Evaluation by molecular biology of enterovirus and hepatitis A virus contamination of shellfish in the Loire river estuary region

Évaluation par biologie moléculaire de la contamination par entérovirus et virus de l'hépatite A des coquillages de l'estuaire de la Loire

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Abstract

Riboprobes developed in our laboratory were used for detection of enteroviruses and hepatitis A virus (HAV) in shellfish (cockles and mussels). After concentration elution, virus nucleic acids were extracted and purified. Hybridization was performed on a solid support according to the dot-blot technique, with (^{32}P) UTP labelling of probes during transcription. Results show high shellfish contamination in samples: 63% enteroviruses and 67% HAV. Cockles were particularly contaminated: 89% enteroviruses and 84% HAV.

Keywords: Enterovirus, hepatitis A virus, molecular hybridization, shellfish, RNA probes.

Résumé

Les sondes génomiques mises au point au laboratoire ont été utilisées pour la détection des entérovirus et de l'hépatovirus (HAV) dans les coquillages (coques et moules). Après concentration les acides nucléiques viraux ont été extraits et purifiés. L'hybridation moléculaire a été réalisée en utilisant un marquage isotopique ^{32} P des sondes durant la transcription. Les résultats montrent une très forte contamination des échantillons : 63 % par les entérovirus et 67 % par l'hépatovirus (HAV). Les coques *Cerastoderma edule* apparaissent particulièrement contaminées : 89 % par les entérovirus et 84 % par l'hépatovirus HAV.

Mots-clés : Entérovirus, virus de l'hépatite A, hybridation moléculaire, coquillages, sondes ARN.

INTRODUCTION

Outbreaks of gastroenteritis or other pathologies after consumption of contaminated food or water are an important public health problem. In most cases, there is no proof of an epidemiologic association since the infectious agent cannot be detected directly. The micro-organisms incriminated are sometimes bacteria (*Salmonella, Shigella,* etc.) but more often viruses such as hepatitis A virus (HAV), Norwalk virus or enteroviruses. For example, a recent severe outbreak of hepatitis A in Shanghai occurred after consumption of clams (Halliday *et al.*, 1991). Other cases of shellfish-related viral pathology have also been

reported in recent years (Gerba *et al.*, 1985 ; Richards, 1987 ; West, 1989 ; De Mesquita *et al.*, 1991).

Virus detection in the environment involves methodological problems (toxicity for cell cultures, low concentrations, etc.), and some enterovirus serovars are difficult or impossible to grow in cell culture (e.g., *Coxsackievirus* and HAV). So, new technics were needed. In recent years, the molecular cloning of certain virus genomes made possible production of genomic probes for detection of viruses like enteroviruses, HAV (Ticehurst *et al.*, 1987 ; Rotbart *et al.*, 1988 ; Kopecka *et al.*, 1988).

In our laboratory we have developed cDNA and RNA probes for the detection of enterovirus and HAV. This paper reports our experience in using the riboprobes to detect these viruses in shellfish samples.

Material and methods

Sampling sites

Our study involved 4 sites near the Loire River estuary in Western France : 3 mussel beds (Roussellerie, Cormorane and Govelle) and one cockle and mussel bed (Benoît beach) (figure 1). Eighty-three samples were obtains between January 1990 and September 1991 by the Direction Departementale des Affaires Sanitaires et Sociales (DDASS: a public health service) during monitoring of beach shellfish-gathering areas. The samples were immediately packed in ice and transported to the laboratory.

Handling of samples

The samples were washed in sterile distilled water and alcohol and then opened in sterile conditions. The meat and intervalvular fluid were collected in 100 g aliquots and frozen at –20°C. The virus concentration protocol of Beril and Schwartzbrod (1989) was used with slight modifications. Virus elution was

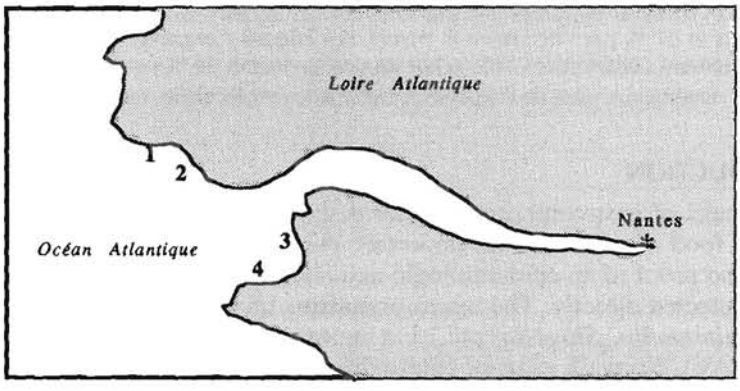

Figure 1: Sampling sites. 1: Govelle, 2: Benoît beach, 3: Roussellerie, 4: Cormorane

performed by mincing (5 mn) in a Virtis blender (Waring Blendor) at high speed and then stirring (15 mn) with 6 volumes of a glycine buffer (Prolabo)/0.1 M NaCl/0.9% NaOH (pH 10). After centrifugation at 1,900 x g for 10 mn, the supernatant was collected and neutralized at pH 7.2 before being concentrated by precipitation in an acid medium (pH 3.5) in the presence of 3% beef extract (AES Laboratory) and stirred for 30 mn. After centrifugation at 1,900 x g for 15 mn, the pellet was resuspended in 30 ml of a sterile phosphate solution (0.15 M Na_2HPO_4) (pH 7.2) (figure 2).

Nucleic acid extraction

One part (3 ml) of the concentrate was treated with proteinase K (200 µg/ml) (Sigma) in the presence of a detergent medium (0.1 M Tris HCl, 1.25 mM EDTA, 0.15 M NaCl, 1% SDS) for 1 h at 56°C (Jiang *et al.*, 1986). Nucleic acids were then purified by one or more phenol/chloroform extractions (V/V). The aqueous phase was collected and precipitated using 2 volumes of absolute ethanol with NaCl at –70°C for 30 mn. After centrifugation at 10,000 x g, for 30 mn, the pellet was washed in 70% ethanol, centrifuged at 10,000 x g for 15 mn and then resuspended in 400 µl of sterile water.

Dot blots

Hybridization was performed on a solid support according to the dot-blot technique. The RNA extract was denatured for 5 mn at 65°C in 3 volumes of a

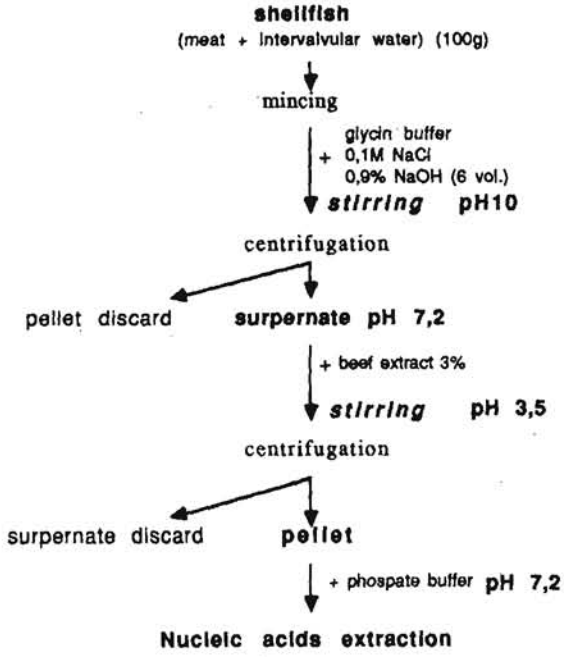

Figure 2: Outline of method for concentrating viruses from samples

mixture containing formamide 500 µl, formaldehyde (37%) 162 µl and MOPS 10X 100µl. It was then placed in ice, and 1 volume of 20X cold SSC was added. Samples serial dilutions (2 fold) were treated in the same conditions.

The Hybond N+ membrane (Amersham) was impregnated with 10X SCC and then placed in a dot-blot apparatus, portions of 100 µl were applied under vacuum suction. Nucleic acids were then fixed by baking the membrane for 2 h at 80°C.

Hybridization

The probe for HAV detection was composed of a conserved sequence (1,500 base pairs) at the 3' end of the genome, coding for the 3D polymerase (Dr. Roberston, CDC, Atlanta). This cDNA was inserted into the pBlueScript SK+ transcription plasmid (Stratagene). For enterovirus, the probe used correspond to a fragment (450 base pairs) at the 5' end noncoding region (5'NC) of poliovirus 1 (Dr. Kopecka, Institut Pasteur, Paris). This cDNA was inserted into the transcription plasmid pGEM 1 (Promega Biotech).

After plasmid linearization, probe transcription and labelling were performed simultaneously using specific SP6, T7 or T3 RNA polymerases and [α^{32}P] UTP (Amersham). The transcribed probes were purified by running the reaction products over a column (Nick column, Pharmacia). After radioactivity measurement, the RNAs were kept at –20°C for use as probes in the hybridization experiments.

Prehybridization and hybridization buffer composition was determined during a preliminary experiment (Apaire-Marchais *et al.*, 1991). Formamide content was 40% for enteroviruses and 50% for HAV. Membranes with immobilized nucleic acids were prehybridized for 2 h at 42°C. Hybridizations were performed at the same temperature and in the same solution but with the addition of [α^{32}P] labelled ssRNA (10^6 to 10^7 cpm/ml) overnight.

After hybridization for the enterovirus research, dots were washed in stringent conditions. Washes were performed at 50°C in 2X SSC-0.1% SDS, 1X SSC-0.1% SDS, 0.5X SSC-0.1% SDS and 0.1X SSC-0.1% SDS, 20 mn each (Kopecka *et al.*, 1988). For the HAV probe, membrane were washed 3 times in 2X SSC-0.2% SDS for 10 min at room temperature and 3 times at 50°C in 2X SSC for 15 mn (Jiang *et al.*, 1987).

The membranes were exposed to MP RPN 6 film (Amersham) in an autoradiography cartridge (Kodak X Omatic) which was then maintained at – 70°C for 24 h.

Results

Enterovirus detection

Fifty-two out of 83 shellfish samples (63%) were found positive by hybridization with the corresponding cRNA probe (table 1).

For the Cormorane site, only 9 mussel samples among the 18 studied were contaminated. Contamination was observed during all the year: in 1990, samples from January, February, March, July, November and December are found positive and during the period studied of 1991, March, June and August.

Table I: Results for shellfish samples

		CORMORANE mussels		GOVELLE mussels		ROUSSELLERIE mussels		BENOIT mussels		BENOIT cockles	
Dates	Sites	EV	HAV	EV	HAV	EV	HAV	EV	HAV	EV	HAV
1990	january	+	–	–	–	–	–	n	n	+	+
	february	+	+	–	–	+	+	n	n	+	+
	march	+	+	–	–	–	–	n	n	+	+
	april	–	–	–	–	–	–	n	n	+	+
	may	n	n	+	–	n	n	n	n	+	+
	june	–	+	–	+	+	+	n	n	+	+
	july	+	+	+	+	+	+	n	n	+	+
	august	–	–	+	–	–	–	n	n	+	+
	september	–	+	+	+	+	+	n	n	+	+
	october	–	+	+	+	+	+	+	+	+	+
	november	+	+	+	+	+	+	=	+	+	+
	december	+	+	–	+	+	+	+	+	+	+
1991	january	–	+	+	+	–	+	–	+	+	+
	february	n	n	n	n	–	+	n	n	n	n
	march	+	+	–	–	–	+	–	–	+	+
	april	–	+	+	–	–	+	–	–	–	+
	may	–	–	+	+	n	n	n	n	n	n
	june	+	–	+	–	+	+	–	–	–	–
	july	–	–	n	n	+	+	–	–	+	–
	august	+	+	+	–	n	n	+	+	+	–
	september	n	n	+	+	n	n	+	+	+	+

Legend:
EV : enterovirus, HAV: hepatitis A virus
+ = sample positive of hybridization with the cRNA probe and negative by hybridization with the vRNA probe
– = sample negative by hybridization with both cRNA and vRNA probes
n = sample not analysed

For the Govelle site, enterovirus was detected in 12/19 mussel samples. In 1990, contamination occurred mainly from July to November and in 1991, from April to September (figure 3).

For the Roussellerie site, results showed 9 positive mussel samples for 17 samples analysed. In 1990, enteroviruses are found during the period between June to December. In 1991, at the beginning of the year samples are found negative, but June and July samples are found positive.

At Benoît beach, for 10 mussel samples analysed during the 2 years, viruses are detected in 5: 3 samples realised at the end of 1990 (October to December) and 2 samples realised in August and September 1991. In cockle bed, a majo-

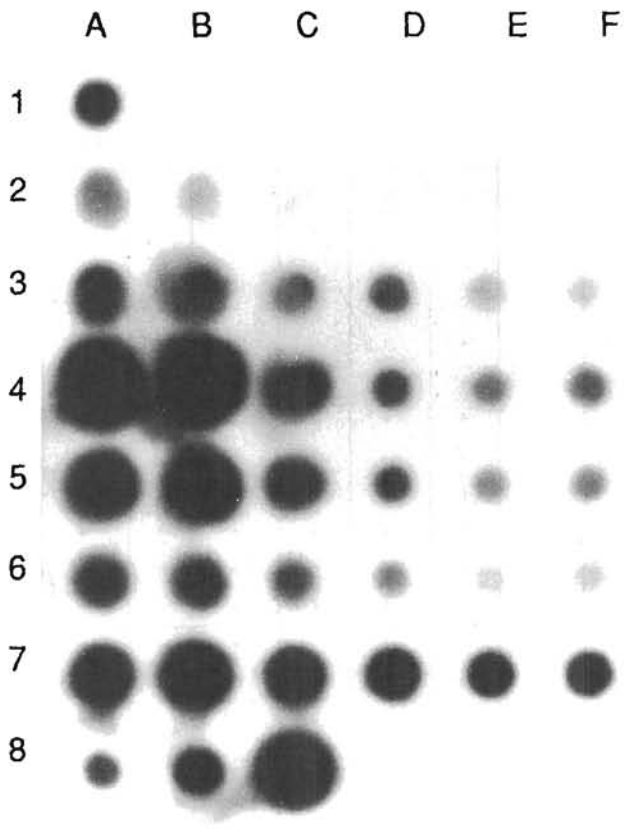

Figure 3: Screening for enterovirus in samples obtained from the Govelle site in 1991. A à F : samples serial dilution (2 fold). 1 : January, 2 : March, 3 : April, 4 : May, 5 : June, 6 : August, 7 : September, 8 : probes

rity of samples are contaminated: 17/19. All samples taken in 1990 and in 1991 only April and June samples are found negative.

HAV detection

Fifty-six out of 83 shellfish samples (67%) were found positive by hybridization with the cRNA probe used to detect HAV (table I).

Studies realised on the Cormorane site showed 12 positive mussel samples (for 18 samples) distributed during all the year 1990: February, March, June, July, September, October, November and December, and 1991: January, March, April and August.

Samples obtained from the Govelle site were less contaminated, 9/19 mussel samples were found positive. These positive samples were obtained in June, July, September, October, November and December 1990; and January, May and September 1991.

For the Roussellerie site, among the 17 mussel samples, 13 were contaminated by the HAV. This contamination was observed mainly from June 1990 to July 1991.

At Benoît beach, 6 mussel samples out of the 10 analysed contained HAV: October to December 1990; January, August and September 1991. The contamination of the cockles was important. As for enterovirus, all samples obtained in 1990 are contaminated. In 1991, samples realised in January, March, April and September are found positive.

Discussion

The purpose of this study was to evaluate the use of genomic probes for characterization of viruses in the marine environment. Near the Loire river estuary (Western France), several beach shellfish-gathering sites are classified as unhealthy areas due to high bacterial contamination of fecal origin (Catherine *et al.*, 1991; DDASS, 1990). The probability to found an associated viral contamination being important (Bosch *et al.*, 1988; Kueh and Grohmann, 1989), we selected for our investigations four of these sites.

From each selected site, viruses were searched for in shellfish present, cockle or mussel. Although viral content of sea water is often low, particles of sediment or shellfish themselves can be highly contaminated (Bosch *et al.*, 1988). Molluscs filter large amounts of water when feeding and are likely to concentrate viruses by retaining the suspended solids (Gaillot *et al.*, 1988; Jehl-Pietri *et al.*, 1990). The viruses are then adsorbed in the digestive tube or the tissues.

Many outbreaks of viral origin have been related to shellfish consumption. *Hepatitis* A virus, *Norwalk* virus and enteroviruses are most often among incriminated viruses. The physicochemical properties of these viruses permit them to escape the effects of depuration techniques and to persist in the environment (Payment *et al.*, 1985; Sobsey *et al.*, 1988; Mbithi *et al.*, 1991). The light cooking that cockles and mussels often receive before consumption is not adequate to inactivate the viruses (Millard *et al.*, 1987). For all these reasons, we have chosen to study shellfish growing in the selected sites.

At the present time, the shellfish salubrity quality is based solely on bacteriological checks. It would thus seem essential to develop a method for detection of viruses in shellfish. Cell culture is the reference method for detecting an infectious virus, however, problems related to the toxicity of environmental samples and the difficulty (sometimes the impossibility) to replicate in cell culture certain viruses led us to apply the molecular hybridization technique in screening for enteroviruses and HAV. The former were detected using a probe provided by Dr Kopecka (Institut Pasteur, Paris) corresponding to the 5'NC part of the genome of *poliovirus* 1 (*Mahomey* strain). This sequence allowed to characterize all enteroviruses except echovirus 22 and HAV. Dr Robertson (CDC, Atlanta) provided us with a sequence corresponding to the 3' end of the genome of HAV (HAS 15 strain) which codes for 3D polymerase. This sequence is specific for HAV, detecting it exclusively.

From these sequences we synthesized DNA and RNA probes. Only our results for RNA probes are presented here since their sensitivity was much greater than that of DNA probes (Apaire-Marchais *et al.*, 1991 ; Kopecka *et al.*, 1988; Shieh *et al.*, 1991). Moreover, the use of RNA probes allows positive samples (cRNA) to be differentiated from non-specific reactions (vRNA). This type of control is of considerable interest for research involving environmental samples. All samples which were positive by hybridization with the cRNA probe proved negative by hybridization with the vRNA probe.

Screening for viruses in environmental samples requires preliminary concentration. The technique used employs elution by a glycine buffer at basic pH and then precipitation by beef extract in an acid medium (Schwartzbrod *et al.*, 1990). This technique is quicker than that using PEG 6000 (Lewis and Metcalf, 1988; Zhou *et al.*, 1991). The nucleic acid extraction phase is an important step since purification must be adequate to avoid non-specific reactions during hybridization ; however, too many extraction steps may lead to the loss of genetic material. The purity check for our extracts was performed by measuring optical densities (verification of the absence of proteins).

Our results show high shellfish contamination since 63% of samples were positive for enteroviruses and 67% for HAV. For the Benoît beach site, 89% of cockles contained enteroviruses and 84% HAV. These results are attributable to the living conditions of these mollusks which lie directly in the sediment and are thus in permanent contact with the particles on which the viruses can be adsorbed. The mussel bed studied at this same site was less contaminated. At the beginning of 1991, samples obtained at the same time from adjoining beds of cockle and mussel were positive for enteroviruses and HAV for cockle samples, whereas mussel samples were negative. Mussel depuration in this case may have been due to tidal action which is an important phenomenon in this region.

Shellfish at the other sites were less contaminated, although some differences were noted. The Govelle site was more contamined by enteroviruses than by HAV, whereas the Cormorane and Roussellerie sites were more contaminated by HAV. The study period was not long enough to evaluate seasonal influences. However, it is noteworthy that all samples from all sites were positive from July to December. Such contamination is probably related to the considerable increase in population in these regions as a result of summer tourism.

Few studies have been concerned with viral contamination of shellfish in the environment. However, HAV has been detected in shellfish by radio immunoassay (RIA), a less sensitive technique than molecular hybridization, and enteroviruses by cell cultures (Schwartzbrod *et al.*, 1990 ; Pietri *et al.*, 1988). Metcalf and Jiang (1988) used molecular hybridization to screen for the presence of HAV in estuarine samples. Only water samples with suspended solids proved to be contaminated, and the rare oyster samples analyzed were negative.

Although molecular hybridization techniques do not differentiate infectious from non infectious particles, they make it possible to detect viruses that are difficult or impossible to obtain in cell culture in a much shorter time and with good sensitivity compared to cell cultures. Moreover, solid support hybridiza-

tion by the dot-blot process allows simultaneous analysis of a large number of samples. The development of polymerase chain reaction will provide lower sensitivity thresholds compatible with environmental levels (particularly in water), thus avoiding problems related to low initial virus concentrations in samples.

The elaboration of these techniques is of considerable importance now that viral contamination is considered to be high in the marine environment. In coming years, it will be essential to perform virological controls in shellfish, particularly in view of the decreased prevalence of anti-HAV antibodies in the human population.

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