

Development of genomic probes for detection of hepatitis A virus and enteroviruses in shellfish

Mise au point de sondes génomiques pour la recherche du virus de l'hépatite A et des entérovirus dans les coquillages

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Abstract

The development of molecular biology techniques has modified research procedures for certain viruses, particularly hepatitis A virus (HAV) and enteroviruses. Accordingly, we designed cDNA and RNA genomic probes to detect viruses in shellfish in the environment. Better sensitivity was noted with RNA than cDNA probes : only 17/83 shellfish samples were positive with HAV and enterovirus DNA probes, where as 42/83 were positive with HAV and enterovirus RNA probes.

Keywords: Hepatitis A virus, enterovirus, cDNA and RNA probes, shellfish.

Résumé

Le développement des techniques de biologie moléculaire a modifié les procédures de mise en évidence de certains virus, en particulier le virus de l'hépatite A (VHA) et des entérovirus. Dans cette voie, nous avons développé des sondes génomiques ADN et ARN pour détecter les virus dans les coquillages provenant du milieu naturel.

La sonde ARN montre une meilleure sensibilité que la sonde ADNc : sur 83 échantillons de coquillages analysés, 17 se sont révélés positifs au VHA et entérovirus avec les sondes ADN, tandis que 42 échantillons se sont révélés positifs au VHA et entérovirus avec les sondes ARN.

Mots-clés : virus de l'hépatite A, entérovirus, sondes ADNc et ARN, coquillages.

INTRODUCTION

Hepatitis A virus (HAV) (classified as Hepatovirus) is like all enteroviruses a member of the *Picornaviridae* family. All these viruses have common structural features: 20 to 30 nm diameter, single-stranded RNA ($2.5 \cdot 10^6$ daltons) and an icosahedral capsid. They have no envelope and are thus resistant to organic solvents, heat and acid pH (Fields 1990). They can persist in the environment over a long period and be transmitted indirectly by water or by shellfish which filter large amounts of water when feeding and accumulate the suspended solids to which the viruses are attached (Jehl-Pietri *et al.*, 1990).

Current evaluations of the microbiological salubrity of seafood are based solely on detection of bacteria. However, viruses are often incriminated in outbreaks due to ingestion of shellfish consumed raw or slightly cooked (Millard *et al.*, 1987).

Replication in cell culture of viruses from environmental samples is hazardous, and even quite difficult for certain species (HAV, Coxsackievirus), it is essential to develop reliable and rapid methods with good sensitivity and specificity. As molecular biology techniques offer such possibilities, particularly through molecular hybridization, we developed DNA and RNA genomic probes to detect HAV and enteroviruses in shellfish.

Material and methods

Probes

Sequences: The sequence used for the development of HAV probes was provided by Dr. Robertson (CDC, Atlanta). This cDNA sequence of the HAV genome (HAS-15 strain), inserted into the plasmid pBR322 (*Pst* I site), is composed of 1,500 base pairs and corresponds to the 3' end coding for the 3D polymerase. For enteroviruses, the cDNA sequence was provided by Dr. Kopecka (Institut Pasteur, Paris). This sequence of *poliovirus* 1 (*Mahoney* strain), inserted into the plasmid pGEM 1 (*Bam*H I site), is composed of 450 base pairs and corresponds to the 5' noncoding (5' NC) region which is quite well preserved in most enteroviruses.

cDNA probes: The method of Ish-Horowicz (1981) was used for minipreparation. After control of cDNA insertion into the plasmid of the isolated bacterial colony, cDNA were amplified (maxipreparation) and obtained by plasmid extraction (Sambrook *et al.*, 1989) (figure 1). The GeneClean TM kit (BIO 101, Inc.) (Ozyme) was used to purify the inserts (employed as cDNA probes).

RNA probes: Riboprobes were prepared by subcloning the cDNA fragment of HAV or poliovirus in transcription plasmids. The insertion of HAV cDNA was performed in the pBlueScript vector (SK+, T3/T7) (Stratagene) by the T4 ligase. A mini- and maxi-preparation were performed as in the case of cDNA amplification. The orientation of the HAV insert in the plasmid was determined in order to obtain complementary RNA (cRNA probes) and RNA with sequences identical to those of viral genomes (vRNA probes). The latter were used as internal controls. The diagrams for synthesis of cRNA and vRNA probes are shown in figures 2 and 3.

Viruses

The strains used were (i) a HAV (strain CF53; 13th passage on PLC/PRF/5 cells; infectious titer $10^{5.5}$ DICT50/ml; Dr Deloince, CRRSA, La Tronche, France); (ii) poliovirus 1, 2 and 3 (*Sabin* strains); (iii) *coxsackievirus* B1 to 6 (strains provided by the Laboratoire National de Référence des Entérovirus, Lyon, France); and (iv) *ECHOvirus* 7, 11, 22 (strains isolated by the Laboratoire de virologie, Nantes, France).

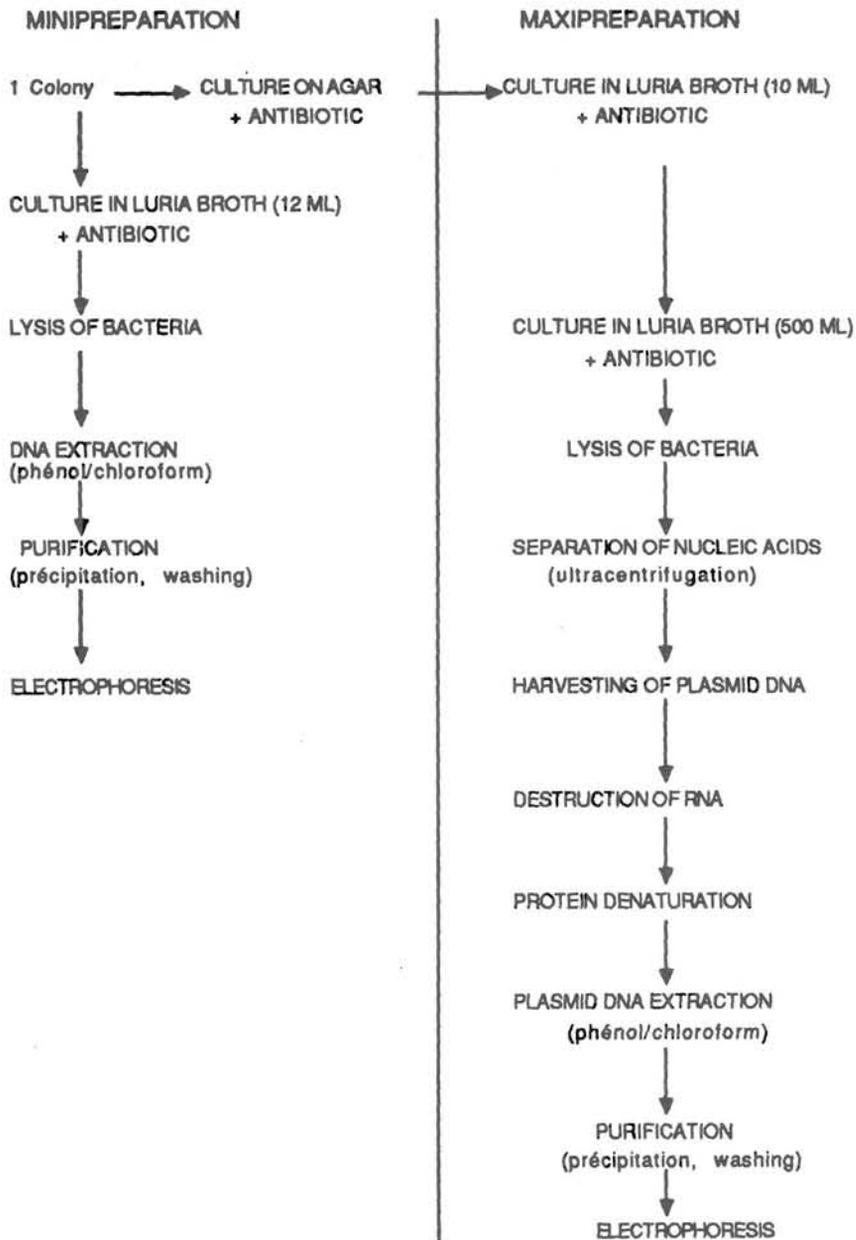


Figure 1: Protocols of mini- and maxi-preparation techniques

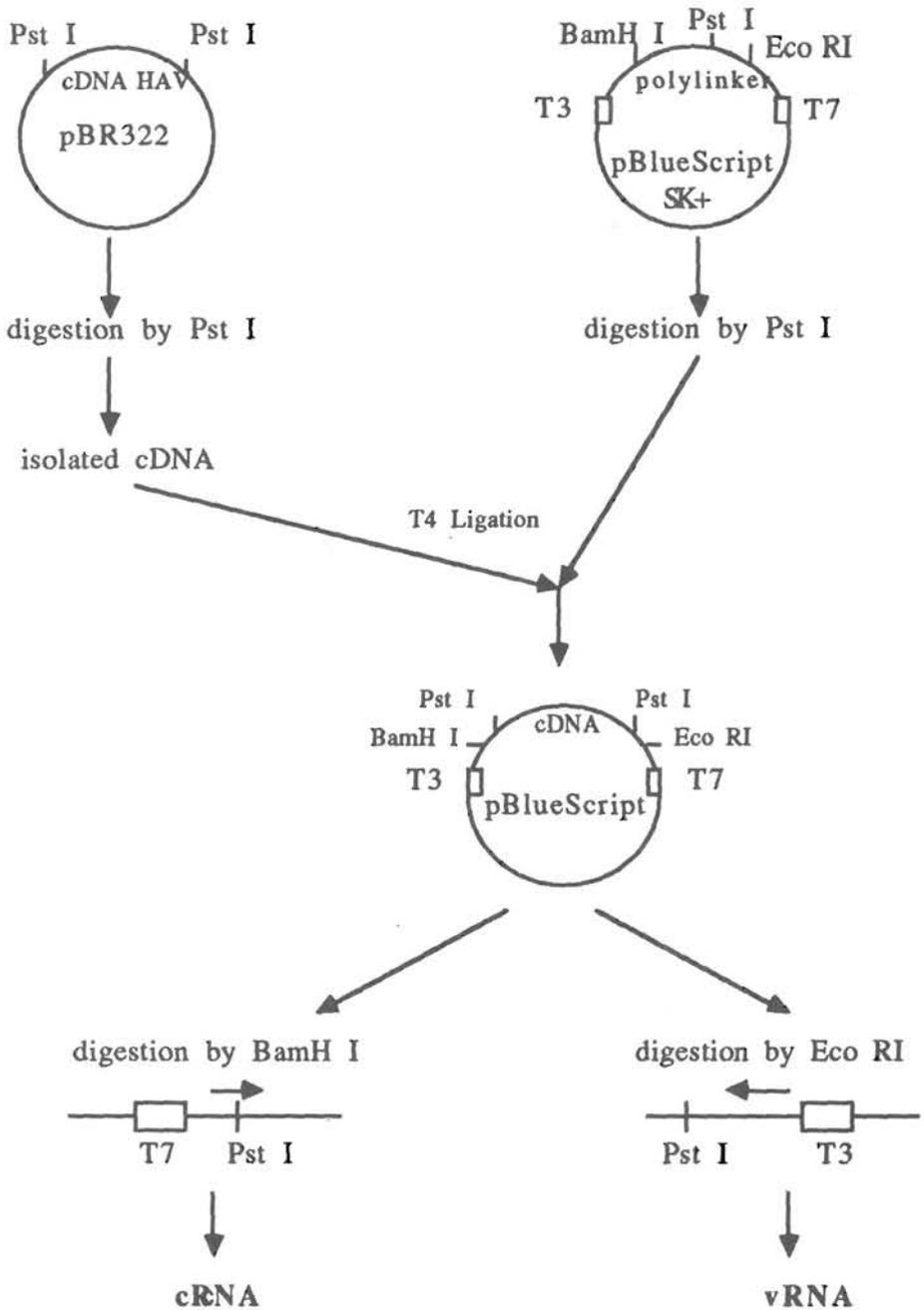


Figure 2: Synthesis of HAV RNA probes

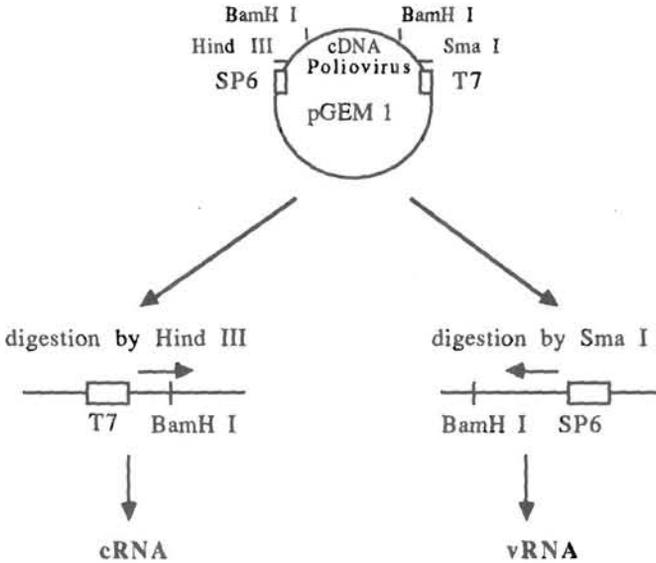


Figure 3: Synthesis of enterovirus RNA probes

Nucleic acid extraction

Nucleic acids were extracted from viruses after cell culture in the presence of proteinase K (100 µg/ml)(Sigma) for 1 h at 56°C. After purification by phenol/chloroform extraction, they were precipitated by absolute ethanol with NaCl, washed in 70% ethanol and then resuspended in 5X SSC (Metcalf et Jiang, 1988 ; Petitjean *et al.*, 1990).

After elution concentration of shellfish (Beril and Schwartzbrod, 1989 ; Le Guyader *et al.*, 1992), viral nucleic acids were extracted by a detergent mixture (1% SDS, 1.25 mM EDTA, 0.1 M TrisHCl, 0.15 M NaCl, 200 µg/ml proteinase K) for 1 h at 56°C (Jiang *et al.*, 1986). After purification by phenol/chloroform extraction, absolute ethanol precipitation with NaCl and washing with 70% ethanol, dried, the nucleic acids were suspended in sterile water.

Hybridization

Dot blotting: After denaturation of the extracts in a formamide-formaldehyde-MOPS solution at 65°C for 5 min, and addition of 20X SSC (V/V), portions of 100 µl were dotted onto Hybond N+ membranes (Amersham). Nucleic acid was then fixed by baking the membranes for 2 h at 80°C.

Hybridization reaction : For cDNA probes, the membranes were first prehybridized in a fast hybridization buffer (Amersham) at 65°C. For RNA probes, the buffer contained 0.9 M NaCl, 2X Denhardt, 0.2 mM EDTA, 0.2 g herring sperm DNA, 5% dextran sulfate, 0.5% phosphate buffer pH 6.5 and 1% SDS.

Only the formamide concentration differed according to the probe used: 40% for the enterovirus RNA probes and 50% for the HAV RNA probes. These two prehybridizations were performed at 42°C.

cDNA probes were labeled with ($\alpha^{32}\text{P}$)dCTP according to the random multipriming technique (Amersham). For RNA probes, transcription with specific RNA polymerases and ($\alpha^{32}\text{P}$)UTP labeling (Amersham) were performed simultaneously. Probes were purified according to the Nick-column protocol (Pharmacia). Hybridization reactions were obtained by adding the labeled probes (10^6 à 10^7 cpm/ml) to the buffers used for prehybridization. The reactions were performed overnight at 65°C for cDNA probes and at 42°C for RNA probes.

Washes: For DNA probes, washing was performed twice for 10 min in 2X SSC-0.1% SDS at room temperature, once for 15 min in 1X SSC-0.1% SDS at 65°C and twice for 15 min in 0.5X SSC-0.1% SDS at 65°C (Amersham). For the enterovirus RNA probe, four washes of 20 min at 50°C were performed in 2X SSC-0.1% SDS; 1X SSC-0.1% SDS; 0.5X SSC-0.1% SDS; 0.1X SSC-0.1% SDS (Kopecka *et al.*, 1988). For the HAV RNA probe, the membranes were washed thrice 10 min in 2X SSC-0.2% SDS at room temperature and thrice 15 min in 2X SSC at 50°C (Jiang *et al.*, 1987).

Detection and revelation: Hybrids were detected by autoradiography using MP RPN-6 film (Amersham) and revealed after 24 h exposure at -70°C.

Results

Probe replication

The maxipreparation reaction enabled us to obtain a large number of copies of plasmid pBR322 containing the HAV cDNA sequence. Using the *Pst* I restriction enzyme, we separated the insert (1.500 base pairs) from vector pBR322. After electrophoretic migration, the band corresponding to the insert was excised, and the purified insert was then used as cDNA probe. The same process was used to amplify plasmid, pGEM 1 containing the *poliovirus* cDNA sequence. The insert (450 base pairs) was separated from the vector using *Bam*H I enzyme and then recovered and employed as a cDNA probe. After insertion of HAV cDNA into the pBlueScript SK+ transcription plasmid, a large number of copies of this vector were obtained by maxipreparation.

The HAV RNA and enterovirus probes were synthesized by specific RNA polymerases during transcription of their respective vectors (pBlueScript SK+ and pGEM 1).

Specificity check

Probe specificity was determined by hybridization with homologous and heterologous nucleic acids (figure 4). cDNA and RNA probes corresponding to the 5' NC region of the *poliovirus* genome enabled the following enteroviruses to be detected: *poliovirus* 1, 2 and 3, *coxsackievirus* B1, B2, B3, B4, B5, B6 and *ECHOvirus* 7 and 11. *ECHOvirus* 22 and HAV were not detected. The HAV riboprobes did not detect the above enteroviruses but only the HAV strain.

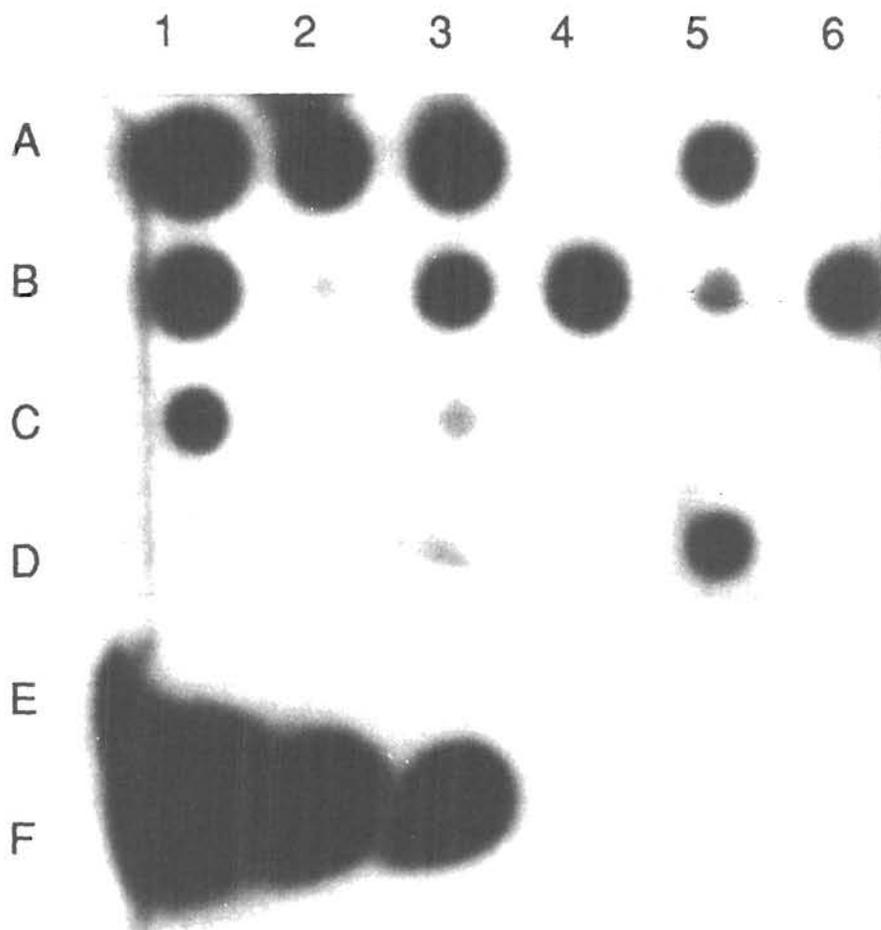


Figure 4: Specificity of cDNA probe relative to selected viral strains.
 A1, A2, A3, A5: *poliovirus* 1, 2, 3, *coxsackievirus* B2.
 B1, B3, B4, B6: *coxsackievirus* B1, B3, B4, B5, B6.
 C1, C2, C4: *ECHOvirus* 11, 22, HAV.
 D1, D5: cell control, *ECHOvirus* 7.
 F1, F2, F3: cDNA probes.

Sensitivity check

Sensitivity was determined using titrated viruses: HAV titrating $10^{6.5}$ DICT50/ml and poliovirus 1, 2 and 3 titrating 10^7 DICT50/ml. Our detection threshold was $10^{4.5}$ for HAV and 10^4 for enteroviruses with RNA probes and respectively $10^{5.5}$ and 10^5 with DNA probes.

Evaluation of shellfish samples

The results in 83 shellfish samples (cockle and mussel) for detection of HAV and enteroviruses with DNA and RNA probes are shown in tables I and II. DNA probes allowed detection of 28/83 (34%) positive enterovirus samples

and 18/83 (22%) positive HAV samples. RNA probes detected HAV in 56/83 (67%) samples and enteroviruses in 52/83 (63%) samples.

Tables III and IV indicate the concordance in detection of shellfish HAV and enteroviruses by RNA-DNA or RNA-RNA hybridizations. The concordance of positive samples was 42/83 for RNA probes and 17/83 for DNA probes. Discordances concerned samples positive for HAV and negative for enteroviruses or negative for HAV and positive for enteroviruses. There was discordance in 12 samples for DNA probes and in 24 for RNA probes.

Discussion

The development of molecular biology techniques has revolutionized the detection of certain viruses, particularly HAV and enteroviruses. Different fragments have been cloned and used as probes, thus providing several sequences

Table I: Results of HAV and enterovirus detection by DNA-RNA hybridization in shellfish samples

	+	-	Total
Enterovirus	28	55	83
HAV	18	65	83

Table II: Results of HAV and enterovirus detection by RNA-RNA hybridization in shellfish samples

	+	-	Total
Enterovirus	52	31	83
HAV	56	27	83

Table III: Concordance of RNA-DNA hybridization results for HAV and enterovirus

		HAV		
		+	-	Total
Enterovirus	+	17	11	28
	-	1	54	55
	Total	18	65	83

Table IV: Concordance of RNA-RNA hybridization results for HAV and enterovirus

		HAV		
		+	-	Total
Enterovirus	+	42	10	52
	-	14	17	31
	Total	56	27	83

for HAV detection. Most of these cloned sequences have been produced by Dr Ticehurst who was the first to establish the restriction map for cDNA clones, which represent at least 99% of the HAV genome of strain HM-175 (Ticehurst *et al.*, 1983). Among these, 4 recombinant plasmids are frequently used : pHAV 1307 (5' end), pHAV 228 and pHAV 148 (central region) and pHAV 207 (3' end). Other riboprobes have been synthesized by Kopecka *et al.* (1988) from sequences provided by Dr Ticehurst and Pr Bercoff. One of these riboprobes corresponds to the 350 nucleotides of the 3' end. Reports on 3' end sequences in the literature also mention pHAV 93 (around 1,500 base pairs), presenting an *Ava* I site (Ticehurst *et al.*, 1983), and a fragment (1,800 base pairs) cloned in the *Eco* RI restriction enzyme site (Fagan *et al.*, 1990). Our sequence, prepared in Dr Robinson's laboratory (Stanford University, Palo Alto, CA) and derived from the viral genome of the HAS-15 strain, codes for 3D polymerase and corresponds to a well-preserved region of the genome within the different HAV strains (Cohen *et al.*, 1987). Sequence comparison has shown that HAV genomes are different from those of other enteroviruses (*poliovirus*, *ECHOvirus*, *coxsackievirus*) (Shieh *et al.*, 1991).

Several *poliovirus* 1 subgenomic riboprobes have been synthesized: 5' NC, VP3, VP1, central region 2C and 3' end. The riboprobe used in this study, corresponding to the 5' NC sequence, allows detection of most enteroviruses, whereas the probe corresponding to a part of VP1 protein is specific for *poliovirus* (Kopecka *et al.*, 1988). The different sequences used as genomic probes are described by Rotbart (1991).

The preparation of HAV riboprobes was facilitated by the use of the pBlueScript SK+ transcription vector which possesses a fraction of the Lac Z gene coding for β -galactosidase and is therefore a better choice than the Gemini vector. This property allows the selection, after transfection, of recombinant bacteria by color. Thus, these bacteria are directly visualized in the agar medium. We used T7 RNA polymerase for synthesis of riboprobes in order to obtain a large number of RNA strands with negative polarity (cRNA) for HAV and enteroviruses. RNA strands with positive polarity (vRNA) obtained through the action of T3 RNA polymerase for HAV and of SP6 RNA polymerase for enteroviruses served as an internal control, permitting detection of nonspecific reactions. This control is of real value for detection of viruses in the environment where there can be various causes of nonspecific reactions. In our samples, no hybridization was observed with vRNA probes.

The specificity of our probes was the same as that reported in the literature (Jiang *et al.*, 1987 ; Kopecka *et al.*, 1988). No nonspecific hybridization was noted with the HAV probe and the different enteroviruses chosen because of their frequency in the population (all of which were detected by the enterovirus probe). This notion of specificity was borne out by our study on shellfish. The results indicated in the concordance tables (III and IV) show that some HAV-positive samples were not necessarily enterovirus-positive, and vice-versa.

The percentage of positive samples detected with DNA probes during our study was 34% for enteroviruses and 22% for HAV. The enterovirus probe corresponding to the 5' NC region recognized around 60 serovars, whereas the HAV DNA probe was only specific for HAV. Moreover, spot intensity was grea-

ter with the enterovirus than the HAV DNA probe, suggesting that enterovirus concentration in shellfish is greater than that of HAV. Thus, it is important to use a more sensitive probe to characterize this virus. In this respect, our HAV RNA probe detected 67% of positive samples vs 22% with the cDNA probe. The increase in sensitivity with the enterovirus RNA probe was lower (63% of positivity vs 34% with the cDNA probe). Our percentage of positive samples with riboprobes was much greater than with cDNA probes. Similar results are reported in various studies. Kopecka *et al.* (1988) noted a sensitivity 100 times greater than during detection of *poliovirus* in infected cells. Shieh *et al.* (1991) observed a signal 5 times greater with RNA probes during detection of enterovirus in water. Metcalf *et al.* (1987) found that sensitivity was at least 8 times greater with RNA probes for HAV detection in stools or in environmental water.

The development of genomic probes thus seems of interest as a rapid, sensitive and specific technique for detection of viruses in the environment, particularly those which are difficult to grow in cell culture. The use of RNA probes provides greater sensitivity, whereas the RNA-RNA hybrids are more stable, allowing stricter hybridization conditions with reduced background. Moreover, the vRNA probes constitute excellent internal reaction controls. The development in our laboratory of polymerase chain reaction technique, controlled by hybridization, will make possible to increase sensitivity even more.

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