

Studies on heat inactivation of hepatitis A virus with special reference to shellfish

Part 1. Procedures for infection and recovery of virus from laboratory-maintained cockles

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SUMMARY

The consumption of bi-valve molluscan shellfish has been associated with outbreaks of viral gastroenteritis and hepatitis A. Investigations were undertaken to determine the heat inactivation conditions necessary to render shellfish such as cockles safe for the consumer. Conditions for the laboratory maintenance of live cockles are described. In preliminary experiments either poliovirus (10^6 TCID₅₀/ml seawater) or hepatitis A virus (HAV) (approx. 10^4 RFU/ml seawater) was introduced into the shellfish tank. Following 48 h filter feeding, virus was recovered from cockles using an adsorption-elution extraction procedure. Titres of virus recovered ranged from 10^4 to 10^5 TCID₅₀/ml of shellfish extract for poliovirus and from 10^3 to 10^5 RFU/ml of shellfish extract for HAV. Active ingestion of the virus from the seawater was demonstrated by recovering virus from within cockle guts.

To quantify recovered HAV, end-point dilutions and an adaptation of a radioimmunofocus assay (RIFA) were compared. The tests were of similar sensitivity but the RIFA has the advantage of being relatively rapid, shortening the time taken to complete an experiment by as much as 4 weeks.

INTRODUCTION

Environmental contamination of shellfish with human pathogens from sewage is a major problem (Gerba & Goyal, 1978; Turnbull & Gilbert, 1982). Unfortunately, the most prolific harvesting areas are often those nearest to large sewage outfalls. Cockles and other bi-valve molluscs are a particular risk to the consumer because they feed by drawing water over their gills and filtering it. Consequently viruses present in water may be taken up and become concentrated in their tissues. Unlike crustacea, these molluscs are not thoroughly cooked. Oysters are frequently consumed raw and cockles after only brief treatment by steaming or immersion in boiling water.

In 1976 a large outbreak of viral gastroenteritis was reported associated with the consumption of cockles harvested from beds on the Thames Estuary in Essex (Appleton & Pereira, 1977). At least 800 people were known to have been ill in

33 separate incidents. This outbreak prompted a review of the general hygiene and processing requirements of the Thames cockle industry which is under the control of the Port of London Health Authority. As a result of this review the implementation of 'double-cooking' was recommended. After an initial brief boil or steam treatment, necessary for the removal of shells, cockle meats were to be boiled for a further 4 min (Ayres, 1979). This procedure is unpopular within the industry because such prolonged cooking of the meats out of the shell makes them shrink, becoming rubbery and less palatable. Gastroenteritis associated with contaminated cockles continued to be reported.

There have been several reports of hepatitis A associated with the consumption of shellfish such as oysters (Portnoy *et al.* 1975), clams (Feingold, 1973), and mussels (Bostock *et al.* 1979). In 1983 O'Mahony and her colleagues reported a series of cases of hepatitis A associated with cockles. Although cockle processing methods have usually proved sufficient to prevent the transmission of bacterial infections to consumers they are clearly still inadequate to ensure that viral infections are averted (Appleton, Palmer & Gilbert, 1981; Sockett, West & Jacob, 1985).

Hepatitis A virus (HAV) has only recently been successfully propagated in cell culture (Provost & Hilleman, 1979; Flehmig, 1980). This development has made it possible to study the association of this virus infection with the consumption of shellfish. A project was undertaken to determine the minimum heat treatment required for the complete inactivation of HAV in cockles so that new recommendations could be made that might result in a safe but marketable product.

Investigations were divided into two parts. Firstly, conditions for the maintenance of live cockles in the laboratory were established. Experiments were undertaken to determine whether cockles would take up virus from artificially contaminated seawater, and methods for recovering and assaying virus were devised. Secondly, the effect of heat treatment on the survival of hepatitis A in cockles was investigated, and these results are presented in part 2 of this paper.

MATERIALS AND METHODS

(a) *Tissue culture*

Foetal rhesus monkey kidney cells (FRhK-4) were obtained from Dr Bertram Flehmig (University of Tübingen). These cells were used throughout for the propagation of HAV, both to prepare inocula and to measure HAV infectivity. The cells were propagated at 37 °C in Eagles minimal essential medium (MEM) (Wellcome Reagents Ltd), supplemented with 6% foetal calf serum (FCS) (Gibco), 0.13% bicarbonate, gentamicin (50 µg/ml) and kanamycin (31.5 U/ml). For cell maintenance the FCS was reduced to 2%. Cells were split 1:4 at weekly intervals.

Continuous African green monkey kidney cells, designated BGM, (Dahling, Berg & Berman, 1974) were obtained from Dr J. Slade, (Thames Water Authority). Cells were grown at 37 °C in 50% MEM, 50% Leibowitz L-15 medium, with 8% FCS, 0.11% bicarbonate and gentamicin (50 µg/ml). Maintenance medium contained Wellcome 199, 5% FCS, 0.22% bicarbonate and gentamicin (50 µg/ml). Cells were split 1:4 twice a week.

(b) *Preparation of inocula*

(i) *Poliovirus*. Monolayers of BGM cells were inoculated with poliovirus type 1 (Sabin strain) and incubated at 37 °C until there was 100 % cell death. The medium and cells from several bottles were pooled and stored in aliquots at -30 °C. The infectivity was 10^9 TCID₅₀ per ml.

(ii) *Hepatitis A virus*. Tissue culture fluid (TCF) removed from FRhK-4 cells in which a chronic infection with HAV (KMW-1 strain) had been established was used as the inoculum. The KMW-1 strain was obtained from Dr F. Burkhardt having been isolated from an outbreak in Wasen, Switzerland in 1979. Harvests of TCF, removed 14 days after the previous passage of the cells, were pooled and stored at -30 °C. This preparation had an infectivity of approximately 10^6 radioimmunofocus units (RFU) per ml and gave a test to negative ratio of 29:1 in a radioimmunoassay (RIA) for HAV antigen. In some instances sonicated infected cells were used as the inoculum rather than the TCF preparation, and occasionally a mixture of the two was employed.

(c) *¹²⁵I-labelled anti-HAV*

The immunoglobulin G (IgG) was prepared from a human serum containing a high concentration of anti-HAV. It was prepared by dialysis against 20 mM phosphate buffer pH 8, followed by fractionation using a column of DE 52 ion exchange gel (Whatman Ltd) equilibrated with the same buffer. Purified IgG was labelled with ¹²⁵I (Salacinski *et al.* 1979). For use in the RIFA ¹²⁵I anti-HAV was diluted to an activity of 100 000 c.p.m./200 μ l in phosphate-buffered saline (PBS) containing 25 % FCS, 2 % pooled human serum (anti-HAV negative), 0.2 % Tween 20 and 0.08 % sodium azide.

(d) *Assaying for virus infectivity*

(i) *Poliovirus*. Shellfish extracts and inocula were assayed for poliovirus by end point titration. Tubes containing monolayers of BGM cells were inoculated, in triplicate, with tenfold dilutions of the test sample prepared in maintenance medium. The TCID₅₀ titre was calculated (Reed & Muench, 1938).

(ii) *HAV*. Shellfish extracts were assayed for HAV infectivity using an adaptation of a radioimmunofocus assay originally described by Lemon, Binn & Marchwicki (1983). FRhK-4 cells were grown in plastic petri dishes containing acetone-resistant film liners (60 mm diameter; Becton Dickinson Labware) and placed at 37 °C in a 5 % CO₂ atmosphere. When the cells were confluent the growth medium was removed and the monolayers were inoculated with 0.25 ml of shellfish extract, seawater or tank inoculum, diluted in growth medium. Inocula were allowed to adsorb for 2 h at 37 °C and were periodically redistributed over the monolayers. The inocula were removed before the monolayers were overlaid with 4.5 ml of maintenance medium containing 0.75 % agar noble (Difco Labs) at 45 °C. After 7 days incubation at 37 °C a second overlay was added and the cells incubated for a further 7 days.

When incubation was complete the overlay was gently dislodged and the monolayers were washed with 5 ml of prewarmed 199 (with 0.22 % bicarbonate, gentamicin (50 μ g/ml) and kanamycin (31.5 U/ml)). The washings were aspirated

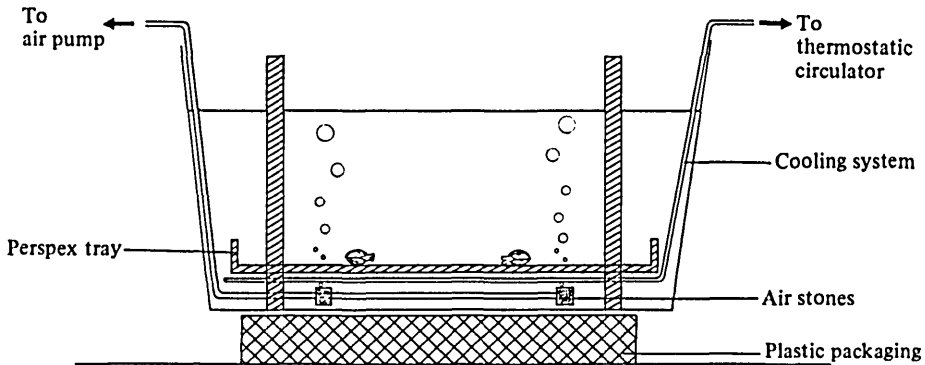


Fig. 1. Shellfish tank system used to maintain live cockles in the laboratory.

and the monolayers air dried and fixed with 2 mls of acetone at room temperature for 2 min. Two ml of diluted radiolabel were added to each monolayer. Following incubation at 37 °C for 4 h the radiolabel was aspirated and each monolayer was washed five times with a total of 10 ml of PBS/T₂₀ (PBS containing 0.05 % Tween 20). The film liners were air dried and the bottom of each was cut out and fixed with sellotape to a card sheet (previously cut to fit an autoradiography cassette). Autoradiography was carried out at -70 °C overnight using Fuji X-ray film and a 'Lightning Plus' intensifier screen. The autoradiogram was processed and examined for foci of infected cells. From the number of foci the amount of infectious virus in the inoculum could be determined and expressed in terms of RFU/ml.

The infectivity of one of the HAV inocula was also assayed by end-point titration to compare its sensitivity with that of the RIFA. Monolayers of FRhK-4 cells were inoculated in triplicate with tenfold dilutions of the HAV inoculum preparation (see (b) ii) from 10⁻⁴ to 10⁻⁸. The cultures were incubated at 37 °C for 6 weeks. TCF was collected weekly and tested for HAV antigen by a sandwich-type RIA.

(e) Maintenance of live shellfish in the laboratory

Live cockles (*Cerastoderma edule*) were harvested from the Thames Estuary and transferred onto a perforated Perspex tray in an open plastic tank (69 cm × 20 cm × 40 cm) containing 20 litres of filtered seawater. The water was aerated through airstones and the temperature maintained at 10–13 °C by a thermostatic circulator (LKB) (Fig. 1). Experiments were carried out in a class 1 safety cabinet. The tank was cushioned on plastic packing material to protect the cockles from vibrations caused by the cabinet. To simulate natural conditions the cockles were protected from the light by covering the front of the cabinet with a black plastic sheet. Before starting a viral contamination experiment cockles were maintained for 24 h after which any dead shellfish were removed. The remainder were fed with 3.5 g of yeast extract dissolved in 500 ml of water.

RESULTS

Experimental infection and recovery of viruses from shellfish

Many of the procedures for the extraction and concentration of viruses from shellfish refer specifically to oysters. For work on cockles several adsorption-elution procedures were evaluated (Johnson, Cooper & Straube, 1981; Sobsey, Carrick & Jensen, 1978; Richards *et al.* 1982). Cockle meats were mixed with either poliovirus or HAV and the method which consistently gave the best rate of viral recovery was determined. The methods of Sobsey, Carrick & Jensen (1978) and Richards *et al.* (1982) gave comparable recovery rates for poliovirus. The Richards method also gave consistent results for the recovery of HAV. However, in the same experiments the Sobsey method proved inconsistent and sometimes failed to recover HAV at all. The procedure of Johnson, Cooper & Straube (1981) was more complex than that of Richards and gave a tenfold lower yield for poliovirus. Therefore, the procedure of Richards and his colleagues proved the most satisfactory for this study and Figure 2 is a flow chart showing the adaptation employed here.

Inoculation of meats

Poliovirus. Cockle meat (10 g) was homogenized in 90 ml of glycine saline buffer (pH 9.5) and inoculated with 1 ml of Sabin strain poliovirus containing 10^8 TCID₅₀/ml. After a further short period of homogenization adsorption was achieved by

(a) adjusting the pH to 4.5 with 6 N HCl, then

(b) pelleting at 3000 rpm for 20 min at 4 °C and discarding the supernatant.

(c) The pellet was resuspended in 90 ml GN buffer pH 9.5 and the extraction procedure continued (stage 3, Fig. 2). The titre of the final extract was 10^6 TCID₅₀/ml of poliovirus.

Hepatitis A virus. Homogenized cockle meat (10 g) was mixed with 1 ml of inoculum (in the form of sonicated FRhK-4 cells, chronically infected with HAV) containing 4×10^8 RFU/ml. The adsorption steps and extraction procedure were carried out as described for poliovirus. The titre of the final extract was 2×10^4 RFU/ml of HAV.

Addition of poliovirus to live shellfish

Preliminary experiments with poliovirus were done as a model for the HAV work since results could be obtained within a few days rather than several weeks.

(a) Cockles filter feeding on 20 l of seawater were given inocula of 37 ml which contained 10^{10} TCID₅₀ poliovirus. Some cockles were sampled preinfection to provide a control. The effect of incorporating extra proteinaceous material into the inoculum on the rate of uptake of poliovirus by the cockles was investigated. This was in the form of bovine serum albumin (BSA, 5 ml) or normal faecal extract (NFE; 25 ml prepared from faeces in which no viruses could be detected). The addition of protein was an attempt to simulate natural pollution and minimize any non-specific binding of virus to the plastic tank and cockle shells and thereby maximise viral uptake. The incorporation of NFE into the inoculum resulted in greater recovery of virus from shellfish extracts and seawater samples than the incorporation of BSA. Filter feeding for 6–24 h led to maximum recovery of

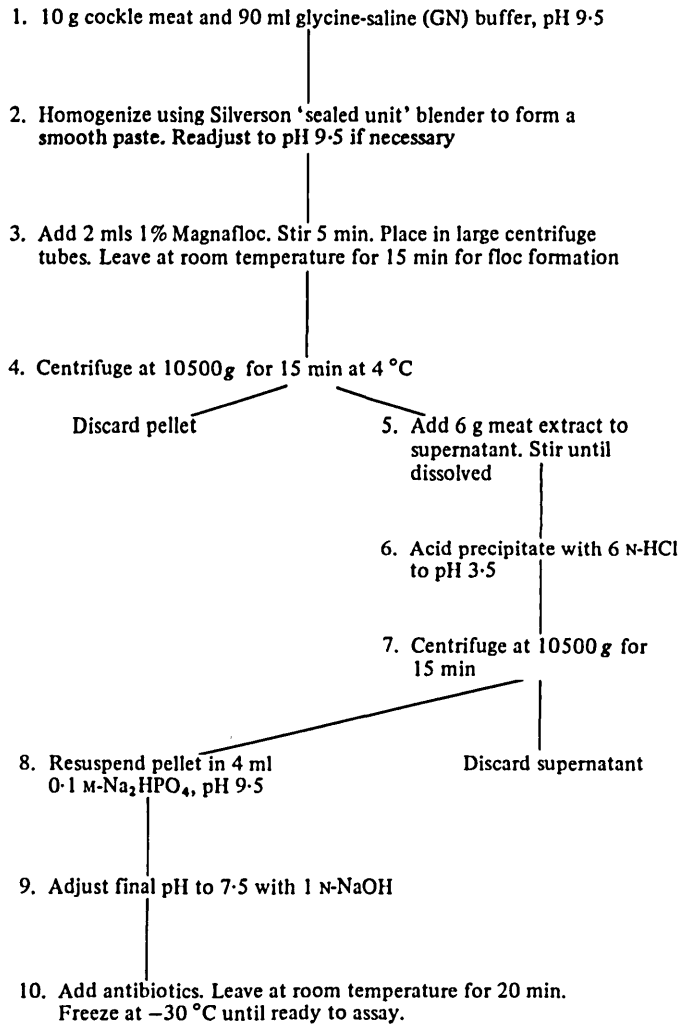


Fig. 2. Procedure for the extraction of hepatitis A virus and poliovirus from cockles (Adapted from Richards *et al.* 1982).

poliovirus from the shellfish meats. After 48 h feeding less virus was recovered (Table 1). Recovery of poliovirus from the seawater declined over the 48 h period, from 10^6 TCID₅₀/ml to 10^3 TCID₅₀/ml with the BSA inoculum and from 10^6 to 10^4 TCID₅₀/ml with the NFE inoculum (Table 1).

(b) In order to investigate the effect of the titre of the inoculum on virus recovery, filter feeding cockles were inoculated on three successive days with 10^5 , 10^7 and 10^9 TCID₅₀ poliovirus made up each time to a total volume of 37 ml with NFE. Before addition of the subsequent inoculum and after filter feeding for 24 h water and shellfish were sampled. Some of the cockles from the final sample were dissected and extractions were carried out on whole cockles, cockle guts and dissected meats.

Virus was found in cockles that had been filter feeding on seawater inoculated with 10^7 TCID₅₀ poliovirus but recovery was greater following an inoculum of

Table 1. Recovery of poliovirus from cockles and seawater: data for two forms of inoculum are shown

Inoculum (37 ml added to 20 l of seawater)	Time after inoculation (h)	Concentration of poliovirus (TCID ₅₀ /ml) in	
		cockle extract	seawater
10 ¹⁰ TCID ₅₀ : poliovirus with BSA	Preinfection	0	0
	0	ND	10 ⁶
	6	10 ⁴	10 ⁵
	24	10 ⁴	10 ⁴
	48	10 ³	10 ³
10 ¹⁰ TCID ₅₀ : poliovirus with NFE	Preinfection	0	0
	0	ND	10 ⁶
	6	10 ⁵	10 ⁶
	24	10 ⁵	10 ⁵
	48	10 ⁴	10 ⁴

ND, not determined.

Table 2. The effect of varying the inoculation dose of poliovirus on its recovery from cockles and the tank water

Inoculum (37 ml added to 20 l of seawater)	Concentration of poliovirus (TCID ₅₀ /ml) in	
	cockle extract	seawater
10 ⁵ TCID ₅₀ in NFE	0	10 ²
10 ⁷ TCID ₅₀ in NFE	10 ²	10 ³
10 ⁹ TCID ₅₀ in NFE	10 ³ (whole shellfish)	10 ⁵
	10 ² (shellfish guts)	
	10 ³ (shellfish meats)	

10⁹ TCID₅₀ poliovirus (Table 2). The presence of poliovirus in shellfish guts indicated that the cockles had actively ingested the inoculum from the surrounding seawater.

Addition of HAV to live cockles

Cockles were sampled before inoculation to provide a control. The HAV inoculum was 100 ml of a mixture of sonicated chronically infected FRhK-4 cells and TCF removed from them. The titre of this preparation was 3.2×10^6 RFU/ml (Fig. 3, Row 2). Whole cockles were sampled after being allowed to filter the infected water (20 l) for 24 or 48 h and extracts from whole shellfish were prepared. Guts and meats dissected from shellfish sampled after 48 h feeding were also extracted. A completed RIFA autoradiogram (Figure 3) shows the recovery of HAV from these cockle extracts. The autoradiogram also shows that virus was present in cockle guts (Row 4, D-F). Thus recovery of HAV does not simply represent superficial contamination of the meats. The titres of viruses in these extracts are given in Table 3.

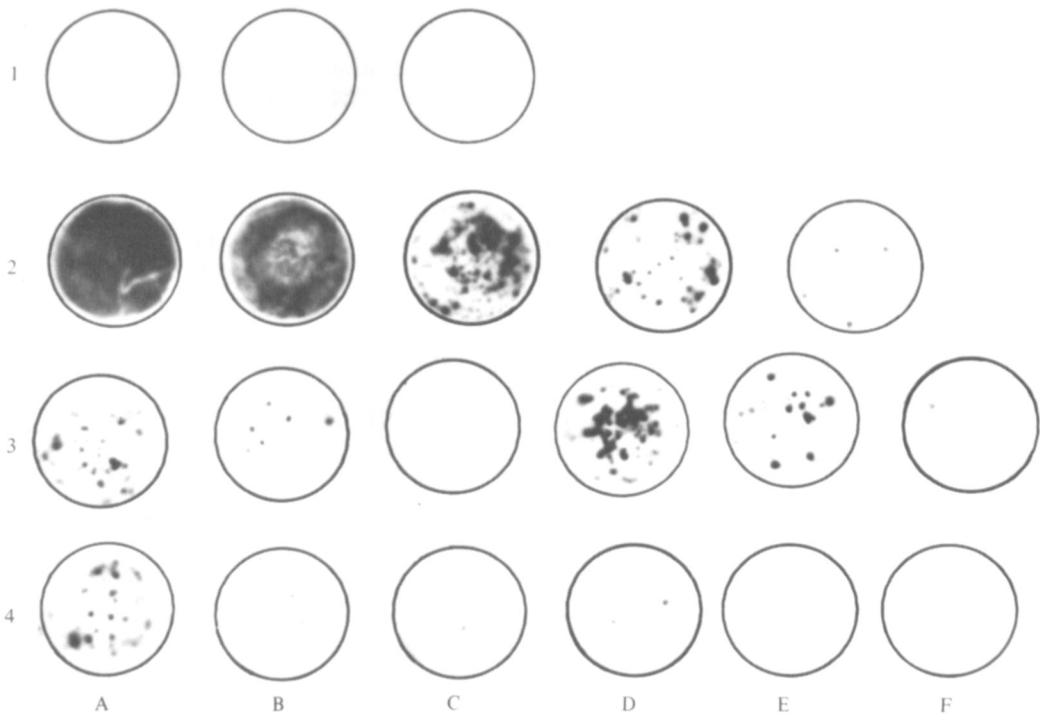


Fig. 3. Quantification of HAV by RIFA. Inocula (cockle extracts or TCF) were adsorbed to FRhK-4 cell monolayers. The cells were overlaid with agar and incubated for 14 days before foci of infected cells were detected by utilizing ^{125}I -antiHAV and autoradiography. Row 1: Controls show no foci of infected cells; 1 A cell control; 1 B and 1 C preinfection cockle extract (10^{-1} to 10^{-2}). Row 2: a titration series (10^{-1} to 10^{-5}) of the HAV inoculum added to the shellfish tank. Row 3: extracts prepared from cockles after filter feeding on HAV contaminated seawater for either (A–C) 24 h or (D–F) 48 h (titrated from 10^{-2} to 10^{-4}). Row 4: recovery of virus from dissected cockles titrated from 10^{-2} to 10^{-4} ; A–C, meats after removal of guts; D–F, cockle guts.

Table 3. Recovery of HAV from live cockles after feeding on inoculated seawater for the times indicated

Inoculum (100 ml added to 20 l of seawater)	Time after inoculation (h)	Concentration of HAV in cockle extract (RFU/ml)
3.2×10^6 (RFU/ml)	Preinfection	0
	24	4.4×10^4
	48 (whole shellfish)	9.2×10^4
	48 (shellfish guts)	2.0×10^3
	48 (shellfish meats)	3.2×10^4

The detection of HAV by RIFA and end-point titration method was compared. An HAV inoculum (Materials and Methods (b) ii) had a titre of 1.2×10^6 TCID₅₀/ml by end-point titration in FRhK-4 cells. TCF from these cells was tested by RIA after incubation for 4 weeks, although the cultures were maintained for a further 2 weeks. The same preparation had a titre of 1.6×10^6 RFU/ml by RIFA after incubation for only 15 days.

DISCUSSION

Oysters and mussels have been maintained under artificial conditions in many laboratories. However, information about keeping cockles under laboratory conditions was not available. Therefore, before experimental work on cockles could begin the requirements necessary to maintain live cockles in the laboratory had to be determined. Initial supplies of cockles were mechanically dredged and many of these shellfish had broken and often incomplete shells. Even those cockles that appeared to be undamaged could not be maintained in the laboratory. Hand picked cockles proved successful and could be maintained, with little mortality, for up to 2 weeks under suitable conditions of temperature, light, aeration and feeding.

We evaluated methods for extracting virus from shellfish and found that for cockles the extraction procedure of Richards *et al.* (1982) was the most satisfactory: it was simple to carry out, took only about 2 h to complete and gave consistently high recovery of both poliovirus and HAV. The final extract was not toxic to BGM or FRhK-4 cells, even in the RIFA where cell monolayers were incubated with the shellfish extract for 2 weeks.

The adaptation of the RIFA technique employed here is similar to a conventional plaque assay: foci only develop in the presence of HAV and each 'radioimmunofocus' is presumed to result from a single infectious virus particle (a RFU). The major advantage of RIFA is its rapidity. A result was obtained in 15 days compared with a minimum of 28 days for the end-point titration method. Therefore, though the two methods were of similar sensitivity, RIFA was a more practical, relatively quick and accurate method of quantifying HAV.

Virus was recovered from the guts of cockles maintained in the laboratory. This demonstrates that virus is not merely attached to the surface of the meats but is actually within the digestive tract of the cockle. The reduction in viable virus observed in seawater samples over time might be the result of viral inactivation due to adverse temperatures and levels of salinity within the tank system. Virus may also attach to plastic surfaces and shells and therefore no longer be available to filter feeding cockles.

The results of these preliminary investigations have provided the basis upon which experiments to determine the heat treatment required for the complete inactivation of HAV in cockles could be undertaken.

Part 2. Heat inactivation of hepatitis A Virus in artificially contaminated cockles

SUMMARY

Before being sold to the public, cockles are subjected to a process of heat treatment to remove microbiological contaminants. Nevertheless, both viral gastroenteritis and hepatitis A have occurred following the consumption of these shellfish and clearly treatments are often inadequate. Investigations into the conditions required for the complete heat inactivation of hepatitis A virus (HAV) in cockles are reported here.

Batches of live cockles, artificially contaminated with poliovirus or HAV, were either immersed in water at temperatures ranging from 85–100 °C or steamed. During inactivation experiments heat penetration into cockle meats was monitored with thermocouples. Heat treated cockles were processed by an adsorption elution extraction method and residual viral infectivity was detected by either end-point titration or a radioimmunofocus assay.

For the small batches of cockles processed in this laboratory immersion for 1 min or steaming for about 1.5 min raised the internal temperature of the meat to 85–90 °C. After maintenance of this temperature for 1 min poliovirus and HAV were completely inactivated.

INTRODUCTION

The association of hepatitis A with the consumption of contaminated shellfish was first reported in 1956 when an epidemic in Sweden was attributed to the ingestion of raw oysters (Roos, 1956). Prevention of such outbreaks is dependent on the elimination of sewage pollution from shellfish harvesting waters or the inactivation or removal of hepatitis A virus (HAV) from shellfish before consumption. Shellfish that maybe eaten raw, such as oysters, are cleansed by relaying or depuration. Other shellfish such as cockles are heat treated. These methods are usually sufficient to prevent bacterial infections in the consumer, but they have proved inadequate on occasions to prevent viral infections (Feingold, 1973; Appleton, Palmer & Gilbert, 1981; Sockett, West & Jacob, 1985).

Koff & Sear (1967) investigated the internal temperature of steamed clams. They found that although shells typically opened during the first minute of steaming, it took 4–6 min for the internal temperature to reach 100 °C. They concluded that clams contaminated with hepatitis virus might not be completely sterilized at or shortly after shell opening and could transmit the disease if consumed. In fact, in 1973, a small outbreak of hepatitis associated with the consumption of inadequately treated clams was reported by Feingold. Peterson and his colleagues (1978) showed that the thermal treatment conditions of 140 °F for 19 min failed to inactivate HAV totally in artificially contaminated oyster homogenates. However, they suggested that pasteurization conditions could be developed that would eliminate hepatitis A whilst retaining the palatability of the shellfish.

In England and Wales, during the period 1980–6, 18 incidents of hepatitis A associated with the consumption of shellfish were reported. Nine of these were both epidemiologically and microbiologically confirmed (PHLS, Communicable Disease Surveillance Centre, unpublished). This demonstrates the inadequacy of present

arrangements for treating shellfish. In recent years there has been a considerable increase in the consumption of all types of shellfish. With modern marketing techniques shellfish such as cockles are no longer just eaten locally, but are distributed to all parts of the country and are frequently served in restaurants as part of mixed seafood dishes. Cockles have been clearly incriminated in the transmission of viral infections and the object of this study was to determine the conditions required for the complete heat inactivation of HAV in cockles.

MATERIALS AND METHODS

Cells and culture conditions, the preparation of poliovirus inocula and assays for virus infectivity have been described in part 1. The conditions for maintaining live shellfish in the laboratory and the method employed for extraction of viruses from them are also detailed there.

(a) *Preparation of hepatitis A virus (HAV) inocula*

Tissue culture fluids (TCF) collected from roller bottles (750 cm²) of foetal rhesus monkey kidney cells (FRhK-4) chronically infected with HAV (KMW-1 strain) were pooled and stored in 100 ml aliquots at -30°C . The cells harvested from one roller bottle were suspended in 5 ml of phosphate buffered saline (PBS) and stored at -30°C . To provide a high titred inoculum for the shellfish tank one aliquot of TCF was mixed with one aliquot of cells. This preparation had an infectivity of 10^7 – 10^8 radioimmunofocus-forming units (RFU) per ml.

(b) *Procedure for heat treating cockles*

Heat treatments were carried out in a large stainless steel electric boiler (Burco Ltd). Batches of shellfish (30–40 cockles) which had been maintained in artificially contaminated seawater were put in a wire basket (10 × 10 × 12.5 cm) which was either immersed in water preheated to the required temperature, or steamed by placing it on an aluminium support above boiling water. After heat treatment the shellfish were rapidly cooled by plunging the basket into cold water. Meats were separated from shells and stored at -30°C until extractions were carried out.

Double cooking experiments involved immersion of shellfish into water at 95°C in two consecutive treatments. Firstly, whole cockles were immersed for 30 s to open the shells and then rapidly cooled in cold water. The meats were then removed from the shells, wrapped in muslin and placed in the basket and immersed at 95°C for a further heat treatment of 30 s to 3 min.

Thermocouples, attached to a digital thermometer (Comark), were inserted into the meats of cockles to monitor the internal temperature throughout all heat treatments. Temperature readings from different positions within each batch were recorded at regular intervals.

RESULTS

Heat treatments and recovery of residual virus

A series of 12 heat inactivation experiments were carried out (3 for poliovirus and 9 for HAV). The tables and figures present a compilation of the results of all these experiments.

Table 1. Heat inactivation of poliovirus in artificially contaminated cockles

Water temperature	Duration of treatment			
	1 min	3 min	4 min	5 min
'boiling'	.	—	—	—
85 °C	+	—	.	.
90 °C	+	—	.	.
95 °C	+	—	.	.

+, Virus detected. —, Virus not detected.

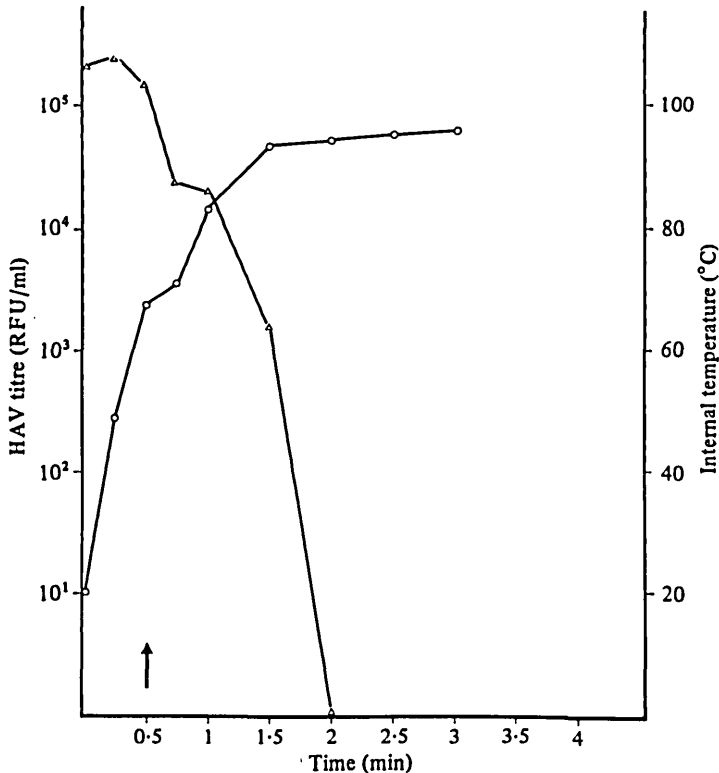


Fig. 1. Relationship between the internal temperature of HAV-contaminated cockles immersed in water at 95 °C (O—O) and the residual infectivity (Δ — Δ). \uparrow , indicates cockle shell opening time.

Poliovirus. Preliminary experiments with poliovirus were used as a model for the HAV work since results could be obtained within a few days rather than several weeks. An inoculum containing 10^{10} TCID₅₀ poliovirus was added to 20 l of seawater in the shellfish tank (i.e. 5×10^5 TCID₅₀/ml of seawater). Cockles were sampled after filter feeding on this water for 48 h. A positive control extract, i.e. prepared from unheated cockles, contained 10^5 TCID₅₀/ml of poliovirus. The remaining cockles were heat treated by immersion in boiling water for 3, 4 or 5 min. No residual virus infectivity could be detected (Table 1).

In further experiments infected cockles were immersed in water at 85, 90 or 95 °C for 1 or 3 min. Virus could still be detected in extracts of cockles that were

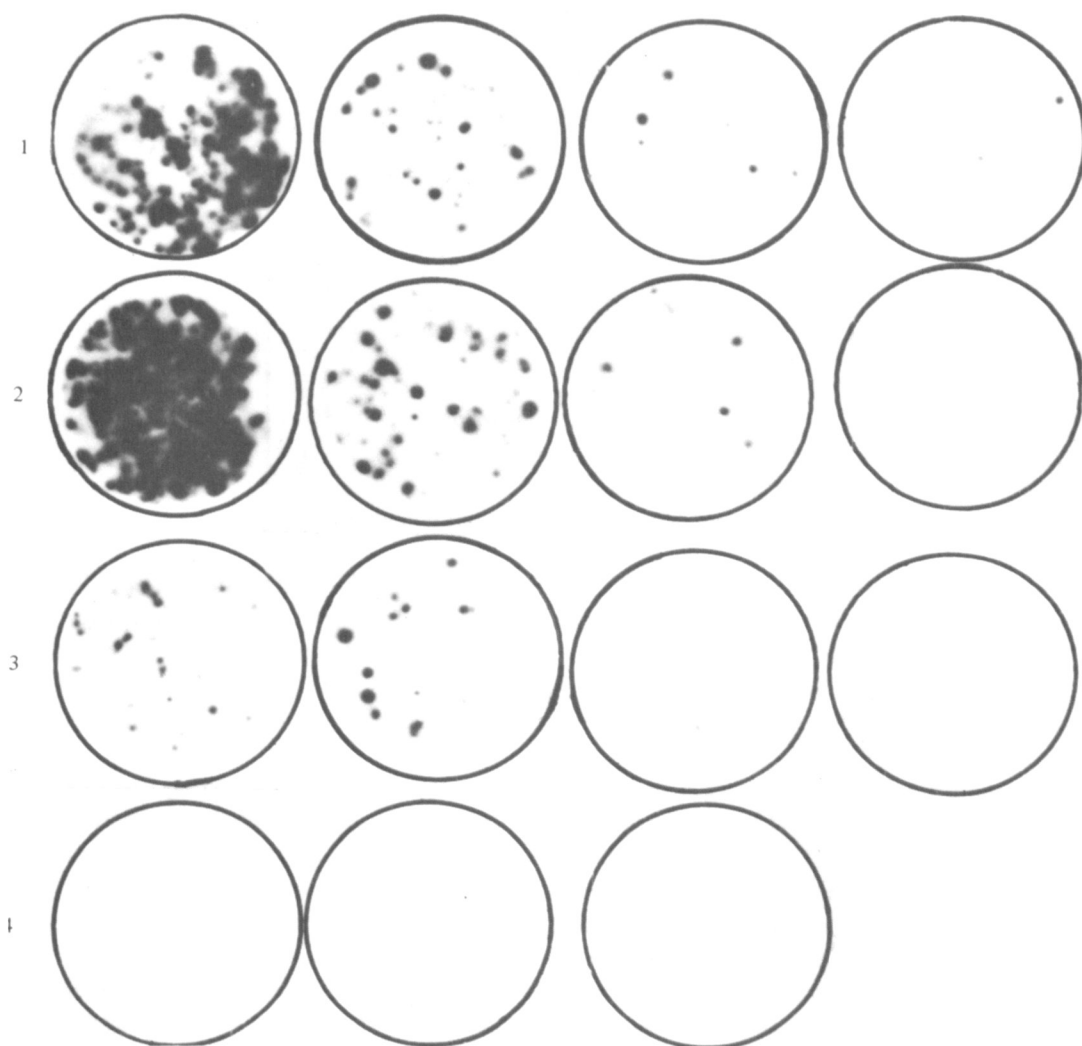


Fig. 2. RIFA autoradiogram illustrating the heat inactivation of HAV in cockles by immersion in water at 95 °C. Row 1: Extract prepared from cockles after filter feeding on HAV contaminated seawater for 48 h. Extract is titrated from 10^{-1} to 10^{-4} . Row 2: Titration (10^{-1} to 10^{-4}) of extract prepared from HAV contaminated cockles after immersion for 30 sec. Row 3: Titration (10^{-1} to 10^{-4}) of extract after immersion of HAV-contaminated cockles for 1 min. Row 4: After immersion for 3 min no residual infectivity could be detected.

treated for 1 min at each of the above temperatures. Some inactivation had occurred, however, as titres of recovered virus were reduced to 10^3 – $10^{3.5}$ TCID₅₀/ml of extract. After heat treatment for 3 min no residual infectivity could be detected at any of the temperatures investigated (Table 1).

Hepatitis A virus (HAV). Cockles were sampled after filter feeding for 48 h on 20 l of seawater artificially contaminated with 100 ml of HAV inoculum containing 2×10^8 RFU/ml (i.e. 10^6 RFU/ml seawater). An extract of unheated cockles

Table 2. *Heat inactivation of HAV in artificially contaminated cockles*

	Immersion							
	Duration of treatment							
Water temp.	15 s	30 s	45 s	1 min	1.5 min	2 min	2.5 min	3 min
85 °C	.	.	.	+	.	.	.	—
90 °C	.	+	.	+	.	.	.	—
95 °C	+	+	+	+	+	—	—	—
Steaming								
Steam temp. (°C)	1 min	2 min	3 min	3.5 min	4 min	5 min	6.5 min	
100/101	+	—	—	—	—	—	—	
Chilling before immersion								
Water temp.	30 s	1 min	2 min	3 min				
95 °C	+	+	—	—				

+, Virus detected. —, Virus not detected.

contained 2.4×10^5 RFU/ml of HAV. The remaining HAV-contaminated cockles were immersed in water at 95 °C for periods ranging from 15 s to 3 min. Following heat treatment residual infectivity was determined. An early, initial rise in virus titre was observed, followed by a steady decrease until at 2 min, when the internal temperature of the cockles had reached 93.5 °C, no virus infectivity could be detected (Figs. 1 and 2, Table 2).

Contaminated cockles were also immersed in water at 85 °C and 90 °C for 30 s, 1 and 3 min (Table 2). At both temperatures infectivity could still be detected after 1 min when internal temperatures ranged from 66–71 °C. However, titres of recovered virus were considerably lower than those of the unheated control. No residual infectivity could be detected after immersion for 3 min, when the internal temperature of the cockles had reached that of the water.

Artificially contaminated cockles were steamed for periods ranging from 1 to 6.5 min. Thermocouple readings taken during these experiments revealed that the internal temperature of steamed cockles did not rise as rapidly as that of immersed cockles. However, after 2 min both treatments produced similar internal temperatures of between 91.5 and 94 °C (Fig. 3). Cockles sampled prior to steaming were extracted to provide a positive control and contained 1×10^5 RFU/ml HAV. Partial inactivation of HAV was observed after steaming for 1 min when the internal temperature had reached 61.5 °C and the titre of virus recovered was reduced to 2×10^4 RFU/ml of extract. Complete inactivation was achieved after steaming for 2 min (Table 2).

Some cockles were chilled on ice for 20–30 min before being immersed at 95 °C. This was done in an attempt to simulate the natural temperature conditions expected during winter months. The rate of increase in internal temperature of these chilled cockles was similar to that observed for steamed cockles, taking almost 2 min to reach 85 °C or greater (Fig. 3). A similar sized batch of cockles maintained at room temperature and immersed at 95 °C required about 1 min to reach 85 °C. Partial inactivation was seen after treatment for 1 min and no residual infectivity could be detected after heating for 2 min (Table 2).

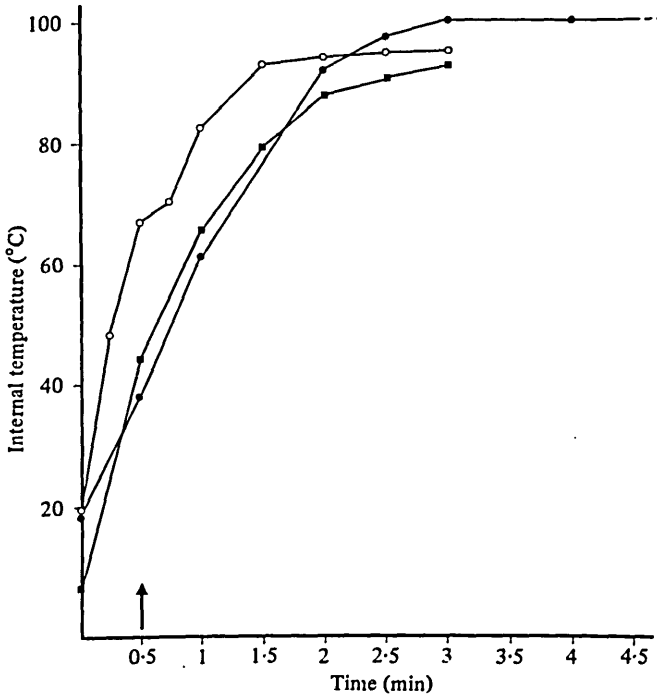


Fig. 3. The rate of increase in internal temperature of whole cockles held at room temperature before immersion in water at 95 °C (O—O); steamed over boiling water (●—●); chilled on ice before immersion at 95 °C (■—■). ↑, indicates shell opening time.

In double cooking experiments the second immersion was performed on meats which had already been treated at 95 °C for 30 s and removed from the shell. In the course of the second immersion internal temperatures of these meats reached at least 85 °C after only 30 s but viable virus could still be detected after 1 min. After 3 min no residual infectivity could be detected. However, cooking of meats out of the shells caused rapid shrinkage and resulted in an unattractive product as compared with the cooking of whole cockles. Heating times necessary for the complete inactivation of HAV in whole cockles left succulent meats with very little shrinkage.

DISCUSSION

The heat stability of hepatitis A virus has only recently been accurately determined. Scheid *et al.* (1982) demonstrated that the infectivity of tissue-culture propagated HAV was reduced by $> 10^4$ times during incubation for 1 min at 85 °C. Frösner (1982) reported $> 10^3$ times reduction after incubation of cell propagated virus for 2 h at 60 °C. Under the same conditions a negligible loss of infectivity in faecal HAV samples was observed. Parry & Mortimer (1984) reported that HAV suspended in phosphate buffered saline was completely inactivated within 4 min at 70 °C, within 30 s at 75 °C, within 5 s at 80 °C and almost instantaneously at 85 °C.

Viruses suspended in protein-rich solutions may be more heat stable. Kaplan

& Melnick (1952) demonstrated that ice cream, cream and milk can exert protective effects on poliovirus when exposed to heat. Parry & Mortimer (1984) found that the stability of HAV was increased when suspended in milk. The steaming or immersion in boiling water of shellfish such as cockles is intended to render them safe for the consumer. The flesh of shellfish may similarly protect any viruses present from the effects of heat. Consequently, suitable conditions for their treatment cannot be defined without data on the heat stability of HAV inside the molluscs and evidence that sufficient heat can penetrate to inactivate any virus present.

In our experiments, HAV in small batches of artificially contaminated cockles was partially inactivated when immersed at 85, 90 or 95 °C for 1 min or when steamed for the same period. For complete heat inactivation the internal temperature of the shellfish should reach 85–90 °C and this temperature must be maintained for 1 min. Our experiments were performed with quantities of virus far in excess of what would be expected as a result of natural contamination, but there are no grounds for thinking that less harsh treatment could be relied upon to inactivate HAV in those circumstances.

A brief heat treatment (20–30 s) results in shell opening, allowing water or steam to penetrate the cockle meat more readily. More virus could be recovered from shellfish treated at 95 °C for 15–30 s than from unheated control cockles (Fig. 1 and 2). This short burst of heat may loosen the attachment between virus and shellfish meat making any virus present more readily available for extraction. Once the shells open fully heat penetrates more readily and a rapid decline in residual viral infectivity is observed.

Many of the immersion heat treatments carried out in this laboratory utilized water at 95 °C rather than at boiling point. On an industrial scale shellfish are treated by immersing large wire baskets packed with cockles into cooking coppers filled with water. Maintenance of such large volumes of water at 100 °C is difficult and heat penetration into the centre of cockle batches is likely to be unreliable. During winter months shellfish may be harvested from seawater which is very cold and temperatures in the cooking sheds are often very low. When external temperatures are extremely low residual seawater in the cockles may even become frozen prior to treatment. Batches of shellfish introduced into the boiler may therefore be very cold and hence reduce the temperature of the water, which then may take some time to regain boiling point. For these reasons the more realistic temperature of 95 °C was chosen for our heat inactivation studies.

On an industrial scale heat penetration could be more effectively achieved by a continuous process in which cockles were exposed to heat in single or at most double layers. Our results indicate that with attention to equipment and plant design the heating times required to render the product safe would be relatively short whilst still attaining a good quality product.

These experiments have determined the heat treatment required for the inactivation of HAV. It cannot be assumed that these conditions would be adequate for the inactivation of other viruses contaminating shellfish such as those causing gastroenteritis.

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