Comparison of different primer sets for the RT–PCR detection of hepatitis A virus and astrovirus in mussel tissues

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Abstract In the present study, the efficiency of several primer sets for the RT–PCR detection of hepatitis A virus (HAV) and astrovirus from both crude viral extracts and experimentally infected shellfish tissues was evaluated. Differences were observed depending on the primer set employed in the sensitivity of amplification of both viral types. For HAV primers, HAV240/HAV68 yielded the higher sensitivity: showing a detection limit of 0.02–0.1 infectious particles/µL or mg of tissue (either crude extracts or seeded mussel tissues). Regarding detection of AsV, a better performance was observed with primer set A1/A2 achieving a sensitivity of 0.1–1 PFU/µL or mg of tissue. The results obtained in this work strongly indicated that selection of primer sets to be employed for the routine detection of enteric viruses was a critical point in the design of the RT–PCR protocols.

Keywords Astrovirus; detection; Hepatitis A virus; primer selection; RT–PCR; shellfish

Introduction
Bivalve shellfish have been implicated as vectors in the transmission of enteric viral diseases for many decades (Richards, 1987), due to the fact that these marine organisms are readily contaminated with viruses present in water because of the concentrating effect of their filter feeding. The periodic occurrence of shellfish-transmitted illness outbreaks has contributed to a public confidence problem over shellfish safety, and has resulted in important economic losses by the seafood industry (Morse et al., 1986).

Hepatitis A virus (HAV) is an environmentally stable, positive stranded RNA virus that is transmitted via the faecal–oral route. Infectious hepatitis, caused by HAV, is one of the most serious viral infections linked to shellfish consumption, causing a serious debilitating disease and even death. Although hepatitis A is a common endemic infection in developing areas, the improvement of sanitary conditions has led to its declining prevalence in developed countries (Koff, 1995; Dal-Ré et al., 2000). A consequence of this decline is that adult populations are more susceptible to potential hepatitis A epidemics from vectors such as food or water or to acquiring infection from travel to endemic areas (Pebody et al., 1998; Lees, 2000).

Astroviruses (AsV) are associated with both sporadic episodes and outbreaks of diarrhoea. The faecal–oral route is the predominant mode of transmission, and contaminated drinking water and shellfish have been implicated as vehicles of transmission (Kurtz and Lee, 1987; Marx et al., 1997). Eight serotypes of AsV have been associated with human infections, with serotype 1 being the most prevalent (Jonassen et al., 1995).

Current control methods of the sanitary quality of marketable shellfish are based on the bacterial indicator faecal coliforms (Anon., 1991, 1993), which may fail to detect viral contamination (Power and Collins, 1989). Therefore, a number of methods for direct detection of enteric virus in this type of sample have been developed (Romalde, 1996). Reverse transcriptase–polymerase chain reaction (RT–PCR) has proved to be a good tool for this purpose, due to its high sensitivity and specificity, being successfully used to detect HAV
and AsV in stools, seawater and shellfish (Kurtz and Lee, 1987; Yamashita et al., 1991; Oishi et al., 1994; Marx et al., 1997). However, these methods are diverse, complex, poorly standardised and restricted to a few specialist laboratories. Quality and standardisation issues, as well as simplification and automation of molecular procedures, are needed before they can be adopted by routine monitoring laboratories.

In a previous work, we compared several commercial kits for nucleic acid extraction and for RT–PCR, evaluating their applicability for viral detection in shellfish (Ribao et al., 2002). This study allowed us to choose the best-performing kits, which yielded higher sensitivities than the commonly used in-house extraction procedures. In addition, the use of such kits would facilitate the standardisation of methods among laboratories. Besides the variability due to the different nucleic acid extraction and RT–PCR procedures, the selection of an appropriate primer set is essential to achieve an accurate detection of enteric viruses from shellfish. Differences in specificity and/or sensitivity of detection procedures dependent on the primers employed have been reported (Marx et al., 1997). Therefore, the selection of an adequate target region within the viral genome, and the design of a reliable primer set are also critical points in the development of a viral detection procedure. The aim of the present study was to evaluate the efficiency of several primer sets described in the literature for direct detection of HAV and AsV in shellfish tissues.

Materials and methods

Viral stocks and titration

HAV was obtained from the ATCC as HM-175/18f, a cell culture-adapted, cytopathic clone of strain HM-175 (Lemon et al., 1991). This clone produces readily visible plaques in foetal rhesus monkey kidney cells (FRhK-4). The HAV stock was titrated by plaque assay (Mullendore et al., 2001; each dilution in duplicate) with the virus titre, expressed as the average value of three independent experiments, being $1 \times 10^6$ PFU/mL. A stock of AsV serotype 4, with a titre of $1 \times 10^7$ infectious particles/mL, was kindly provided by Dr A. Bosch (University of Barcelona, Spain).

Sensitivity of the primer sets

To evaluate the sensitivity of the RT–PCR with the different primer sets (Table 1), serial dilutions of the HAV and AsV stocks were prepared in sterile distilled water and RNA was extracted by heat treatment (95°C, 5 min). Extracted RNA (10 µL) was then subjected to RT–PCR using the Superscript™ One-Step RT–PCR System (Life Technologies, Barcelona, Spain).

Table 1  Primers employed in this study for the detection of hepatitis A virus and astrovirus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Hepatitis A virus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAVp3</td>
<td>5'-GGAATGTCTCAGGTACCTTTCTTG-3'</td>
<td>Atmar et al., 1995</td>
</tr>
<tr>
<td>HAVp4</td>
<td>5'-GTTTTGCTCCTCTTATCATGCTATG-3'</td>
<td></td>
</tr>
<tr>
<td>HAV240</td>
<td>5'-GGAGAGCCTGGAAGAAAGA-3'</td>
<td>Bosch et al., 2001</td>
</tr>
<tr>
<td>HAV68</td>
<td>5'-TCACCGCCGTTTGCCCTAG-3'</td>
<td></td>
</tr>
<tr>
<td>HAV1</td>
<td>5'-TTGGAAAGTGCACCTTGCAAG-3'</td>
<td>Torrado et al., 2003</td>
</tr>
<tr>
<td>HAV2</td>
<td>5'-CTGACTACCTCAGAAGCAAAC-3'</td>
<td></td>
</tr>
<tr>
<td><strong>Astrovirus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>5'-CCTGCCCCGAGAAACAACCAAGC-3'</td>
<td>Pintó et al., 1996</td>
</tr>
<tr>
<td>A2</td>
<td>5'-GTAAGATTCCAGGGTGTGC-3'</td>
<td></td>
</tr>
<tr>
<td>AV15</td>
<td>5'-CTTTTCTGTCCTGTGTTAGTA-3'</td>
<td>Jonassen et al., 1995</td>
</tr>
<tr>
<td>AV12</td>
<td>5'-TTTTTTTTTTTTTTTTTTGTC-3'</td>
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RT–PCR amplifications of HAV RNA were performed using the primer pairs HAVp3/HAVp4 (Atmar et al., 1995) targeting the VP1 gene, HAV240/HAV68 (Bosch et al., 2001) which amplifies a fragment of 174 bp within the 5’ non-coding region, and HAV1/HAV2 (Torrado et al., 2003). RT–PCR conditions for primers HAV240/HAV68 and HAV1/HAV2 were fixed as previously described by Bosch et al. (2001) and Torrado et al. (2003) respectively. The program conditions utilised for the primers HAVp3/HAVp4 were those reported by Atmar et al. (1995), with minor modifications. Briefly, after an RT step (43°C, 1 h) and an initial heat denaturation (94°C, 5 min), 40 cycles of template denaturation (94°C, 1 min), primer annealing (49°C, 80 s) and primer extension (72°C, 50 s) were performed followed by a final extension (72°C, 15 min). RNA obtained from FRhK-4 cells infected with HAV strain HM-175 was employed as positive control in all the RT–PCR assays. Negative controls containing water instead of RNA extract were included in all reactions.

For AsV, two primer sets were evaluated, including primers A1/A2 (Pintó et al., 1996) which amplify a fragment corresponding to nucleotides 2363 to 2599 (OFR 1a) of AsV serotype 2 (L13745), and AV15/AV12 (Jonassen et al., 1995) yielding an amplicon of 77 bp within the ORF 2. For each primer pair, RT–PCR conditions described by the respective authors were employed.

In all cases, 10 µL of the RT–PCR products were analysed by electrophoresis on 1.2% (w/v) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA) electrophoresis buffer. Amplified products were visualised with a UV transilluminator after ethidium bromide staining.

Application to spiked shellfish samples
Mussels (Mytilus galloprovincialis), obtained from local markets, were shucked and the stomachs/hepatopancreas removed from the shellfish by dissection and kept on ice. Serial dilutions of the HAV or the AsV stocks were adsorbed to these mussel tissues (1 g) as previously described (Atmar et al., 1995). Contaminated tissues were subjected to the extraction protocol employing the Total Quick RNA Cells & Tissues kit (Talent, Trieste, Italy), which is based on the use of resin columns, following the manufacturer’s instructions. Extracted RNA (10 µL) was subjected to amplification as mentioned above for viral stocks. Amplified products were analysed by electrophoresis on agarose gels and visualised by ethidium bromide staining and UV transillumination.

Results and discussion
The fact that molluscan shellfish can serve as vectors of important viral human pathogens, including HAV and AsV, has led to a widely recognised need for improvement of the sanitary control measures of these marine products (Lees, 2000). Therefore, in recent years the development of RT–PCR techniques for rapid and reliable detection of these viral pathogens has become an important research goal (Yamashita et al., 1991; Le Guyader et al., 1994; Atmar et al., 1995; Abad et al., 1997; Marx et al., 1997; Croci et al., 1999; Kingsley and Richards, 2001; Mullendore et al., 2001; Yokoi et al., 2001). It is clear that the RT–PCR detection of HAV and AsV in shellfish is feasible and the most sensitive method available at present. However, the primer selection constitutes a critical step in order to obtain the required characteristics of sensitivity and specificity (Marx et al., 1997; Lees, 2000). This is of particular importance in cases of great viral strain heterogeneity (with the consequent nucleotide sequence variation), such as AsV.

Results obtained in this work showed great differences in the effectiveness of amplification in relation to the primer pair used. Thus, the best results for HAV detection, from both dilutions of the viral stock and seeded mussel tissues, were obtained using the primer
set HAV240/HAV68 with a sensitivity of 0.02–0.1 PFU/µL (Table 2; Figure 1). This sensitivity was higher by 1-log than that obtained with primer set HAVp3/HAVp4 (Table 2; Figure 1). On the other hand, no positive amplifications were obtained when primer set HAV1/HAV2 was utilised, which implied a detection limit >10^4 PFU/µL (Table 2). However, since this set of primers is usually employed in a nested-PCR protocol (Torrado et al., 2003), its use in a one-step RT–PCR procedure could have been the reason for lack of amplification products. On the other hand, the diverse primer pairs amplify different regions of the HAV genome, which may have some effect on their sensitivity.

Regarding the AsV detection, the best sensitivity was observed with the primer set A1/A2 (0.1–1 infectious particles/µL), showing a difference in sensitivity greater than 3-log in relation to set AV15/AV12 (Table 2; Figure 2). It should be pointed out that the latter primer pair was designed to amplify all the AsV serotypes (Jonassen et al., 1995). As in the case of HAV, sensitivity could be affected by the genome region amplified by each primer set. In fact, it has been reported by Willcocks et al. (1995) that the 3′ end of the ORF2 was more variable among different AsV strains than the 5′ end of ORF2 or the non-structural protein regions. In addition, the possibility of non-specific amplification has been reported for primers AV15/AV12 (Jonassen et al., 1995) as the primer AV12 is basically an oligo

**Table 2** Sensitivity achieved with the different primer sets evaluated in the amplification of HAV and AsV from viral crude extracts and seeded tissues

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sensitivity (range obtained from four independent experiments)</th>
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<tr>
<td></td>
<td>Viral extracts (PFU/µL)</td>
<td>Seed mussel tissue (PFU/g)</td>
</tr>
<tr>
<td><strong>Hepatitis A virus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAV240/HAV68</td>
<td>0.02–0.1</td>
<td>0.02–0.1</td>
</tr>
<tr>
<td>HAVp3/HAVp4</td>
<td>0.1–1</td>
<td>0.2–1</td>
</tr>
<tr>
<td>HAV1/HAV2</td>
<td>&gt;10^4</td>
<td>&gt;10^4</td>
</tr>
<tr>
<td><strong>Astrovirus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1/A2</td>
<td>0.1–1</td>
<td>0.1–1</td>
</tr>
<tr>
<td>AV15/AV12</td>
<td>&gt;10^3</td>
<td>&gt;10^3</td>
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</table>

**Figure 1** Sensitivity achieved for the amplification of HAV RNA using the primer sets HAV240/HAV68 (A) and HAVp3/HAVp4 (B) [Lanes: A, molecular size marker (PCR marker 50–2,000 bp, Sigma); B to I, amplification of RNA extracted from dilutions of the HAV strain at concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0.1 PFU/µL respectively; J, negative control (no RNA); K, positive control (HAV RNA from undiluted viral stock). Numbers on the left (size markers) and right (specific amplicon) are expressed in base pairs (bp)]
d(T). Therefore, this primer set not only possessed a lower sensitivity but also less specificity. Similar sensitivity results were obtained for both primer sets in the experiments employing dilutions of the AsV stock and in those using spiked mussel tissues (Table 2).

Conclusions
The results obtained in this work confirm, once again, that selection of primer sets to be employed for the routine detection of enteric viruses in monitoring laboratories is a critical point in the design of RT–PCR protocols. Primers have to be carefully selected for both sensitivity and specificity, and their use has to be standardised among laboratories, before recommendation for their inclusion in a routine detection method.

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References


