Thermal inactivation of foot and mouth disease virus in extruded pet food

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Summary

The risk of importing foot and mouth disease, a highly contagious viral disease of livestock, severely restricts trade and investment opportunities in many developing countries where the virus is present. This study was designed to investigate the inactivation of foot and mouth disease virus (FMDV) by heat treatments used in extruded commercial pet food manufacture. If extrusion could be shown to reliably inactivate the virus, this could potentially facilitate trade for FMDV-endemic countries. The authors found that there was no detectable virus following: *i)* treatment of FMDV-spiked meat slurry at 68°C for 300 s; *ii)* treatment of FMDV-spiked slurry and meal mix at 79°C for 10 or 30 s, or *iii)* treatment of homogenised bovine tongue epithelium, taken from an FMDV-infected animal, at 79°C for 10 s.

This corresponds to an estimated 8 $\log_{10}$ reduction in titre (95% credible interval: 6 $\log_{10}$–13 $\log_{10}$). Furthermore, the authors found that the pH of the slurry and meal mix was sufficient to inactivate

FMDV in the absence of heat treatment. This demonstrates that heat treatments used in commercial pet food manufacture are able to substantially reduce the titre of FMDV in infected raw materials.

Keywords

Cat – Dog – Extrusion – FMDV – Foot and mouth disease virus – Heat treatment – Pet food.

Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, characterised by rapid onset and high morbidity (1, 2). It is one of the world's most important animal diseases, and is responsible for substantial global losses to livestock production and trade, as well as frequent and highly disruptive large-scale epidemics (3, 4). The risk of importing foot and mouth disease virus (FMDV) restricts trade in livestock and their products where the virus is present, reducing trade and investment opportunities in many developing countries (3, 5, 6).

Many commercial pet foods use animal-based raw materials derived from livestock (including FMDV-susceptible species such as cattle, sheep and pigs) to provide protein, fat and other nutrients for a nutritionally complete and balanced pet food (7). In commercial operations the animal-derived ingredients normally undergo a preconditioning heat treatment prior to extrusion, during which temperatures normally exceed 68°C. The preconditioned ingredients are then pumped into an extruder, where the material is extruded at temperatures exceeding 100°C. The residence time in the extruder is typically 30–45 seconds (8).

The aim of the present study was to quantify the effect of heat treatment on the levels of FMDV in extruded pet food (i.e. dry kibble). The virus has already been shown to be inactivated by high temperatures (> 50°C) or by acidic or alkaline conditions (pH < 6.0 or > 9.0) (9). This study evaluated the inactivation of FMDV in three pet food matrices (uncooked meat slurry, dry rendered meal, and a meat

slurry and meal mix) spiked with FMDV and in homogenised bovine tongue epithelium taken from FMDV-infected cattle.

Materials and methods

Pet food matrix

The pet food matrix was supplied by Nestlé Purina PetCare (Sudbury, United Kingdom [UK]) in two components, both of bovine origin. One component was an uncooked wet pet food slurry mixture (meat slurry) which was aliquoted and stored in 10 ml volumes at –20°C until required. The second component (8% moisture content) was a dry rendered meal (dry meal) which was stored at room temperature. The average pH of the two matrices under test conditions was determined to be 6.6 and 5.2, respectively. A third matrix (meat slurry mixed with dry meal) was also prepared for testing using a combination of the meat slurry and dry meal to represent the components of pet food that undergo a typical pet food extrusion manufacturing process. This third matrix had 28% moisture content and was prepared using 790 g dry meal, 210 g meat slurry and 97 g hard water (prepared by adding 0.402 g/l [w/v] calcium chloride [$CaCl_2$] and 0.138 g/l [w/v] magnesium chloride [$MgCl_2$] to distilled water). The average pH of the 28% matrix was 5.7; when adjustment of the pH of this matrix was required, it was achieved through the replacement of the 97 g hard water with 1 M sodium hydroxide. The average adjusted pH for this matrix was 6.7.

In all experiments the matrix mixtures were pre-heated to 72°C for 5 min prior to addition of virus. This was required to inactivate endogenous microbial activity, which would have adversely affected the plaque assay performance. On each occasion the pre-treatment was immediately followed by cooling the matrix on ice to 4°C.

Growth and titration of foot and mouth disease virus

Stock virus pool

The virus strain used for the *in vitro* spiking experiments was FMDV O1 BFS 1860/UK 67. This was derived from a British field strain

isolated during the FMD outbreak in the UK in 1967. The working stock was prepared in accordance with standard procedures, and a sufficient volume of working stock was prepared as a single homogeneous pool for the purpose of the study. The working stock pool was aliquoted in volumes appropriate for each assay and stored at –80°C to avoid the deleterious effects of freezing and thawing on the virus titre. The titre of this working stock pool was confirmed to be 7.7 $\log_{10}$ plaque-forming units (pfu)/ml. In all assays, unless otherwise specified, the cell line used was BHK-21 (baby hamster kidney) originally sourced from the American Type Culture Collection (ATCC-CCL10).

In vitro assay and determination of residual virus activity

Following pre-treatment, a 100 mg aliquot of matrix was spiked with 100 µl FMDV and thoroughly mixed prior to heat treatment. Three replicates were included in each assay and, following serial ten-fold dilutions, each replicate and associated dilution was plated in triplicate on a six-well (35 mm diameter) cell culture plate for plaque assay. Each assay was repeated on three separate occasions. The plaque assay was incubated for 48 h and then fixed and stained prior to quantification of the plaques. In every assay a virus control (FMDV plus matrix) was also used to determine any detrimental effect of the matrix alone on FMDV.

Small-scale heat inactivation study

Equipment used for thermal inactivation and calibration of the equipment

All heat inactivation studies were carried out using a programmable 12-well aluminium heating block, designed and supplied by D. Foster (Gas-X Ltd, UK) (8). The heating unit consisted of a rectangular, interchangeable aluminium block capable of holding 12 × 2 ml screw cap polypropylene tubes (VWR International, Lutterworth, UK). A Peltier cartridge supplied the heat, and the temperature was monitored in each well using thermocouples inserted into individual tubes. The temperature readings of the thermocouples were validated using a

reference thermometer. This thermometer was certified by comparison against a calibrated probe and the thermometer was used in each experiment to determine the temperature reached within the matrix. When required, the target temperature of the block was adjusted to ensure that the appropriate temperature was reached within the matrix. Where time measurements were required for the assay, a calibrated timer was used. Ramp rates were held at the maximum rate for the heating block in each experiment and the ramp time was measured from the initial temperature point to the target temperature, at which time the tubes were removed and placed on ice.

Temperature and ramping time parameters

The effect of temperature on virus inactivation was determined by placing the pre-treated matrices plus FMDV into a pre-heated block and holding them there for the prescribed time interval. At the end of the time period the tubes were immediately cooled in an ice bath at 4°C prior to serial dilution and inoculation on to cell culture plates for plaque assay. The temperatures, matrices and times used were as specified in Tables I, II, III and IV and reflect those used in the pet food extrusion process.

The effect of the ramping temperatures on virus inactivation was determined by placing the pre-treated matrices plus FMDV into the block pre-heated to 25°C until the target temperature had been reached and then immediately removing them to an ice bath at 4°C. The ramping temperatures were set to 40, 50, 60 and 70°C. These ramping conditions were applied to the meat slurry alone and then to the slurry and meal mix that had been pH-adjusted using 1 M sodium hydroxide as described (Table III). Control samples with FMDV-spiked matrices were held at 25°C in the ramping experiments without additional heating to evaluate any detrimental effect of the matrix on virus titre.

Infected tissue homogenate and the effect of heat treatment

The thermal inactivation assays described above were carried out using pet food matrices spiked with virus. This was to replicate raw materials used in the pet food manufacturing process. To further

evaluate the effect of heat inactivation of FMDV in infected tissue, a homogenate of bovine tongue epithelium from an infected animal was used as a surrogate for an infected matrix in the pet food process. The animal from which the tongue was derived had been infected intranasally (via mask and nebuliser) with FMDV O UKG 34/2001 as a positive control in a vaccine study. The virus was derived from a British field strain isolated during the FMD outbreak in the UK in 2001. The tissue sample used was a 10% (w/v) homogenate stored in 50% glycerol, and the pH was determined to be 7.6. The tongue homogenate was heat treated to 79°C for 10 s prior to the serial dilution and plaque assay process, as described above. The cell line used in this case was renal swine cells (RSB), a continuous cell line, rather than BHK-21. The RSB cell line was used in this circumstance because the isolate produced a higher titre in this cell line than in BHK-21. This result is characteristic for tissue isolates or early passage material.

Statistical analysis

The reduction in titre due to each of the heat treatments was estimated for three matrices (meat slurry, pH-adjusted slurry and meal mix, and FMDV-infected bovine tongue epithelium) using a generalised linear model (GLM) for the number of plaques, assuming Poisson errors and a log link function. More specifically, the number of plaque-forming units (pfu) (y_{jk}) for the jth replicate following heat treatment k was assumed to follow a Poisson distribution, with the expected titre (μ_{jk}) given by

$$\log_{10}(\mu_{jk}) = \log_{10}(V_0) + b_k - D_j,$$

where V_0 is the initial titre, b_k is the reduction in $\log_{10}$ titre due to heat treatment k and D_j is the dilution (in ten-fold dilutions) for replicate j.

Parameters were estimated in a Bayesian framework using OpenBUGS (www.openbugs.net; version 3.2.3). Non-informative priors were assumed for all parameters: diffuse exponential for the log initial titre ($\log_{10}(V_0)$) and diffuse normal for the $\log_{10}$ reduction in titre for each heat treatment (b_k). Two chains each of 6,000 samples

were run, with the first 1,000 iterations of each chain discarded to allow for burn-in. Convergence was monitored visually and using the Gelman–Rubin statistic in OpenBUGS.

Results

For the experiments in which samples were treated at a constant temperature for a fixed period of time, there were no detectable plaques following treatment for any of the combinations of matrices and heat treatment (Tables I, II, III and IV). This shows that the heat treatments applied were able to reduce the level of virus from around 7 $\log_{10}$ pfu/ml to no detectable virus. In the case of meat slurry spiked with FMDV (Table I) and FMDV-infected bovine tongue epithelium (Table IV), FMDV was detectable at high titres in the matrix without heat treatment. However, for the dry meal or the slurry and meal mix spiked with FMDV, there were no detectable plaques in the samples which were not heat treated, which is probably a consequence of the relatively low pH of these matrices (Table II). If the pH of either matrix was adjusted (using sodium hydroxide), however, there was a high titre of virus without treatment, but no detectable plaques following heat treatment at 79°C for either 10 or 30 s (Table III).

The titre was estimated to be reduced by approximately 8 $\log_{10}$ (95% credible interval [CI]: 6 $\log_{10}$–13 $\log_{10}$) following treatment at 68°C for 300 s (meat slurry) or at 79°C for 30 s or for 10 s (pH-adjusted slurry and meal mix). It was estimated to be reduced by 7.6 $\log_{10}$ (95% CI: 5 $\log_{10}$–12 $\log_{10}$) in FMDV-infected bovine tongue epithelium following treatment at 79°C for 10 s (Table V). This difference reflects the slightly lower titre in the FMDV-infected bovine tongue epithelium when compared with the FMDV-spiked matrices (Table IV; cf. Tables I, II and III).

Insert Table V

The ramping experiments were designed to provide additional information on the temperature (and time) required for inactivation of FMDV. For both the meat slurry and the mixture of meat slurry and dry meal, the reduction in titre increased with the temperature at

which the samples were removed from the block (Tables I and III), though the reduction at each temperature was significantly higher in meat slurry compared with the pH-adjusted meat slurry and dry meal mixture (Table V). In particular, there was no detectable virus in the meat slurry by the time the matrix reached 60°C (Table I). By contrast, there was still detectable virus (though at a 5 $\log_{10}$ lower titre) in the pH-adjusted meat slurry and dry meal mixture, even by the time the matrix had reached 70°C (Table III).

The estimated reduction in titre was similar for those experiments in which no plaques were detected for any of the samples (meat slurry: ramping to 60 or 70°C, 68°C for 300 s; meat slurry and dry meal mixture: 79°C for 30 s or 10 s; FMDV-infected bovine tongue epithelium: 79°C for 10 s) (Table V). The slightly lower reduction estimated for FMDV-infected bovine epithelial tissue is primarily a consequence of the lower initial titre in this matrix when compared with the meat slurry or the meat slurry and dry meal mixture.

Discussion

Dog and cat food (pet food) forms part of the globalised economy because raw materials and finished products enter international commerce from many countries. The importation requirements for pet food are often determined through bilateral agreements between trading nations and incorporate the animal health requirements of the importing country as well as relevant international standards recognised under the Sanitary and Phytosanitary Agreement of the World Trade Organization. For example, the *Terrestrial Animal Health Code* (Article 8.8.31) of the World Organisation for Animal Health (OIE) states that meat shall be subjected to heating so that an internal temperature of > 70°C is maintained for a minimum of 30 minutes (10). Fundamental to ensuring the safe trade of extruded pet food is the underlying effectiveness of the heat treatment used to inactivate or destroy pathogenic microbes and viruses that potentially could be present in raw materials sourced from livestock.

There are only limited data on the inactivation of FMDV in meat by commercially used heat treatments (6). This study has demonstrated

the effectiveness of heat treatment in inactivating FMDV in extruded pet food. Treatment of samples with an initial viral titre of around 7 $\log_{10}$ pfu/ml using temperatures and times reflecting those used to precondition components during a representative manufacturing process (68°C for 300 s and 79°C for 30 s) resulted in no detectable virus post treatment. In addition, further heat treatment of the pet food components occurs in the extruder, in which the temperature is typically 100°C and the residence time is 30 s. This suggests that the overall reduction in titre due to the manufacturing process will be even greater than the estimated 8 $\log_{10}$ reduction (95% CI: 6 $\log_{10}$– 13 $\log_{10}$) based on preconditioning treatments. Furthermore, the pH of the dry meal and of the slurry and meal mix was sufficient to inactivate FMDV even without heat treatment.

When examining thermal inactivation of FMDV in this study the authors primarily made use of virus-spiked matrices, though bovine epithelial tissue derived from a naturally infected animal was also used. Some previous studies have shown a difference in thermal inactivation between spiked and naturally infected matrices. For example, with FMDV in milk (11) and avian influenza virus in chicken meat (12), virus was inactivated more slowly in the naturally infected matrix compared with the artificially infected one. By contrast, no difference in the rate of inactivation was identified between chicken meat spiked with Newcastle disease virus and meat from chickens that were naturally infected with the virus (12). In this study there was no detectable virus following treatment at 79°C for 10 s in either FMDV-spiked slurry and meal mix or FMDV-infected bovine tongue. This suggests that if there is a difference in inactivation rates between these two matrices, it does not have a substantial impact on the reduction in viral titre for this heat treatment.

In addition to potential differences in inactivation rates between spiked and naturally infected matrices, several studies have reported a biphasic response (or tailing effect) during thermal inactivation of FMDV (9, 13, 14, 15, 16). Such an effect can be the result of a (small) proportion of the viral population being resistant to thermal inactivation (9, 13). In addition, inactivation of virus may be reduced

in cells and fat globules because these have a protective effect (14, 15). In the present study, there was no detectable virus following any of the heat treatments representative of those used in pet food manufacture. This suggests that the effect of a biphasic response, if there is one, on the reduction in viral titre due to treatment is minimal, or that any thermal-resistant virus occurs at a level below the detection threshold of the assays used.

Following treatment at temperatures and times typical of extrusion pet food manufacturing processes, the authors were unable to detect FMDV by cell culture in any of the four matrices considered in this study. An earlier study on thermal inactivation of FMDV in milk from infected cattle identified some samples that were negative by cell culture, but which caused disease when inoculated into cattle (15). However, cattle inoculation is a very sensitive, but artificial, means of detecting FMDV and is of limited relevance when assessing the risk of natural FMDV infection (6).

This study demonstrates that heat treatments used in extruded pet food manufacture are able to substantially reduce the titre of FMDV in infected raw materials. This reduction in titre will translate into a reduction in the risk of livestock infection.

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Table I

Effect of heat treatment on the inactivation of foot and mouth disease virus in FMDV-spiked meat slurry

Matrix	Heat treatment	log_{10} pfu/ml[a]	pH[b]
Meat slurry + FMDV	68°C, 300 s	NDP	6.6
Meat slurry + FMDV	4°C	7.1 ± 0.2	
FMDV control	4°C	7.4 ± 0.1	
Meat slurry + FMDV	25°C, 0 s[c]	6.9 ± 0.2	6.5
Meat slurry + FMDV	40°C, 120 s[c]	5.1 ± 0.3	
Meat slurry + FMDV	50°C, 181 s[c]	2.4 ± 0.2	
Meat slurry + FMDV	60°C, 243 s[c]	NDP	
Meat slurry + FMDV	70°C, 348 s[c]	NDP	
FMDV control	4°C	7.5 ± 0.1	

a) mean ± standard deviation

b) pH of matrix under test conditions

c) ramping experiment: time is the mean time taken to reach temperature

pfu: plaque-forming units

NDP: no detectable plaques

Table II

Effect of heat treatment on the inactivation of foot and mouth disease virus in FMDV-spiked dry meal or meat slurry and dry meal mixture

Matrix	Heat treatment	log10 pfu/ml[a]	pH[b]
Slurry and dry meal + FMDV	79°C, 30 s	NDP	5.7
Slurry and dry meal + FMDV	4°C	NDP	
FMDV control	4°C	7.4 ± 0.3	
Dry meal + FMDV	79°C, 30 s	NDP	5.2
Dry meal + FMDV	4°C	NDP	
FMDV control	4°C	7.7 ± 0.1	

a) mean ± standard deviation

b) pH of matrix under test conditions

pfu: plaque-forming units

NDP: no detectable plaques

Table III

Effect of heat treatment on the inactivation of foot and mouth disease virus in FMDV-spiked meat slurry and dry meal mixture following pH adjustment

Matrix	Heat treatment	log10 pfu/ml[a]	pH[b]
pH-adjusted slurry and dry meal + FMDV	79°C, 30 s	NDP	6.7
pH-adjusted slurry and dry meal + FMDV	79°C, 10 s	NDP	
pH-adjusted slurry and dry meal + FMDV	4°C	7.3 ± 0.2	
FMDV control	4°C	7.7 ± 0.2	
pH-adjusted slurry and dry meal + FMDV	25°C, 0 s [c]	6.9 ± 0.2	6.7
pH-adjusted slurry and dry meal + FMDV	40°C, 171 s [c]	6.1 ± 0.4	
pH-adjusted slurry and dry meal + FMDV	50°C, 227 s [c]	4.5 ± 1.1	
pH-adjusted slurry and dry meal + FMDV	60°C, 307 s [c]	1.9 ± 0.3	
pH-adjusted slurry and dry meal + FMDV	70°C, 452 s [c]	2.3 ± 0.5	
FMDV control	4°C	7.6 ± 0.1	

a) mean ± standard deviation

b) pH of matrix under test conditions

c) ramping experiment: time is the mean time taken to reach temperature

pfu: plaque-forming units

NDP: no detectable plaques

Table IV
Effect of heat treatment on the inactivation of foot and mouth disease virus in FMDV-infected bovine tongue epithelium

Matrix	Heat treatment	log10 pfu/ml[(a)]	pH[(b)]
FMDV-infected bovine tongue epithelium	79°C, 10 s	NDP	7.6
FMDV-infected bovine tongue epithelium	4°C	6.1 ± 0.7	
Uninfected bovine tongue epithelium control	4°C	NDP	

a) mean ± standard deviation

b) pH of matrix under test conditions

pfu: plaque-forming units

NDP: no detectable plaques

Table V

Estimated reduction in log_{10} plaque-forming units of foot and mouth disease virus in different matrices following heat treatment

Heat treatment and matrix	Estimate	95% credible interval
Meat slurry + FMDV		
40°C, 120 s [(a)]	2.07	(2.00, 2.15)
50°C, 181 s [(a)]	5.15	(4.93, 5.38)
60°C, 243 s [(a)]	8.34	(6.13, 12.83)
70°C, 348 s [(a)]	8.34	(6.14, 12.80)
68°C, 300 s	8.75	(6.61, 13.16)
pH-adjusted slurry and dry meal + FMDV		
40°C, 171 s [(a)]	1.01	(0.91, 1.13)
50°C, 227 s [(a)]	3.06	(2.96, 3.15)
60°C, 307 s [(a)]	4.16	(4.08, 4.25)
70°C, 452 s [(a)]	4.96	(4.79, 5.16)
79°C, 30 s	8.76	(6.62, 13.00)
79°C, 10 s	8.77	(6.64, 13.08)
FMDV-infected bovine tongue epithelium		
79°C, 10 s	7.59	(5.27, 12.23)

a) ramping experiment: time is the mean time taken to reach temperature