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Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish

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SUMMARY

Commercial heat treatment procedures for molluscan shellfish are based on data obtained for the inactivation of hepatitis A virus (HAV) in cockles. However, the most frequently reported illness associated with consumption of bivalve molluscs is gastroenteritis caused by small round structured viruses (SRSVs) of the Norwalk group. Conditions for inactivation of SRSVs are unknown. In this study a feline calicivirus was used as a model for the SRSV group and conditions for its heat inactivation determined. Experiments showed that feline calicivirus is more readily inactivated in shellfish than HAV, and confirmed that current heating recommendations to the UK shellfish industry are adequate. A reverse transcription polymerase chain reaction (RT-PCR) assay for the detection of calicivirus in shellfish was developed and results compared with isolation in cell culture. The RT-PCR detected virus in some samples that failed to yield virus on culture. This has important implications if molecular virology techniques are to be used in the design and monitoring of shellfish treatment procedures and for routine testing of food samples.

INTRODUCTION

Consumption of bivalve molluscs has been associated with two viral illnesses, namely hepatitis A and viral gastroenteritis caused by the small round structured viruses (SRSVs) of the Norwalk group [1–4]. Bacterial pathogens are rarely associated with commercially treated bivalve shellfish, and nor are other viruses that cause gastroenteritis. Rotavirus has never been reported as a cause of shellfish associated gastroenteritis and astroviruses on only very rare occasions [5, 6].

Before sale, molluscs from all but the cleanest class A waters are subjected to cleansing treatments under the terms of the EC Directive on Shellfish Hygiene [7]. This is either heat treatment or relaying and depuration. Such treatments effectively remove bacterial contaminants, but viruses pose more of a problem. In an earlier study, the heat inactivation of hepatitis A virus (HAV) in cockles was investigated [8]. Results from this study led to recommendations from the Ministry of Agriculture, Fisheries and Food (MAFF) to the UK shellfish industry for the heat treatment of bivalve molluscs, and the design of new heat processing equipment. Epidemiological monitoring by the Public Health Laboratory Service Communicable Disease Surveillance Centre (CDSC) has indicated that since the implementation of the recommendations at the end of 1988, illness from shellfish heat treated according to the recommendations has ceased, whilst illness from other shellfish such as oysters undergoing depuration treatment continues (CDSC, unpublished data).

Unlike HAV, SRSVs cannot be cultured in the laboratory, and thus the effect of heat on infectivity cannot be investigated directly. Lack of laboratory confirmed data on the conditions required for inactivation of SRSVs is of some concern, as the most frequently reported illness associated with consump-

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tion of bivalve molluscs is gastroenteritis caused by SRSV (CDSC, unpublished data).

Norwalk virus, the prototype strain of the SRSV group, has been classified as a member of the Caliciviridae, and genomic data for other SRSV strains indicate they are also caliciviruses [9–11]. Although human caliciviruses cannot be grown in cell culture, some veterinary caliciviruses can. In this study a feline calicivirus (FCV) was used as a model, to investigate the heat inactivation of calicivirus in shellfish.

SRSVs were first detected and identified by electron microscopy [12]. Although this is still the most commonly used diagnostic method for SRSV, electron microscopy is a time consuming and relatively insensitive technique, which cannot be sensibly applied to food or environmental samples. The trend is increasingly towards using PCR assays to detect SRSVs. A PCR assay however, does not detect whole infectious virus particles, and there is concern that if these techniques are used for testing food samples, positive results may not necessarily mean that the food is unsafe. An RT-PCR assay for FCV was developed and applied to shellfish samples from this study. The results of the PCR assay and virus culture were compared.

METHODS

Virus and cells

Feline calicivirus strain F9 (kindly supplied by Dr E. O. Caul, Public Health Laboratory, Bristol) was propagated in monolayers of a feline kidney cell line (F cells) grown in Eagle's Minimal Essential Medium (MEM). Advanced cytopathic effect was observed after 24 h incubation at 37 °C and stocks of FCV inoculum prepared by freezing/thawing (xl) and sonication in a water bath (3 × 30 s). The inoculum was dispensed into aliquots and stored at −70 °C.

Maintenance of live cockles in the laboratory

Live cockles were maintained at 10–13 °C in the laboratory essentially as described in our previous study [8], with the difference that 20 l filtered natural sea water was replaced with artificial seawater (specific gravity 1·0195, prepared from 'Seamix' salt kindly supplied by Peacock Salt Ltd, Glasgow), and the cockles were fed with 4 g yeast extract (Difco). After equilibration of the cockles, a known titre (1×10^{11} $TCID_{50}$) of FCV inoculum was added to the water and mixed carefully without disturbing the cockles. No faecal material nor further protein was added as carrier or nutrient supplement. The cockles were removed after 24 h and stored at −30 °C prior to heat treatment and/or FCV extraction.

Heat treatment of cockles

Heat treatments were carried out in a stainless steel electric boiler (27 l capacity, Burco Ltd). Batches of 6–8 cockles were removed from frozen storage and allowed to reach 15–17 °C by standing at ambient temperature in a wire basket. Internal temperatures of the cockles were monitored by Cu/Ni thermocouples (Comark) inserted into the cockle meat and connected to a SP25 dataprinter (Digitron Instrumentation, UK). In cooking experiments, batches of cockles were immersed in boiling water for periods of 30 s, 1 min, 1·5 min, 2 min, 2·5 min and 3 min. Temperature readings were ignored where the thermocouples had detached from the cockle meat and were clearly registering water temperature. After each heat treatment the cockles were immersed in ice-cold water for rapid cooling and then frozen at −30 °C until required for extraction.

Extraction of infectious FCV from cockle meat

Several different methods for the extraction of FCV from cockle meat were evaluated. The procedure finally chosen for this study was based on the method of Atmar and colleagues [13], originally developed for recovering Norwalk virus from seeded oysters. Approximately 5 g of cockle meat was homogenized in 16 ml GN buffer (50 mM glycine (pH 9·5), 140 mM sodium chloride) containing 0·4 ml antifoam B (Sigma) for 2 × 30 s bursts (Silverson sealed unit blender). Three millilitres ice-cold arklone (1,1,2-trichloro, 2,2,1-trifluoroethane) was added and homogenization repeated. The pH was adjusted to 9·5 and Magnafloc (2%) (Allied Colloids Ltd) added to a final concentration of 0·007%. This was stirred at room temperature for 15 min and centrifuged at 12000 r.p.m. in a Sorval SS-34 rotor for 20 min at 4 °C. The supernatant was recovered, the pH adjusted to 7·2 and solid PEG 6000 added to give 8% (w/v). This was stirred for 2 h at 4 °C, centrifuged as above for 20 min and the pellets resuspended in 4 ml 0·15 M sodium phosphate buffer (pH 9·3) and the pH adjusted to 9·0. The suspension was agitated on a rotary shaker at room temperature for 10 min, centrifuged at

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12000 r.p.m. for 10 min at 4 °C and the supernatant recovered. The pH was adjusted to 7·0–8·0 and the extract stored at −70 °C.

Extraction of RNA from shellfish concentrates

This was a modification of the method of Boom and colleagues [14] which has been used to extract RNA from SRSVs [15]. One ml L6 buffer (6·6 M guanidinium isothiocyanate, 1·7% Triton X-100, 70 mM Tris-HCl (pH 6·4), 30 mM EDTA) was added to 120 μl cockle extract plus 10 μl suspension of size-fractioned silica (Sigma), vortexed for 15 s and incubated for 15 min at room temperature with occasional agitation and a final vortex. Silica was pelleted in a microcentrifuge (15 s) and the supernatant discarded. The pellet was washed first in 1 ml L2 buffer (5 M guandinium isothiocyanate, 0·1 M Tris-HCl (pH 6·4), then in 1 ml 70% ethanol and finally with 1 ml acetone. The silica was dried at 56 °C for 10 min, resuspended in 40 μl RNase-free distilled water (USB, Amersham) containing 80 units RNase inhibitor (rRNasin, Promega) and incubated for 10 min at 56 °C to elute RNA. The suspension was pelleted for 2 min and the supernatant carefully withdrawn.

Reverse transcription (RT) of RNA

Thirty microlitres eluted RNA was mixed with 1 μl random primers (mostly hexamers, 22·5 OD_{260} units/ml, Life Technologies), incubated at 70 °C for 5 min and cooled on ice. This was added to an RT reaction mix (50 μl final volume) containing 50 mM Tris-HCl (pH 8·3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0·5 mM each dNTP (Pharmacia) and 200 units Moloney Leukaemia Virus Reverse Transcriptase (MMLV RT, Life Technologies). After cDNA synthesis for 1 h at 37 °C, the RT reaction was inactivated by heating to 95 °C for 5 min and cooled on ice.

Polymerase chain reaction (PCR) to detect FCV

'Hot start' PCR was employed to amplify the cDNA produced in the RT reaction by using wax beads (GEM100, Perkin Elmer Applied Biosystems) to partition the reaction mixes [16]. Each 50 μl PCR volume consisted of 60 mM Tris-HCl (pH 8·5), 15 mM $(NH_4)_2SO_4$, 1·5 mM $MgCl_2$, 0·2 mM each dNTP, each primer at 250 nM, 1·25 units *Taq* polymerase (Amplitaq, Perkin Elmer Applied Biosystems) and 2·5 μl inactivated RT reaction (cDNA). The primers (originally designed by Dr M. J. Carter, University of Surrey; LH: GTCCCATGACTAAGTTAT, RH: TTTTTTCCCTGGGGTTAGGC) amplify a 386 nucleotide portion of the FCV genome which encompasses the 3′ open reading frame (ORF) and adjacent sequences [17]. PCR mixes were heated to 94 °C for 30 s, and cycled at 94 °C (1 min), 55 °C (45 s), 72 °C (1 min) (×40), with the final 72 °C extension being extended to 5 min.

RESULTS

Temperature monitoring of cockle meat immersed in boiling water

Results of internal temperature measurements are summarized in Figure 1, and clearly demonstrate an increase in temperature with duration of immersion. On average it took 60 s for the internal temperature of cockle meat to reach 78 °C, and 88 °C was attained after a further 30 s.

Recovery of FCV from contaminated cockle meat and seawater

Extraction of 10 g contaminated unheated cockle meat yielded a titre $3{\cdot}2 \times 10^4$ $TCID_{50}$/g meat. Seawater specimens were taken after initial contamination, 1·5 h and 24 h incubation, and infectious FCV titres were $2{\cdot}5 \times 10^6$, 1×10^5 and $1{\cdot}2 \times 10^5$ $TCID_{50}$/ml respectively. This indicated an immediate 20-fold reduction of FCV infectivity upon its addition to seawater, most probably due to the presence of salts. However, FCV infectivity in the seawater did not significantly decrease further in the course of the 24 h incubation.

Heat treatment of contaminated cockles

Following FCV extraction, virus could not be cultured from cockles which had been immersed in boiling water for 1 min or longer, i.e. had reached an average internal temperature of 78 °C or higher. However, virus was recovered from the four cockle batches that had been immersed for 30 s (Table 1). The virus titre was reduced ×100 fold after heat treatment for 30 s.

PCR testing of heat-treated cockle extracts

Extracts from the same heat-treated batches of cockles were subjected to RT-PCR. A 'hot-start' PCR was

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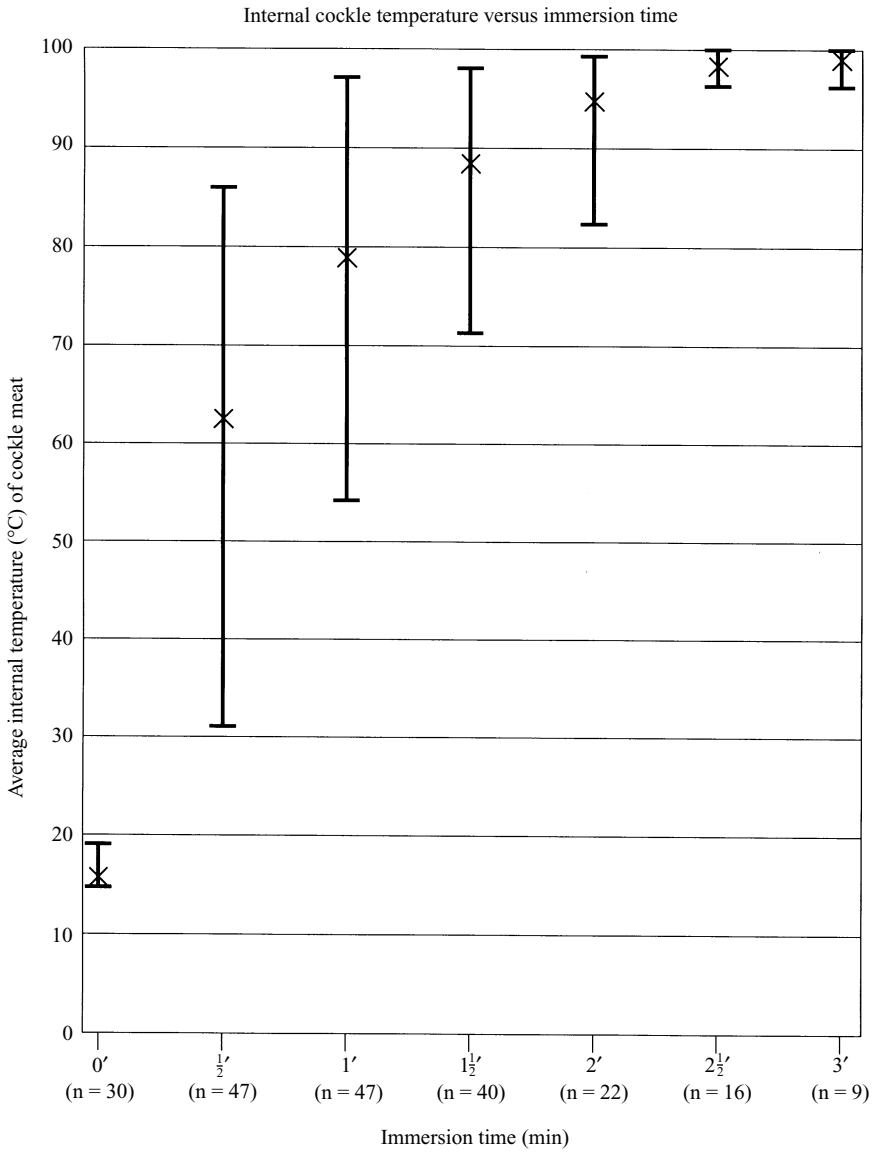

Fig. 1. Average internal temperatures of cockle meat plotted against duration of immersion time. Each point was calculated as the average of $n = 9$ readings (3 min heat-treatment), $n = 16$ (2·5 min) $n = 22$ (2 min), $n = 40$ (1·5 min), $n = 47$ (30 s). The vertical bars delineate the scatter of the internal temperatures for a given duration of heat treatment.

Table 1. *Heat treatment of contaminated cockles*

Duration of immersion in boiling water	30 s	1 min	1·5 min	2 min	2·5 min	3 min
Number of batches FCV positive by culture	4*/4	0/7	0/7	0/3	0/3	0/3
Number of batches FCV RNA positive by RT-PCR	4/4	5/7	5/7	0/3	0/3	0/3

* The FCV tires were approximately 700, 750, 300, 700 $TCID_{50}$ per g cockle meat.

chosen, in order to eliminate spurious PCR products from mis-priming of total nucleic acids from shellfish. No FCV amplimers were detected in any of the batches that had been immersed for 2 min or longer (Fig. 2). There were five RT-PCR positive specimens in each of the batches immersed for 1·5 min and 1 min (Fig. 2), but virus was not isolated in tissue culture from these specimens (Table 1). All four batches that

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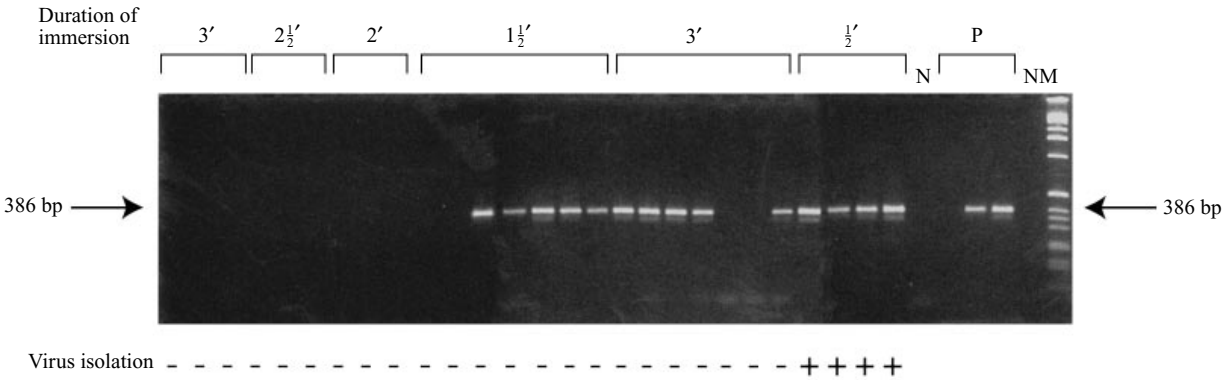

Fig. 2. Detection of FCV RNA by RT-PCR. Cockle batches were immersed for the duration indicated, extracted and amplified. Arrows indicate migration positions of FCV amplimers (386 bp). M, kilobase ladder (size markers, Life Technologies); N, amplified extracts from uncontaminated cockle meat (negative controls); P, FCV-contaminated cockle meat (unheated) extracted and diluted in water to provide positive controls containing 0·15, 1·5, 15 $TCID_{50}$ infectious FCV per RT-PCR (left to right, respectively).

had been heat-treated for 30 s were FCV positive both by RT-PCR and culture (Table 1, Fig. 2).

DISCUSSION

In our earlier study on the inactivation of HAV in cockles [8], it was found that if the internal temperature of cockle meat was raised to 85–90 °C and maintained for 1 min, HAV was completely inactivated. These conditions were also shown to inactivate poliovirus [8]. Since inactivation conditions were not known for other viruses, namely the gastroenteritis viruses, and also as an added safety margin to ensure complete inactivation of HAV, MAFF recommended to the shellfish industry that the internal temperature of the meat should be raised to 90 °C and be maintained for 1·5 min.

In the current study, in infectivity assays FCV was inactivated in cockles more readily than HAV, which indicates that caliciviruses would readily be inactivated during commercial processing. Epidemiological monitoring by CDSC has already shown that since the introduction of the heat processing recommendations by MAFF at the end of the 1988, there has been no viral illness from shellfish heat treated according to the recommendations. The results of the heat inactivation experiments together with the epidemiological data support the view that the current recommendations are adequate to produce a microbiologically safe product for the consumer.

Extraction of virus from shellfish is laborious and inefficient, and no published method is entirely satisfactory. At the outset of the study four methods were evaluated for the extraction of infectious FeCaV from cockle meat. The method of Richards and colleagues [18] had been used in our earlier study with HAV and poliovirus [8], but was not satisfactory for use with FCV. Likewise two methods of Yamashita and colleagues [19] failed to achieve adequate recovery of FCV from cockle meat. The three methods had all been developed for the extraction of infectious enteroviruses and HAV from contaminated shellfish meat. These approaches utilize a low pH step, and it is possible that under such conditions enteroviruses and HAV remain stable, but the more labile FCV may have become inactivated. The method of Atmar and colleagues [13] did not involve low pH. It consistently yielded $\geqslant 10\,\%$ FCV from spiked cockle meat, and was selected as the method of choice for this study.

Since SRSVs cannot be cultured and culture of HAV is lengthy and unreliable, there has been widespread interest in developing assays such as PCR to directly detect these viruses in shellfish, and indeed in water and other environmental samples. PCR assays have been used successfully for detecting viruses in clinical specimens, but there have been considerable technical difficulties in applying these techniques to environmental samples. However, these difficulties, such as interference by naturally occurring inhibitors are being overcome [20]. Reservations remain though, that detection of viral nucleic acid does not necessarily represent detection of infectious virus, which from a public health point of view is the key factor. HAV and SRSVs are RNA viruses. There is argument that as free RNA is unstable, any RNA detected must be packaged in complete virions, and hence be infectious.

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The PCR assay developed for this study did detect FCV RNA in some heat treated shellfish samples that had failed to yield virus on culture. However, PCR positive results were not obtained for any samples that had been sufficiently heat treated to meet the MAFF recommendations. It is unclear whether, detection of virus RNA by PCR in samples that were negative on culture, was due to the presumed greater sensitivity of the PCR assay over culture, or whether the assay was detecting non-viable viral RNA. This is why it was considered essential for this study that an infectivity assay was used, which necessitated the use of a model virus system. This issue of whether PCR detects infectious virus or not has important implications. As SRSV cannot be cultured, there appears to be a role for techniques such as PCR to be used in selecting effective depuration procedures for products such as oysters, which are not normally heat treated before consumption, and for determining the inactivation parameters of SRSVs directly. An RT-PCR has been developed in our laboratory, which successfully detects SRSV in shellfish, and is already being applied to the investigation of outbreaks of SRSV gastroenteritis [21]. The use of this PCR is producing useful epidemiological data, but it cannot be assumed that the test is detecting viable virus. For shellfish producers, it is important that the viral assays used are in fact detecting infectious virus, so that products that are microbiologically safe are not prevented unnecessarily from reaching the market. This also has wider implications if testing for human viral pathogens becomes part of the monitoring process for the wider food industry and the Water Utilities.

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