepatitis E virus in pigs: the way forward to limit consumer exposure.	Rose N	2014	Journees de la Recherche Porcine en France	46; 159-168	3
Seroprevalence of Hepatitis E Virus in Pigs from Different Farming Systems in The Netherlands	Rutjes S	2014	J Food Protection	77; 640-642	2
Persistent viremia and presence of hepatitis E virus RNA in pig muscle meat after experimental co-infection with porcine reproductive and respiratory syndrome virus	Salines M	2019	Int J Food Microbiology	292; 144-149	2
Prevalence of IgG against hepatitis E virus, Salmonella spp., and Toxoplasma gondii in meat juice samples from wild boars hunted in Southern Italy	Sarno E	2014	J Food Safety Food Quality	65; 141-144	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Estimated exposure to hepatitis E virus through consumption of swine liver and liver sausages	Sarno E	2017	Food Control	73; 821-828	2
Prevalence and Antimicrobial Susceptibility of Foodborne Bacteria in Wild Boars (Sus scrofa) and Wild Deer (Cervus nippon) in Japan	Sasaki Y	2013	Food Paths Dis	10; 985-991	1
Capacity of pasteurization to inactivate a wide range of non-enveloped viruses	Schaefer W	2001	Blood	98; 110B	1
Thermal stability of hepatitis E virus assessed by a molecular biological approach	Schielke A	2011	Virol J	8; 487	Accepted
Food associated viruses and their detection - a review	Scrader C	2011	Archiv Fur Lebensmittelhygiene	62; 36-51	2
Seasonal and regional prevalence of norovirus, hepatitis A virus, hepatitis E virus, and rotavirus in shellfish harvested from South Korea	Seo DJ	2014	Food Control	41; 178-184	2
Prevalence and evaluation strategies for viral contamination in food products: Risk to human health-a review	Shukla S	2018	Critical Reviews in Food Science and Nutrition	58; 405-419	2
New trends in emerging pathogens	Skovgaard N	2007	Int J Food Microbiology	120; 217-224	1

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Genetic variability and the classification of hepatitis E virus	Smith DB	2013	J Virol	87; 4161-4169	2
Foodborne infections during pregnancy	Smith JL	1999	J Food Protection	62; 818-829	2
A review of hepatitis E virus	Smith JL	2001	J Food Protection	64; 572-586	2
Emerging and Re-Emerging Foodborne Pathogens	Smith JL	2018	Food Paths Dis	15; 737-757	2
Hepatitis E virus: the latest public health scenario	Soare C	2017	Game meat hygiene. Food safety and security (Paulsen, Bauer and Smulders, eds)	Book	5
Hepatitis E virus: reasons for emergence in humans	Sooryanarain H	2019	Cur Opin Virol	4; 10-17	4
Foodborne pathogens and their risk exposure factors associated with farm vegetables in Rwanda	Ssemanda JN	2018	Food Control	89; 86-96	2
Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers	Stine SW	2005	J Food Protection	124; 1352-1358	1

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Detection of hepatitis E virus RNA in raw sausages and liver sausages from retail in Germany using an optimized method	Szabo K	2015	Int J Food Microbiology	215; 149-156	2
Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype	Takahashi M	2011	J Gen Virol	92; 902-908	2
Development of reverse transcriptase polymerase chain reaction enzyme-linked immunosorbent assay for the detection of hepatitis A virus in vegetables	Takh H	2012	Food Control	23; 210-214	1
Development and evaluation of an efficient cell-culture system for Hepatitis E virus	Tanaka T	2007	J Gen Virol	88; 903-911	4
Much meat, much malady: changing perceptions of the epidemiology of hepatitis E	Teo GC	2010	Clinical Microbiology and Infection	16; 24-32	3
Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy	Terio V	2017	Int J Food Microbiology	249; 61-65	2
Epidemiological study of hepatitis E virus infection in the general population of Okinawa, Kyushu, Japan	Toyoda K	2008	J Gastroenterol Hepatol	23; 1885-1890	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Hepatitis E door het eten van rauw vlees.	van der Poel W	2003	Tijdschr Diergeneeskd	128; 601-602	0
Knowledge gaps and research priorities in the prevention and control of hepatitis E virus infection	van der Poel W	2018	Transboundary and Emerging Diseases	65; 22-29	2
Hepatitis E virus: a review	Vasickova P	2007	Veterinarni Medicina	52; 365	2
Viruses as a cause of foodborne diseases: a review of the literature	Vasickova P	2005	Veterinarni Medicina	50; 89-104	3
Absence of zoonotic hepatitis E virus infection in Flemish dairy cows	Vercouter AS	2018	Int J Food Microbiology	281; 54-59	2
Detection of IgM and IgG Against Hepatitis E Virus in Serum and Meat Juice Samples from Pigs at Slaughter in Bavaria, Germany	Wacheck S	2012	Food Paths Dis	9; 655-660	2
Seroprevalence of Anti-Hepatitis E Virus and Anti-Salmonella Antibodies in Pigs at Slaughter in Switzerland	Wacheck S	2012	J Food Protection	75; 1483-1485	2
Low-dose irradiation improves microbial quality and shelf life of fresh mint (Mentha piperita L.) without compromising visual quality	Wei-Yea H	2010	J Food Sci	75; M222-M230	1

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Influence of RNA secondary structure on HEV gene amplification using reverse-transcription and nested polymerase chain reaction	Wei, S	2003	J Clin Virol	27; 152-161	2
Achievement V - Methods for breaking the transmission of pathogens along the food chain Detection of viruses in food	Widen F	2011	Trends Food Sci Tech	22; S49-S57	1
Survey of Canadian retail pork chops and pork livers for detection of hepatitis E virus, norovirus, and rotavirus using real time RT-PCR	Wilhelm B	2014	Int J Food Microbiology	185; 33-40	2
Stability of hepatitis E virus at different pH values	Wolff A	2020	Int J Food Microbiology	325; 108625	Accepted
Effect of sodium chloride, sodium nitrite and sodium nitrate on the infectivity of hepatitis E virus	Wolff A	2020	Food and Environmental Virology	12; 350-324	Accepted
Other viral food poisoning (hepatitis A and E)	Yano K	2012	Nihon Rinsho	70; 1386-1390	2
Hepatitis E virus was not detected in feces and milk of cows in Hebei province of China: no evidence for HEV prevalence in cows	Yanshen G	2019	Int J Food Microbiology	291; 5-9	2

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
High seroprevalence of hepatitis E virus in rabbit slaughterhouse workers	Yanshen G	2019	Transboundary and Emerging Diseases	66; 1085-1089	2
First reported outbreak of locally acquired hepatitis E virus infection in Australia	Yapa CM	2016	Med J Aust	204; 274	2
Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food	Yazaki Y	2003	J Gen Virol	84; 2351-2357	2
Investigation of Hepatitis A and E Viruses in Mussels Collected from the Bosphorus, in Istanbul, Turkey - Short Communication	Yilmaz J	2018	Czech Journal of Food Sciences	36; 215-220	2
Homology model and potential virus-capsid binding site of a putative HEV receptor Grp78	Yu H	2011	J Mol Model	17; 987-995	2
Extent of hepatitis E virus elimination is affected by stabilizers present in plasma products and pore size of nanofilters	Yunoki M	2008	Vox Sanguis	95;-94-100	Accepted
Hepatitis E virus derived from different sources exhibits different behaviour in virus inactivation and/or removal studies with plasma derivatives	Yunoki M	2016	Biologicals	44; 403-411	Accepted

Title	First Author	Date	Journal	Volume / Page Number	Rejected at Checklist Step
Acidic pH enhances structure and structural stability of the capsid protein of hepatitis E virus	Zafrullah M	2004	Biochem Biophys Res Communication	313; 67-73	4
Expression of thermal stable, soluble hepatitis E virus recombinant antigen	Zhang M	2002	Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi	16; 20-22	2
Comparison of effects of hepatitis E or A viral superinfection in patients with chronic hepatitis B	Zhang X	2010	Hepatology Intermnational	4; 615-620	2
Epidemiology of zoonotic hepatitis E: a community-based surveillance study in a rural population in China	Zhu FC	2014	PLoS One	9; e87154	2
Effects of treatments used in food processing on viruses	Zuber S	2013	Foodborne viruses and prions and their significance for public health	6; 376	4

Appendix 2: Literature Database and information for the selected manuscripts described in Table 2, Genotype 3 and 4.

Table 1: Assay - Infectivity assayed by counting infected cells by immunofluorescence microscopy; 2 replicates. Matrix - cell culture supernatant (5% fetal calf serum). Strain/source of virus - Genotype 3 strain 47832c; isolated from patient and propagated on A549 cells. Reference - Johne R (2016) Appl Env Micro 82; 4225-4331.

Time	Temperature (˚C)	Starting input of virus	Log Reduction
10 d	4	~4 log	1.60
56 d	4	~ 4 log	2.70
7 d	RT (~22)	~ 4 log	1.70
28 d	RT (~22)	~ 4 log	3.70
4 d	37	~ 3.5 log	2.00
21 d	37	~ 3.5 log	3.40
1 min	60	~ 3.5 log	<1.3
1 min	65	~ 3.5 log	2.60
50 sec	70	~ 4 log	2.90
1 min	70	~ 3.5 log	2.90
90 sec	70	~ 4 log	>3.0
2 min	70	~ 4 log	>3.9
1 min	75	~ 3.5 log	3.40
1 min	75	~ 3.5 log	>3.5

Time	Temperature (˚C)	Starting input of virus	Log Reduction
1 min	80	~ 3.5 log	>3.5

Table 2: Assay - Genome equivalents measured by RT-PCR; no information on replication. Matrix - Naturally infected pig liver, homogenised with added "spices", nitrite salt (2%, containing 99.4% NaCl and 0.5% NaNO2 E250), dextrose (0.5%) and sodium caseinate (2%). Strain/source of virus - Genotype 3 subtype 3e; from experimentally infected pig. Reference - Barnaud E (2012). Appl Env Micro 78; 5153-5159

Time	Temperature (°C)	Starting number of virus	Log Reduction
5 min	62	10^8 GE g^{-1}	1.19
10 min	62	10^8 GE g^{-1}	1.83
120 min	62	10^8 GE g^{-1}	2.17
5 min	68	10^8 GE g^{-1}	2.28
10 min	68	10^8 GE g^{-1}	2.26
20 min	68	10^8 GE g^{-1}	2.31
5 min	71	10^8 GE g^{-1}	2.93
10 min	71	10^8 GE g^{-1}	2.58
20 min	71	10^8 GE g^{-1}	2.69

Table 3: Assay - Genome equivalents measured by capsid integrity / RT-PCR assay; no information on replication. Matrix - Naturally infected wild boar liver, in PBS pH7.4. Strain/source of virus - Genotype 3i; homogenised and filtered infected liver suspension. Reference - Schielke A (2011) Virol J 8; 487

Time	**Temperature (°C)**	**Starting number of virus**	**Log Reduction**
15 min	56	5.8×10^8 GE ml^{-1}	0.59
30 min	56	5.8×10^8 GE ml^{-1}	4.00
60 min	56	5.8×10^8 GE ml^{-1}	3.00
60 min	60	5.8×10^8 GE ml^{-1}	3.22
1 min	70	5.8×10^8 GE ml^{-1}	0.48
1 min	75	5.8×10^8 GE ml^{-1}	0.00
1 min	80	5.8×10^8 GE ml^{-1}	2.47
1 min	85	5.8×10^8 GE ml^{-1}	2.58
1 min	90	5.8×10^8 GE ml^{-1}	3.58
1 min	95	5.8×10^8 GE ml^{-1}	3.67

Table 4: Assay - Genome equivalents measured by RT-PCR; no information on replication. Matrix - PBS containing 2 M NaNO3, 1% beef extract, and 0.1% Triton X-100. Strain/source of virus - Genotype 3f; Human faecal suspension. Reference - Randazzo W (2018). Front Microbiol 9; 1973; (Fig 1 indicates very approx 6.7 GE ml-1)

Time	**Temperature (°C)**	**Starting number of virus**	**Log Reduction**
15 min	60	approx 6 log IU ml-1 stated	<1.0
15 min	72	approx 6 log IU ml-1 stated	2.00
15 min	95	approx 6 log IU ml-1 stated	2.00

Table 5: Assay - Infectivity assayed by calculation of the 50 % tissue culture infectious dose (TCID50); no information on replication. Matrix - PBS pH7.5 (1hour time point); 12.5% human serum albumin (3hour time point). Strain/source of virus - Isolate from pig liver (genotype not stated). Reference - Farcet M (2016) Transfusion 56; 383-391

Time	**Temperature (°C)**	**Starting number of virus**	**Log Reduction**
1 h	58	4.5 log TCID50 ml^{-1}	>3.7
3 h	58	4.5 log TCID50 ml^{-1}	>3.7

Table 6: Assay - Infectivity measured by titration of suspension followed by cell culture / RT-PCR end-point detection; no information on replication. Matrix – PBS; +25% foetal bovine serum at 5hour time points. Strain/source of virus – i-Genotype 3 strain JPa; ii-Genotype 3 strain SP; faeces of naturally infected pig; faeces of experimentally infected pig; iii- Genotype 3 strain US; feces of naturally infected pig; iv-Genotype 4 strain JP; faeces of naturally infected pig. Reference - Yunoki M (2008) Vox Sang 95; 94-100.

Time	**Temperature (°C)**	**Strain/source of virus**	**Starting number of virus**	**Log Reduction**
30 min	60	i	3.8 log	>2.7
5 h	60	i	3.8 log	2.00
30 min	60	ii	~ 5 log	>3.7
5 h	60	ii	~ 5 log	2.00
30 min	60	iii	4.8 log	>3.7
5 h	60	iii	4.8 log	1.00
30 min	60	iv	3-4 log	>2.4
5 h	60	iv	3-4 log	>2.2

Table 7: Assay - Infectivity measured by titration of suspension followed by cell culture / RT-PCR end-point detection; 3 replicates. Matrix – refer to table column. Strain/source of virus – Gentotype 3b; from faeces of experimentally infected pig. Reference - Yunoki M (2016) Biologicals 44; 403-411.

Time	**Temperature (°C)**	**Matrix**	**Starting number of virus**	**Log Reduction**
0.5	60	PBS + 5% albumin	4.72 log TCID ml^{-1}	0.30
0.5	60	PBS + 5% albumin	4.72 lo TCID50 ml^{-1}	0.70
0.5	60	haptoglobin (commercial preparation)	5.07 log TCID50 ml^{-1}	0.60
0.5	60	antithrombin (commercial preparation)	4.42 log TCID50 ml^{-1}	>1
0.5	60	5% venoglobulin (commercial preparation)	4.87 log TCID50 ml^{-1}	1.70
1	60	PBS + 5% albumin	4.72 log TCID ml^{-1}	0.50
1	60	PBS + 25% albumin	4.72 log TCID ml^{-1}	0.50
1	60	haptoglobin (commercial preparation)	5.07 log TCID50 ml-1	1.20
1	60	antithrombin (commercial preparation)	4.42 log TCID50 ml^{-1}	>1.2
1	60	5% venoglobulin (commercial preparation)	4.87 log TCID50 ml^{-1}	>1.9
5	60	PBS + 5% albumin	4.72 log TCID50 ml-1	1.60

Time	Temperature (°C)	Matrix	Starting number of virus	Log Reduction
5	60	PBS + 25% albumin	4.72 log TCID50 ml-1	1.50
5	60	haptoglobin (commercial preparation)	5.07 log TCID50 ml-1	>2.7
5	60	antithrombin (commercial preparation)	4.42 log TCID50 ml^{-1}	>1.2
5	60	5% venoglobulin (commercial preparation)	4.87 log TCID50 ml^{-1}	>1.9
10	60	PBS + 5% albumin	4.72 log TCID ml-1	3.10
10	60	PBS + 25% albumin	4.72 log TCID50 ml-1	2.80
10	60	haptoglobin (commercial preparation)	5.07 log TCID50 ml-1	≥2.9
10	60	antithrombin (commercial preparation)	4.42 log TCID50 ml-1	≥3.2
10	60	5% venoglobulin (commercial preparation)	4.87 log TCID50 ml-1	≥2.4

Table 8: Assay - Infectivity assayed by counting infected cells by immunofluorescence microscopy; 8 replicates. 0.125 day is equivalent to 3 hours. Matrix – refer to table column. Strain/source of virus – Genotype 3 strain 47832c; isolated from chronically infected patient and propagated on A549 cells. Input of virus - 1.3 x 10*4 FFU ml-1. Reference - Wolff A (2020) Int J Food Micro 325 108625.

Time (days)	**Temperature (°C)**	**Log Reduction**	**Matrix**
7	4	0.00	PBS, adjusted with DL-lactic acid to pHs from 4.5 to 6.5
7	23	0.00	PBS, adjusted to pH 4.7 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 4.9 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.1 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.3 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.5 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.7 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.9 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 6.1 with DL-lactic acid

Time (days)	Temperature (°C)	Log Reduction	Matrix
7	23	0.00	PBS, adjusted to pH 6.3 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 6.5 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 4.5 with DL-lactic acid
7	23	0.50	PBS, adjusted to pH 4.7 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 4.9 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.1 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.3 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.5 with DL-lactic acid
7	23	-1.00	PBS, adjusted to pH 5.7 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 5.9 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 6.1 with DL-lactic acid
7	23	0.00	PBS, adjusted to pH 6.3 with DL-lactic acid

Time (days)	Temperature (°C)	Log Reduction	Matrix
7	23	0.00	PBS, adjusted to pH 6.5 with DL-lactic acid
0.125	23	>3.0	PBS, adjusted to pH 1.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.50	PBS, adjusted to pH 2.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.50	PBS, adjusted to pH 3.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.50	PBS, adjusted to pH 4.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.50	PBS, adjusted to pH 5.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.00	PBS, adjusted to pH 6.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.00	PBS, adjusted to pH 7.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.00	PBS, adjusted to pH 8.0 with 0.1-0.01M HCl / NaOH
0.125	23	0.00	PBS, adjusted to pH 9.0 with 0.1-0.01M HCl / NaOH
0.125	23	3.00	PBS, adjusted to pH 10.0 with 0.1-0.01M HCl / NaOH

Table 9: Assay - Infectivity assayed by counting infected cells by immunofluorescence microscopy; 8 replicates. Matrix – refer to table column. Strain/source of virus – Genotype 3 strain 47832c; isolated from chronically infected patient and propagated on A549 cells. Input of virus – 2.9 x 10*4 FFU ml-1. Reference - Wolff A (2020) Int J Food Micro 325 108625.

Time (days)	**Temperature (°C)**	**Log Reduction**	**Matrix**
1	23	0.60	PBS (0.8% NaCl)
1	23	0.60	PBS + 0.015% sodium nitrite
1	23	0.60	PBS + 0.03% sodium nitrate
1	23	0.60	PBS + 2% NaCl
1	23	0.60	PBS + 0.015% sodium nitrite + 2% NaCl
1	23	0.60	PBS + 0.03% sodium nitrate + 2% NaCl
1	23	0.60	PBS + 10% NaCl
1	23	0.60	PBS + 0.015% sodium nitrite + 10% NaCl
1	23	0.60	PBS + 0.03% sodium nitrate + 10% NaCl
1	23	0.60	PBS + 20% NaCl
1	23	0.60	PBS + 0.015% sodium nitrite + 20% NaCl

Time (days)	Temperature (°C)	Log Reduction	Matrix
1	23	0.60	PBS + 0.03% sodium nitrate + 20% NaCl
6	22	1.60	PBS
6	22	1.60	PBS + 2% NaCl
6	22	1.60	PBS + 0.015% sodium nitrite + 2% NaCl
56	16	1.80	PBS
56	16	1.80	PBS + 2% NaCl
56	16	1.80	PBS + 0.015% sodium nitrite + 2% NaCl
56	16	1.80	PBS + 0.03% sodium nitrate + 2% NaCl

Appendix 3: Literature Database and information for the selected manuscripts, Genotype 1 and 2.

Table 9: Assay - Infectivity measured by counting infected cells by immunofluorescence microscopy. Matrix – PBS PH7.4. Strain/source of virus – Genotype 1; patients' faecal samples. Input of virus – 204IU. Reference - Emerson S (2005) J Infect Dis 192; 930-933

Temperature (˚C)	**Time**	**Log Reduction**
56	15 min	1.27
56	30 min	1.83
56	1 h	1.83

Table 10. Assay - Genome equivalents measured by RT-PCR; no information on replication. Matrix – soil. Starting number of virus - 6.47 x 107 GE. Strain/source of virus - Genotype 1; patients' faecal samples. Input of virus - 6.47 x 10^7 GE. Reference - Parashar D (2011) Clin Microbiol 17; E1–E4

Temperature (°C)	**Time (weeks)**	**Log Reduction**
37	1	<1
37	2	0.56
37	3	3.02
37	4	3.09
37	5	3.49
37	6	3.56
37	7	4.49
37	8	4.62
37	9	4.75
37	10	4.83

Appendix 4 User guide

Introduction

The HEVTimes project has developed a thermal death model for Hepatitis E Virus (HEV), to investigate the relationship between cooking times, temperatures, and the resulting reduction in virus particles when HEV is heated, within the context of food preparation.

The model uses existing data from the literature, alongside new data from experiments carried out at Glasgow Caledonian University, both of which describe the log-reduction in HEV over time, for various times and temperature combinations. A Bayesian approach is then used to fit a suitable predictive microbiology model to the data. Data is contained within a .csv type file, which can be easily updated to include new data, as it becomes available.

The model has been coded into a user-friendly Shiny app that runs within the RStudio software, and the following user manual is intended to guide the user through the operation of the resulting HEVTimes app. The user manual contains three main sections:

1. **Quick Start Guide**: To get up and running quickly.

2. **Notes: User Manual**: Some more in depth notes about the app processes.

3. **Mathematical Model**: Brief summary of the predictive microbiology model.

4. **Updating the main Data File (hevtimes_Data.csv)**:
   Brief guide to using, editing, and updating with additional data.

Quick Start Guide:

1. Install **R software**: www.r-project.org . (Choosing correct version for your computer's operating system) Software download links can be found at cran.r-project.org/mirrors.html .
2. Install **RStudio Desktop software** (user interface for R): rstudio.com . Software download can be found at rstudio.com/products/rstudio/download/ .
3. Install **JAGS software** (Just Another Gibbs Solver mcmc-jags.sourceforge.net This handles the Bayesian simulations. Download at sourceforge.net/projects/mcmc-jags/files/ .
4. Install required **R Packages**:
   Simply open "HEVTimes_Install_Packages.R" in RStudio, highlight all (crtl+a) and hit "run" in top toolbar.

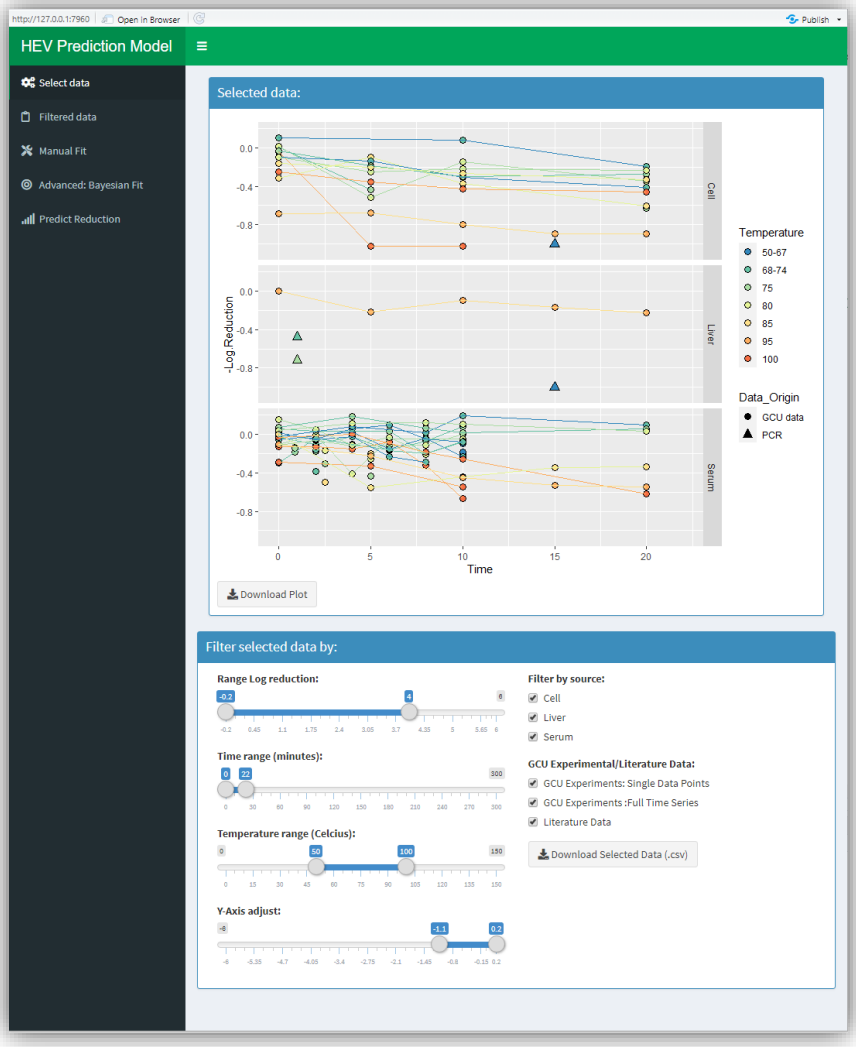

Figure 1 Selected data tab

5. Provide **HEVTimes data** in appropriate .csv file format:
   This must be labelled “HEVTimes_Data.csv” and placed in the same folder as “HEVTimes_App.R” code.
6. Open “**HEVTimes_App.R**” in Rstudio. (Main code for the Shiny app):
   Hit “Run App” in the top toolbar. This should launch the app, as per Figure 1.
7. On the “**Select Data**” Tab (Figure 1):
   Use the sliders/check boxes to select the data you want to visualise/fit the model to. The figures will automatically adjust to show you the data you have chosen. Whilst the two “Download” buttons let you download this selected data as a .csv file or save the current plot. The data selected within this tab is then used by all the other tabs within the app.

8. Move to the “**Filtered Data**” Tab (Figure 2): This tab presents a searchable table, containing all of the data selected in the “Select Data” tab.

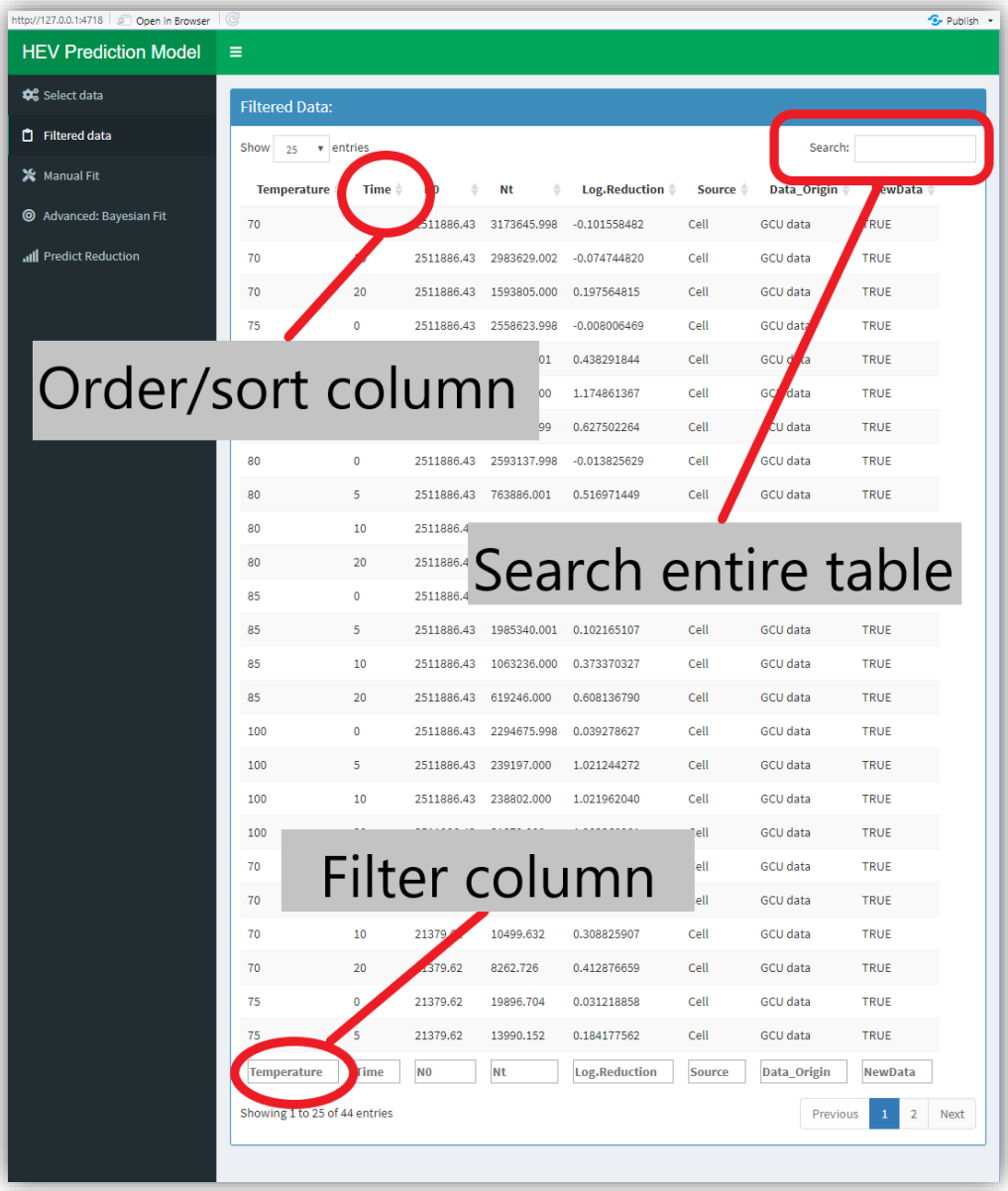

Figure 1: Filtered Data Tab

Allowing you to see exactly which data points you are working with, and search/filter them by Temperature, Log Reduction, Time, etc. to gain further insights.

9. Move to the “**Manual Fit**” Tab (Figure 3):

[Note: This manual fit step is entirely optional. If desired, users can skip straight to Step 10.

This manual fit is intended as useful visual aid for both visualising the effect each model parameter has on the curve, as well as selecting suitable “initial guesses” for the true value of each parameter].

The model equation is presented at the top of this page, for reference.

Two sliders let you manually adjust the model parameters (A and β), to instantly see the effect they have upon the log-reduction curve produced by the model equation (Plotted in black).

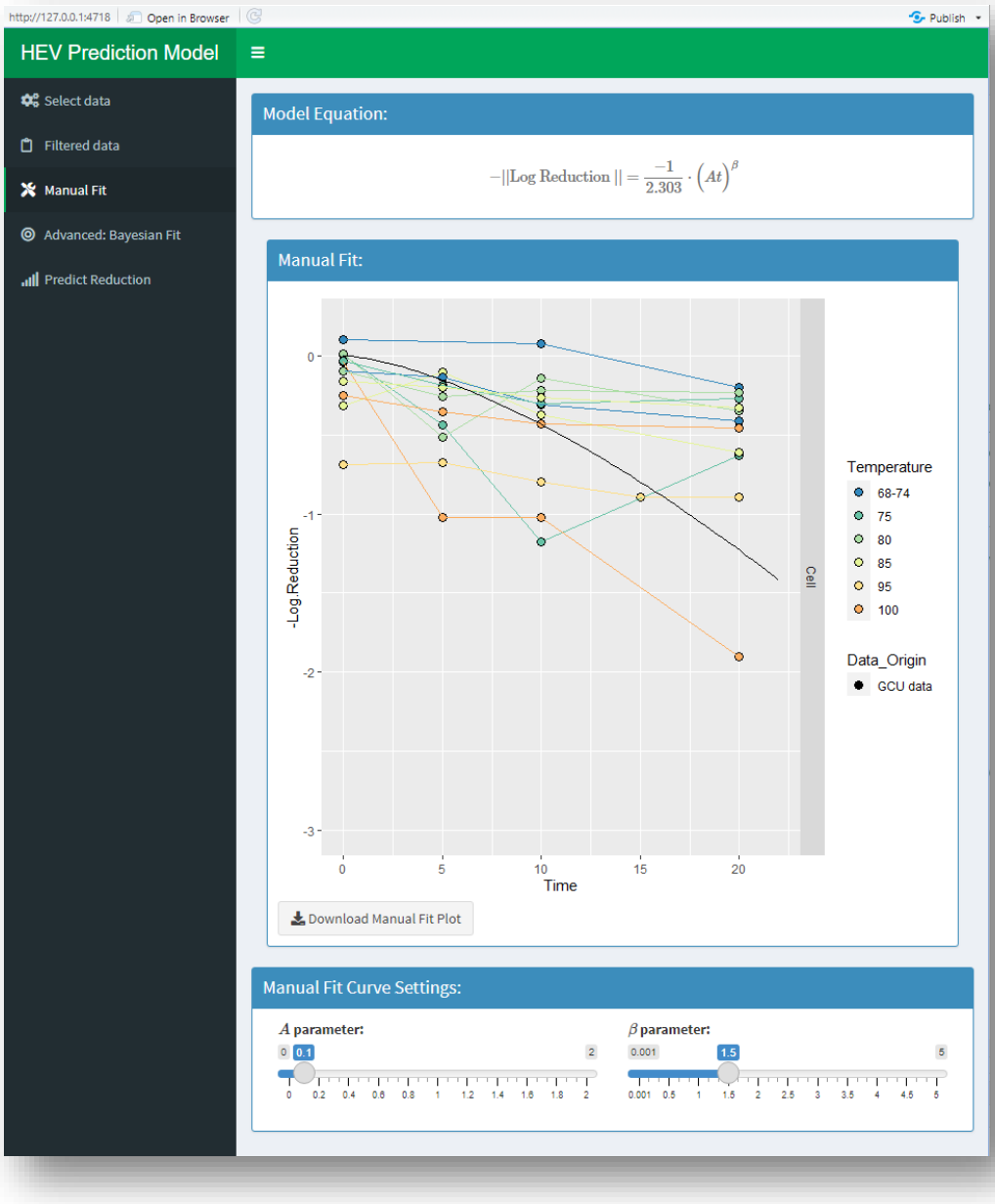

Figure 2: Manual fit tab

Here, A is a constant frequency parameter and β is a constant shape parameter, and **these two parameters will be different for each temperature T .**

In its standard form, the model equation is a two-parameter Weibull equation, that takes the form:

$$N_t = N_0 \, exp\left[-(A\,t)^{\beta}\right]$$

where, N_t is the number of surviving virus particles left after a sample has been exposed to a particular temperature T (Celsius), for time t (minutes). N_0 is the number of virus particles initially, before any heating has taken place.

Adjusting the values of A and β on this page, automatically updates the corresponding model prior on the following “Advanced: Bayesian Fit” tab. (“Priors” are essentially informed guesses, as to what the true value of these parameters should be). This feature allows you to easily focus on a particular temperature, find parameter values that fit the data points for this temperature, and in turn send sensible priors to the next tab, for use within the Bayesian simulations.

The “Download” button lets you save any figures you create on this page.

10. Next the “**Advanced: Bayesian Fit**” Tab (Figure 4):

    This tab fits the model equation/parameters to the data points **for the selected temperature**.

    First, select a temperature (using the slider) to estimate A and β for, then select sensible priors (or feel free to leave these alone, if you have already estimated them using the “Manual fit” tab). (Prior values inserted here are the mean value of the prior distributions used within simulations).

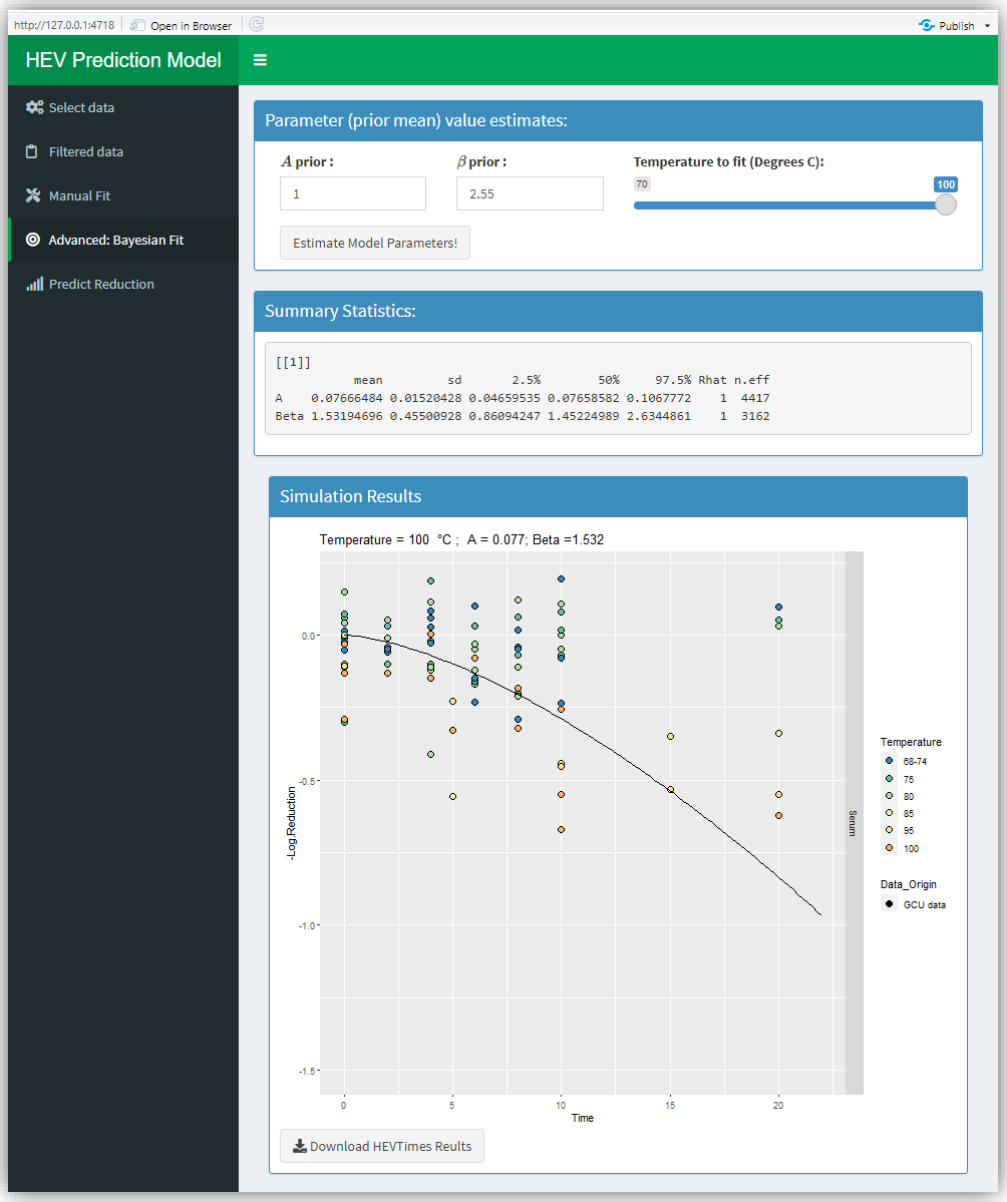

Figure 4: Advanced BayEsian Fit

Finally: Simply hit the **“Estimate Model Parameters!”** button, and R/RJAGS will take care of the rest. The simulations may take a while to run (Usually sub –

30seconds).

CAUTION: Whilst each viral source is plotted individually; the Bayesian parameter estimation combines ALL selected data, from ALL selected sources, to fit A SINGLE pair of parameters (A and β), to ALL selected data. This allows data from different viral sources to be combined (if desired) to produce a single pair of parameter estimates, based on mixed viral sources.

To calculate unique parameters for each individual viral source, you must only select one single source on the initial "select data" tab, and then repeat the process for each viral source, individually.

11. **Simulation Results:**

Once the simulations have finished running, summary statistics will appear,

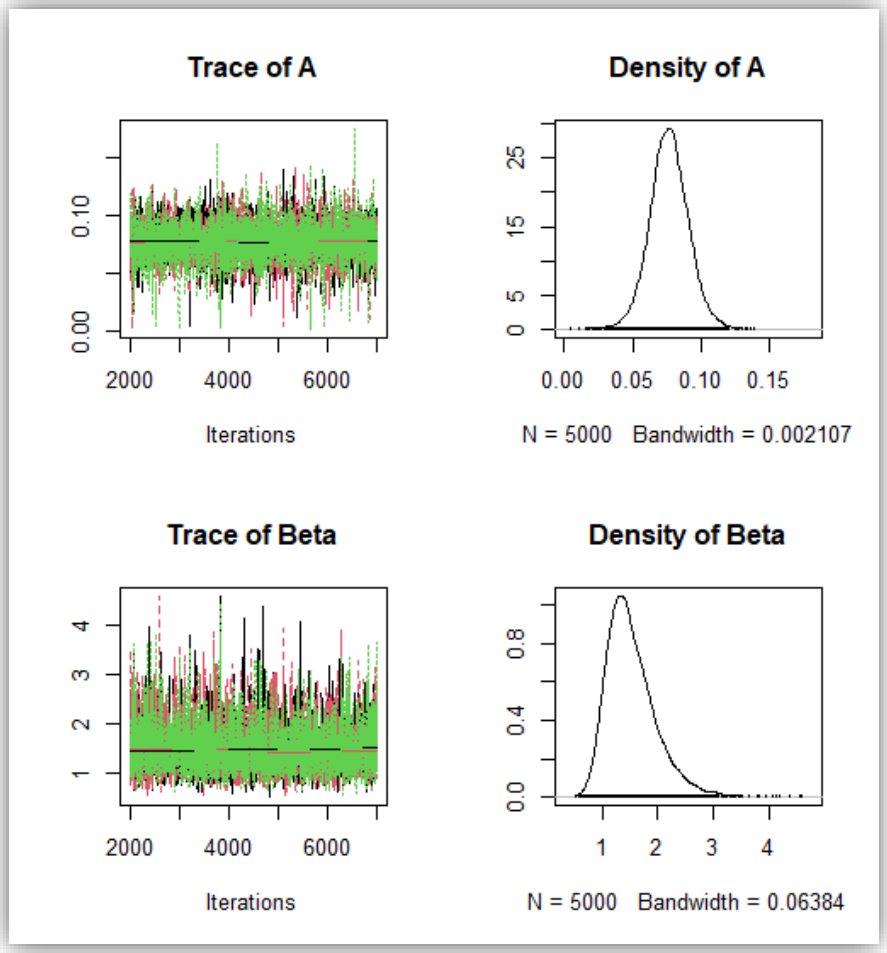

Figure 3: Trace And Density Plots For Bayesian MCMC Parameter Simulations

alongside a plot of the fitted model curve.

Summary statistics include the **mean** value, **standard deviation**, **2.5% quantile**, **median** (50% quantile), and **97.5%** quantile, for the estimated model parameters A and β.

"**Rhat**" value: Convergence diagnostic statistic. If we have R-hat close to 1, it indicates convergence.

Whilst if R-hat is greater than 1.05, this indicates we don't have good convergence.

"**n.eff**" value: This is the effective sample size, kept from the posterior distribution.

As a default rule, for this particular model, any n.eff value greater than 30 is good (This corresponds to 10 times the number of MCMC chains used by the code). This should typically correspond to stability of the convergence (Gelman , 2013, pp. 284-290).

Note: The model uses three "Markov chains", in its simulations. These three chains simultaneously estimate the two model parameters, at each timestep. So "convergence" refers to all three chains converging upon approximately the same value (And can be seen in the corresponding trace plots).

12. **Trace and density plots** of the MCMC simulation that has estimated the model parameters are shown within the main Rstudio window, as pictured in Figure 5/Figure 6. Depending on your current RStudio settings, you may need to click on "Plots" tab in the bottom right pane, for these to become visible.

    The density plots are essentially smoothed histograms of the estimated values of each parameter (Usually termed the "posterior distribution") and indicate the likelihood of observing the parameter value on the x-axis.

    The trace plots show the convergence of the three Markov chains used to estimate the model parameters. These contain three different coloured lines, one for each chain, and we are ideally looking for these three chains to converge to the same value. This implies out estimates are good.

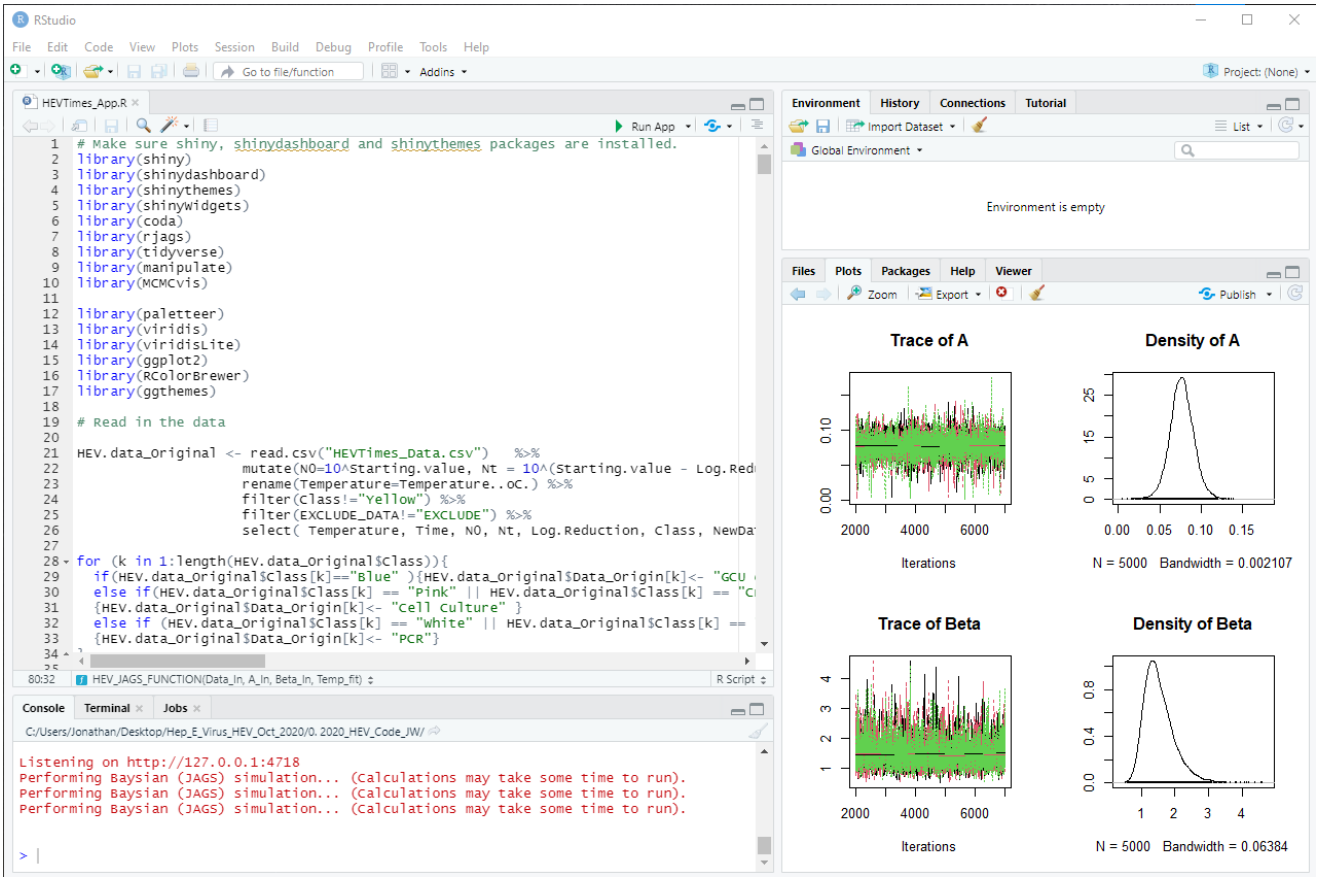

Figure 4: Main Rstudio window for reference

Notes: Model User Guide

1. Install “R” Statistical Software

The model has been coded into a Shiny application, using the R programming language, and thus an installation of the R software (R Project, 2021) is required to run the simulations. This is an open-source programming language and software, supported by the R Foundation for Statistical Computing: R-project.org

R software is available for all the major operating systems (Windows, MacOS, Linux, etc.,), so please make sure the software you install matches to your current computer’s operating system. The same is true for all other software listed below.

2. Install Rstudio Software

R itself is a command line interface. The third party “RStudio” interface is required to run the Shiny app. “RStudio” is available in both an open source, as well as a commercial version for organizations not able to use AGPL software (Affero General

Public License). The following page should guide you through installation: RStudio.com

3. Install “JAGS” Software

The model makes use of a Bayesian analysis technique known as Markov Chain Monte Carlo (MCMC) simulation, to remove some of the random noise found in the data whilst fitting the model equations. These are often carried out numerically using various Gibbs Sampling software variants, that have all stemmed from the original BUGS (Bayesian inference Using Gibbs Sampling) software. Here, we make use of the JAGS (Just Another Gibbs Sampler) variant of this software. This is a is a cross-platform alternative with a direct interface to R using the “rjags” R package. The model requires a working installation of the JAGS software, the most recent version is JAGS-4.3.0.exe.

4. Install Required R Packages

Running the attached “HEVTimes_Install_Packages.R“ R-script should install all the r-packages that are required to run the app successfully. Simply open the script in RStudio, “select all”, and hit “run” in the top toolbar.
You only need to run this script once per computer/R installation, and the packages will always be available. You may see a message “do you want to install from sources the package which needs compilation”. If this appears, you can simply choose “no”, and the required packages should still install correctly.

5. Save Data in Appropriate .csv Format

If updating the “HEVTimes_Data.csv” file with additional data. Take care to ensure the new data is saved in the exact same format as the current file, and with the exact same filename, as this filename is hard coded into the R code.

An additional, colour coded, .xlsx format Excel spreadsheet is also included, to make it easy to view the current data. It can be used as a template for the .csv file, **however, requires the removal of some surplus additional columns that contain working/notes (Columns N-R)**. Simply open the .xlxs sheet, add new data, click “Save as…”, and select “.csv”. Since the surplus columns that need to be removed (Columns N-R) contain excel formulas, you will have to close the newly

saved .csv file, reopen this new .csv file, and then remove additional columns. Otherwise, you may unintentionally end up with zero values in the log-reduction column (Column D).

6. Bayesian Parameter simulation notes

Model prior distributions are specified within lines 82-100 of the current version of the code; See Figure 7.

NOTE: These are written using the JAGS language, which uses slightly different syntax than the R language. So, take care if editing these!

```
HEV_JAGS_model <- " model {
 for(i in 1:length(NLR) ){
  NLR[i] ~  dnorm(Mu[i],tau )              # ASSUMES NEGATIVE LOG REDUCTION IS NORMALLY DISTRIBUTED,
                                           #  with mean Mu, given by the model equation, and precision tau
                                           # Here, this "Precision" is equivalent to (1/variance).
  Mu[i] <- (-1/2.303)*((  A*Time[i]  )^ Beta )
            }

  A  ~      dunif( 0 , 2*mu1 )   #  A prior distribution = uniform distribution, with centre point given by
                                 #   the prior estimate within the app (ie. mu1 here).


  Beta ~   dunif( 0 , 2*mu2 )     # Beta prior distribution = uniform distribution, with centre point given by
                                  #   the prior estimate within the app (ie. mu2 here)

                                 # Be careful: dgamma, gamma distribution parameters are Shape and rate here.
 sigma2 ~    dgamma(1 , 4)
 tau <- 1 / ( sigma2 )           # Precision for Normal distribution used for Negative log reduction (NLR).
 } "
```

Figure 5: RJAGS code extract – specifying THE model for the JAGS solver, along with the chosen model priors

Mathematical Model

In the above, we demonstrated the use of the Weibull mathematical model (Smith R. , 1987), The classic Weibull equation takes the form $N_t = N_0\, exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right]$, where α and β are the scale and shape parameters, respectively (Cunha, 1998). The equation used to successfully predict the thermal death times for Hepatitis E virus (HEV) in foodstuffs. Commonly used in survival analysis, to predict the time till death of biological organisms, and within engineering applications to predict time till failure of components parts (Smith R. L., 1991), the Weibull model has also recently been used to describe the thermal inactivation of Hepatitis A virus (HAV) in blue mussel (Bozkurt, 2014) .

The Weibull model equation takes the form:

$$N_t = N_0\, exp\left[-(A\, t\,)^{\beta}\right] \qquad \textbf{1}$$

where, N_t is the number of surviving virus particles left after a sample has been exposed to a particular temperature T (Celsius), for time t (minutes). N_0 is the number of particles initially, before any heating has taken place. Here, A is a constant frequency parameter and β is a constant shape parameter. These will be different for each temperature T .

Rearranging equation (1), the negative of the log reduction, (Note: Using rules of logs: $-\left|\left|\text{Log Reduction}\right|\right| = [log_{10}[N_0] - log_{10}[N_t]] = \log_{10}[\,N_t\,] - \log_{10}[N_0] = \log_{10}\left[\frac{N_t}{N_0}\right]$), in the number of virus particles, at time t is given by the equation:

$$-\left|\left|\text{Log Reduction}\right|\right| = log_{10}\left[\frac{N_t}{N_0}\right] = log_{10}\left(exp\left[-(A\, t\,)^{\beta}\right]\right)$$

$$= \frac{1}{2.303}\, ln\left(exp\left[-(A\, t\,)^{\beta}\right]\right)$$

$$= \frac{-1}{2.303}(A\, t\,)^{\beta} \qquad \textbf{2}$$

The model parameters A and β have then been estimated using a Bayesian approach, within the R software. This is achieved using the JAGS (Just another Gibbs Sampler) package, which uses Markov chain Monte Carlo simulation (MCMC) to estimate the parameters. Doing so allows us to account for some of the uncertainty and noise found within the literature/GCU trial data.

Updating the main Data File (HEVTimes_Data.csv)

All data used by the app, is contained within the file “HEVTimes_Data.csv”. This contains all literature data, plus new experimental data, and is designed to be easily edited, to allow additional data to be added to the model (or to exclude existing data).

The main HEVTimes_Data.csv file contains the following columns:

1. **Temperature (°C):**
   The temperature at which the viral sample was heated (°C).

2. **Time (HOURS):**
   The time the viral sample was heated for (Hours).

3. **Time (Mins):**
   The time the viral sample was heated for (Minutes).

   This is the main time data, that the app uses. However, as some of the literature data is recorded in hours, it is useful to have both time columns for easy conversion to minutes.

4. **Log Reduction:**
   The log-reduction observed upon heating the viral sample, for the corresponding time and temperature combination. This is calculated as:

   log-reduction = Log_{10} (Initial virus particles) – Log_{10} (Virus particles remaining at time t).

5. **Matrix:**
   Denotes the matrix the virus was obtained from.
   This is simply for reference and is not used within the app itself.

Subsequently, any desired information can be included here, and no specific format is required.

6. **Other information:**
   Denotes any other information, about the data/virus used, that may be useful. For example Genotype.
   Again, this is purely for reference and is not explicitly used within the app itself, so no specific format is required.

7. **Starting value (Log!):**
   This is the log of the initial number of virus particles. I.e. Log_{10} (No. initial virus particles).

8. **Source:**
   This denotes the **viral source** that the virus was obtained from.
   All data points, with the same source, should have the same source entry in this column.
   Currently, the source options are: "**Cell**", "**Liver**", "**Soil**", "**Faecal**" and "**Serum**", and these are the names that will be displayed in the corresponding plots and tables within the app itself.
   However, the app is designed to automatically adapt, if a new source appears in this column. Subsequently, it should be possible to add new sources, other than those already present.

9. **NewData:**
   This column specifies whether the **data origin** is new experimental data, or existing literature data.
   Subsequently, there are only two values this column should take:
   a. **"TRUE" –** To denote that the data is **Experimental**.
   b. **"FALSE" –** To denote that the data is from the **Literature**.

10. **EXPERIMENT_DATASET:**
    This column is **intended to group all data from the same experimental dataset together**.

For example If repeated experiments were carried out, using the same experimental setup, in the same lab session, these will all be grouped together with the same “EXPERIMENTAL_DATASET” label.
Subsequently, if the data is from the literature (not experimental), the value recorded is simply “NA”. Similarly, if no experimental grouping is desired, this can also be set to “NA”.

The values used in this column are:

a. **“NA”** – Literature data (or other misc. data).
b. **“DATASET0”**
   – “Single point” experimental data.
   These are data points that are not part of a wider “time-series”: In a time-series, the log reduction is measured at multiple time points in time, during the same experiment.
c. **“DATASET1”**
   – Contains the first batch of time-series experimental results.
   This contains multiple time-series (runs), carried out, using the same experimental setup, in the same lab session.
   (Each individual time-series is then grouped by run number - see “RUN_NUMBER” below).
d. **“DATASET2”, “DATASET3” …**
   - Denotes the subsequent second and third batch of experiments.
e. **“… DATASET100, DATASET101… “–** Future dataset labels should keep the same format.

Note: The information in this column is mainly used to distinguish single point data (DATASET0) from timeseries data (DATASET1 and above).

11. RUN_NUMBER:

This column distinguishes between different time series runs and is used to link time series points together within the plots produced by the app.

In a time-series run, the log reduction is recorded at multiple time points in time, during the same experiment (Same virus, constant temperature). These

can then be plotted together, to see the reduction in virus over time. Subsequently, ALL data from the same time series should be given the same number here, as this is the value the app uses to link them together.
RUN_NUMER can take any whole number value in the range [1, ∞].
If the data is not from a time-series, this can simply be left empty.

12. **EXCLUDE_DATA:**
    Easily include or exclude data from being fed into the app/model:
    a. **"KEEP" –** to use the data in the model.
    b. **"EXCLUDE" –** to exclude the data from the model.

Additional notes on updating the .csv file:

The main HEVTimes_Data.csv file can easily be edited in Microsoft Excel (or similar).

After editing the HEVTimes_Data.csv file, if you currently have the Rstudio Shiny app open, you will have to close RStudio and **restart Rstudio**, before the changes to the data will be recognised.

Similarly, **care should be taken to select the correct format of ".csv"** when saving the data as a .csv file. This is mentioned, to highlight the fact **that there are multiple variations upon the standard ".csv"** listed in the "Save as" menus within excel. You should select the option "CSV (Comma delimited) (*.csv)", as illustrated in **figure 8**.

Figure 6: Correct File Format From "Save As" Drop Down Menu In Microsoft Excel.

There is also an **additional, colour coded spreadsheet**, containing identical data, that is simply intended as an easier to read option. It is currently called

“**HeVtimes_Data_update_24_2_2021.xlsx**”, to reference the date the most recent data was added, and to distinguish it from the main data file. It can act as a useful template, that can subsequently be saved as a .csv file, and named “HEVTimes_Data.csv”, to replace the existing one.

The filename for the main .csv data file, is hard coded into the app in line 21 of its code:

```
HEV.data_Original <- read.csv("HEVTimes_Data.csv") %>%
```

Subsequently**, it is important to ensure that the file name (HEVTimes_Data.csv), and .csv format, both stay the same,** if adding new data. Alternatively, line 21 of the code must be edited accordingly.

Part of the HEVTimes Project:

Thermal death model FOR HEPATITIS E virus (HEV)