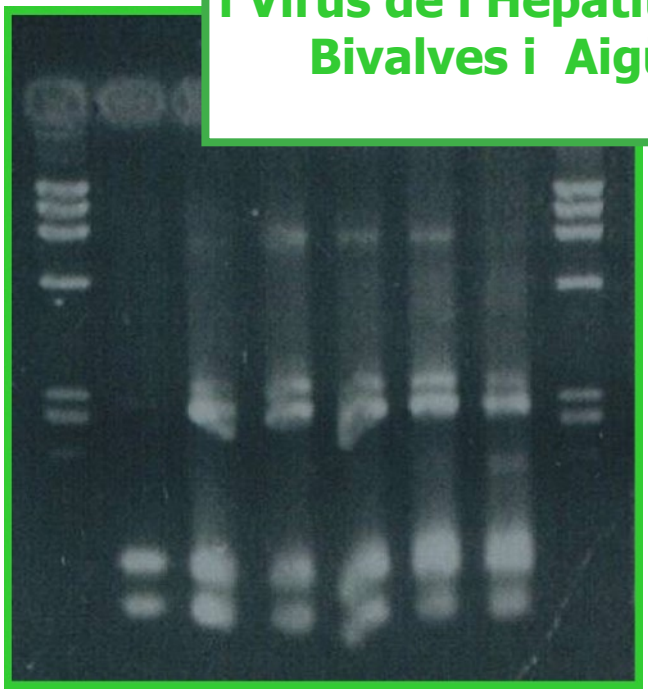
CAPÍTOL III. Desenvolupament d'una RT-PCR Multiplex per a la Detecció d'Adenovirus, Enterovirus i Virus de l'Hepatitis A en Mol·luscs Bivalves i Aigües Residuals



Nested Multiplex PCR Assay for Detection of Human Enteric Viruses in Shellfish and Sewage.

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RESUM DEL CAPÍTOL

La utilització de la PCR als laboratoris pot veure's limitada pel seu cost i de vegades, també per la disponibilitat d'un volum de mostra adequat per a realitzar el test. Aquestes limitacions poden superar-se amb la reacció en cadena de la polimerasa múltiple (PCR multiplex). La PCR multiplex és una variant de la PCR on s'amplifiquen dues o més seqüències diana en una mateixa reacció. Aquesta tècnica pot suposar un gran estalvi en temps i esforç al laboratori. Des de la seva primera descripció el 1988 per Chamberlain i col., s'ha aplicat amb èxit en diverses àrees d'anàlisi d'ADN, entre elles, la identificació de virus (Elnifro i col., 2000), bacteris (Hendolin i col., 1997) i paràsits (Harris i col., 1998).

L'objectiu del present capítol és desenvolupar una PCR multiplex que permeti la detecció d'adenovirus, enterovirus i virus de l'hepatitis A en mostres tant de mol·luscs bivalves com d'aigües residuals d'una forma ràpida, més econòmica i altament sensible. La tria d'aquests virus com a diana de la PCR multiplex es deu d'una banda, al potencial paper d'adenovirus i enterovirus com a models de contaminació vírica d'origen humà en mol·luscs bivalves i altres mostres ambientals i, d'altra banda, a la gran importància epidemiològica del virus de l'hepatitis A.

El desenvolupament de la PCR multiplex es dugué a terme en tres fases. Primer, s'establiren les concentracions de reactius i les temperatures d'amplificació òptimes per a la retro-transcripció, primera i segona PCR utilitzant suspensions control de les tres espècies de virus. Un cop establerts aquests paràmetres, es feren experiments de dopatge de musclos amb concentracions conegudes dels tres virus. Finalment, s'analitzaren mostres de mol·luscs bivalves i d'aigua residual.

S'observà que, de vegades, en presència d'elevades concentracions d'un dels tres virus (>10^4 equivalents genòmics) podia inhibir-se la detecció dels altres virus, sobretot en el cas dels virus RNA. Aquest problema fou en part superat a l'ajustar les concentracions dels iniciadors externs dels virus RNA. A més, es compararen els resultats obtinguts en les anàlisis de mostres naturals per PCR multiplex i per les PCR monoplex amb resultats satisfactoris.

Així doncs, les conclusions extretes en el present capítol són:

1. S'ha desenvolupat una PCR multiplex per a la detecció d'AdH, EV i VHA en mol·luscs bivalves i aigua residual, que redueix el temps i el cost de l'anàlisi, a més de mantenir una elevada sensibilitat.

2. La RT-PCR multiplex niada desenvolupada presenta un funcionament òptim quan les concentracions de virus són iguals o inferiors a 1000 equivalents genòmics o no difereixen més d'un logaritme, nivells observats pel nostre grup en mol·luscs bivalves en el Capítol I d'aquesta tesi.

Nested Multiplex PCR assay for detection of human enteric viruses in shellfish an sewage

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Abstract

We have developed a nested multiplex RT-PCR for the detection of adenovirus, enterovirus, and hepatitis A virus in different kinds of environmental samples (shellfish and sewage). This assay not only will save time and cost in the process for the detection of these enteric viruses, but also it will reduce the volume of sample tested, which can be a limiting factor in routine analysis. Additionally, our multiplex assay has been found to be highly sensitive in the several experiments performed. Thus, the limit of detection of this multiplex assay goes down to 1 genome equivalent for adenovirus and to 10 genome equivalents for enterovirus and hepatitis A virus. This multiplex PCR has been optimized for detecting all three viruses when present in levels equal or lower than 1,000 genome equivalents in shellfish and environmental samples, which from our experience are the most prevalent levels in the environment.

Key words: multiplex, adenovirus, enterovirus, hepatitis A virus, shellfish, sewage

1. Introduction

Viral pathogens are the most common cause of gastroenteritis in industrialized countries (Lopman et al., 2003). Enteric viruses, which are excreted in large numbers in feces even by asymptomatic carriers, can cause other outbreaks of illness such as hepatitis A. Food- (particularly, shellfish) and waterborne infections are of particular importance since these outbreaks may involve large number of people and wide geographical areas (Halliday et al., 1991; Sánchez et al., 2002).

The traditional detection of enteric viruses involves cell culture, which is expensive, labor-intensive and time-consuming. Moreover, there is a lack of efficient cell lines to isolate some of the epidemiologically most important enteric viruses such as hepatitis A virus (HAV), adenovirus 40 and 41 (Ad40, Ad41) and norovirus (NV). For these reasons, nucleic acid-based methods such as PCR and hybridization have been extensively applied. However, in routine laboratories the use of PCR is limited by cost and sometimes the availability of adequate test volume sample. To overcome these limitations and also to increase the detection capability of PCR, the multiplex PCR assay was developed. Since its first description by Chamberlain et al. (1998), multiplex PCR has been successfully applied mainly in clinical diagnostics (Elnifro et al., 2000). However, its application to the analysis of human viruses in environmental samples is, so far, quite limited (Rosenfield and Jaykus, 1999; Cho et al., 2000; Fout et al., 2003).
Enteroviruses have been used as target of PCR assay for the assessment of viral pollution, since they are well characterized for the nucleic acid-based methods, and have been shown to be abundant in sewage and shellfish (Kopecka et al., 1993; Puig et al., 1994; Pina et al., 1998; Formiga-Cruz et al., 2002). However, some reports have shown the lack of correlation between the presence of enterovirus and the presence of important pathogens such as hepatitis A virus in some environmental samples (Dubrou et al., 1991; Pina et al., 1998).

In the last years, the detection of human adenoviruses by PCR has attracted considerable attention in relation to the evaluation of viral quality of environmental samples, because the adenovirus genome is well characterized, adenovirus are more stable in various environments and more resistant to some disinfection treatments (UV, chlorine) than other enteric viruses (Gerba et al., 2002, 2003; Thurston-Enriquez et al., 2003a, 2003b), and finally, are the most prevalent human viruses detected by PCR in sewage and shellfish (Puig et al., 1994; Pina et al., 1998; Vantarakis et al., 1998; Hernroth et al., 2002; Formiga-Cruz et al., 2002). The detection of human adenoviruses has been proposed as a molecular index of viral contamination of human origin (Pina et al., 1998).

On the other hand, HAV is one of the most important pathogenic viruses in water and shellfish. HAV can be transmitted from person to person, or indirectly via food, water, or fomites contaminated with virus-containing feces or vomit. The burden of hepatitis A may increase following hygienic control measures, due to a decreasing percentage in the population of naturally immune individuals and a concurrent increase in the population at risk. Unfortunately, little information is available on preventive measures specific for HAV (Koopmans et al., 2002).

Therefore, the simultaneous detection of adenovirus (Ad), enterovirus (EV), and hepatitis A virus (HAV) could improve the feasibility of the control of viral contamination

in shellfish and water. Hence, the aim of this study is to describe the development and application of a nested multiplex RT-PCR, which provides a highly sensitive, rapid and cost-reduced way for the detection of Ad, EV and HAV.

2. Materials and Methods

2.1. Human virus suspensions

Adenovirus type 41 (Ad41) Tak prototype strain (ATCC VR-930) was cultivated on A549 cells. Cells were grown in 75-cm^2 plastic flasks in Dulbecco's minimum essential medium (D-MEM) supplemented with 2% fetal calf serum. The Ad41 suspensions were quantified by real-time Taqman PCR as described elsewhere (Formiga-Cruz et al., 2002). For enterovirus, a patient strain representing coxsackievirus type B5 was inoculated on Green Monkey Kidney (GMK) cells under the same conditions as the adenovirus strains.

Hepatitis A virus vaccine strain HM175 was inoculated on Vero cells. These cells were grown in D-MEM supplemented with 10% fetal bovine serum, 20U/ml of penicillin and 20µg/ml of streptomycin. During this infection, no cytopathic effect (CPE) was detected. Approximately 15-20 days post infection, HAV was harvested and the infected cell suspensions were freeze-thawed 4-5 times to release the virus particles. All viral dilution suspensions were divided into 60 µl batches to be used only once, since repeated freeze-thawing can reduce viral content up to 50-80 % (Formiga-Cruz et al., 2002).

2.2. Samples

2.2.1. Shellfish samples

Two sets of commercial mussel samples (*Mytilus galloprovincialis*) were used in experiments to determine the multiplex PCR sensitivity. These mussels were kept at –80ºC before being processed. Additionally, we analyzed the natural viral pollution in three cockle samples (*Tapes decussatus*) from an outbreak of hepatitis A occurred in Spain and, three commercial samples of stripped venus [*Venus (Chamelea) gallina*], and three more mussel samples. After harvest, shellfish were shipped directly to the laboratory via cold storage and processed within a 24-hour period.

Shellfish were washed, scrubbed under clean running water, and opened with a sterile shucking knife. Cockles and stripped venus flesh and liquor, and mussels digestive glands were collected into a sterile beaker and diluted with glycine buffer 0.25 N at pH 10 (1:5, w/v) according to the method previously described (Pina et al., 1998; Muniain-Mujika et al., 2000; Formiga-Cruz et al., 2002). The mixture was homogenized by magnetic stirring for 15 min. Once the pH was adjusted to 7±0.2, the treated homogenate was clarified by centrifugation at 2, 170 × *g* for 15 min at 4ºC. The supernatant was centrifuged at 39, 800 × *g* for 45 min at 4ºC. To pellet all viral particles, the supernatant was ultracentrifuged at 100,000 × *g* for 1 hour at 4ºC. The final pellet was resuspended in 200-400 µl PBS with a maximum volume of viral concentrate of 500 µl. The viral concentrate was stored at −80ºC prior to nucleic acid extraction.

2.2.2. Sewage samples

Ten independent raw domestic sewage samples collected from September 2002 to August 2003 in the sewers of Barcelona (Spain) were tested for the multiplex assay developed in this study. This water treatment plant receives 670,000 m^3/day of waste products from approximately 1.8 million inhabitants. Each sample was collected in a sterile 500-mL polyethylene container, kept at 4ºC for <8h until processed. Forty-two ml of each sample were ultracentrifuged at 100, 000 × *g* at 4ºC for 1 h to form pellets of all viral particles with any suspended material. The viruses retained in the pellet were eluted by mixing it with 3.5 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min and then 3.5 ml of PBS 2× (Phosphate Buffer Saline, double concentration) were added and the suspended solids separated by centrifugation at 12, 000 × *g* for 15 min. The virus suspensions obtained were ultracentrifuged at 100, 000 × *g* for 1 h at 4ºC to pellet all viral particles, which were resuspended in 100 µl of PBS and kept at -80ºC until processing for nucleic acid extraction and PCR detection.

2.3. Nucleic acid extraction

Nucleic acids were extracted by the method described by Boom et al. (1990) with minor modifications using guanidinium thiocyanate as the principal component of the lysis buffer and the adsorption of nucleic acids to silica particles. Briefly, 50 µl of viral concentrate were added to a mixture of 50 µl of silica particle suspension and 900 µl of lysis buffer. The mixture was incubated at room temperature for 10 min and washed twice in 1 ml of washing buffer (12 g of guanidine thiocyanate in 10 ml of Tris-EDTA), twice more in cold ethanol 70% and once in acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 50 µl of elution buffer [49.4 µl of dithiothreitol 1mM (DTT) and 12 U of RNase inhibitor, Applied Biosystems]. The extracted nucleic acids were then used for nested multiplex RT-PCR of Ad, EV and HAV, and each individual nested (RT)-PCR.

2.4. Oligonucletotide primers

The sequence, specificity, and sensitivity of the oligonucleotides primers used were described in previous studies (Puig et al., 1994; Pina et al., 1998, Formiga-Cruz et al., 2002). In Table 1, a summary of all primers characteristics is presented. All oligonucleotide primers

have been tested for primer-dimer formation using the PubMed NCBI Blast software.

2.5. Monoplex nested (RT)-PCR amplification

The amplification conditions of the nested monoplex (RT)-PCR methods used for detecting enterovirus, adenovirus and hepatitis A virus have been described elsewhere (Pina et al., 1998). Briefly, cDNA synthesis was performed with 5 µl of the extracted nucleic acids in a final 10-µl mixture of 10 mM Tris-HCl (pH 8.3 at 25 ºC), 0.6 µl $MgCl_2$ 25 mM, 200 µM of each deoxynucleotide triphosphate (Genotek) and 20 µM of the corresponding reverse primer. The reaction mixture was incubated at 95ºC for 5 min before the addition of 50 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems), 10 U of RNase inhibitor (Applied Biosystems) and 0.5 µl of DTT 0.2M. Reverse transcription (RT) was performed at 42ºC for 30 min, followed by 5 min of reaction termination at 95ºC. The tubes were then chilled on ice, and 10 µl of the RT mixture was added to 40µl of PCR mixture in a final concentration of 10 mM Tris-HCl (pH 8.3 at 25ºC), 1.5 mM of MgCl2, 0.4 µM (each) reverse and forward primers, and 2 U of Ampli Taq polymerase (Applied Biosystems). Ten of silica extracted nucleic acids were directly used for PCR amplification of Ad DNA under the conditions already described for PCR. In the PCR assays for Ad, HAV, and EV, the first cycle of denaturation was carried out for 2 min at 95ºC. The conditions during the 30 cycles of the amplification were denaturing at 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min. For the nested PCR amplification, 1 µl of the amplified DNA from the first PCR reaction was added to a new batch of 50 µl of PCR reaction mix containing 9 µM of each inner primer. The amplification cycles were as described before.

2.6. Multiplex nested RT-PCR amplification

The optimal conditions to perform the multiplex assay were determined after several trials (data not shown). Initially, equimolar concentrations of each primer were assayed. However, it was necessary to change empirically the proportions of various primers to obtain the best amplification results. Regarding the thermocycling temperatures, although all the viral genomes can be specifically amplified at 55ºC by monoplex PCR, our experience showed that lowering the annealing temperature to 51ºC in first-round PCR and to 53ºC in nested-PCR was required for the same genomes to be coamplified in the multiplex mixture. In addition, the extension temperature was also lowered to 68ºC. Finally, to improve the retrotranscription of the RNA viruses, temperature was lowered to 40ºC.

Therefore, the reaction mixture for reverse transcription contained 5 µl of the extracted nucleic acids, 1µl PCR Gold Buffer 10× (Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3 at 25 ºC), 0.6 µl $MgCl_2$ 25 mM, 200 µM of each deoxynucleotide triphosphate (Genotek), 25 µM of the external right primer for HAV and 20 µM of the right primer for EV. The reaction mixture was incubated at 95ºC for 5 min before the addition of 50 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems),

Table 1
Oligonucleotide primers used for PCR amplification of human adenovirus, enterovirus and hepatitis A virus

Virus type (region)[a]	Position	Reaction	Primer	Product size (bp)	Sequence
Ad 40 (hexon)	18858-18883[b]	First left	hexAA1885	301	5'-GCCGCAGTGGTCTTACATGCACATC-3'
Ad 41 (hexon)	19136-19158[b]	First right	hexAA1913		5'-CAGCACGCCGCGGATGTCAAAGT-3'
Ad 2 (hexon)	18937-18960[b]	Nested left	nehexAA1893	143	5'-GCCACCGAGACGTACTTCAGCCTG-3'
Ad 2 (hexon)	19051-19079[b]	Nested right	nehexAA1905		5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'
CV B4 (5'NTR)	64-83[c]	First left	Ent 1[d]	540	5'-CGGTACCTTTGTACGCCTGT-3'
Polio 1 (5'NTR)	578-597[c]	First right	Ent 2		5'-ATTGTCACCATAAGCAGCCA-3'
Polio 1 (5'NTR)	430-450[c]	Nested left	neEnt 1	123	5'- TCCGGCCCCTGAATGCGGCTA-3'
CV B4 (5'NTR)	547-567[c]	Nested right	neEnt 2		5'-GAAACACGGACACCCAAAGTA-3'
HAV (5'NTR)	332-352	First left	HAV 1	368	5'-TTGGAACGTCACCTTGCAGTG-3'
HAV (5'NTR)	680-700	First right	HAV 2		5'-CTGAGTACCTCAGAGGCAAAC-3'
HAV (5'NTR)	371-391	Nested left	neHAV1	290	5'-ATCTCTTTGATCTTCCACAAG-3'
HAV (5'NTR)	641-661	Nested right	neHAV2		5'-GAACAGTCCAGCTGTCAATGG-3'

[a] Ad, adenovirus; CV, coxsackievirus; HAV hepatitis A virus; 5'NTR: 5' non-translated region
[b] Sequence position refers to the Ad2 hexon region
[c] Sequence position refers to the coxsackievirus B4 5' NTR
[d] Modified from Gow et al. (1991)

10 U of RNase inhibitor (Applied Biosystems) and 0.5 µl of DTT 0.2M. Temperature was cycled as follows: 45 min at 40ºC and 5 min at 95ºC.

We performed all PCR reactions with AmpliTaqGold polymerase (Applied Biosystems), which is activated only after heating at 95ºC for 10 min the reaction mixture in order to eliminate potential nonspecific reactions (i.e. primer-dimers formation). Thus, the first PCR amplification was carried out in 50 µl of reaction mixture with 4 µl of PCR Gold Buffer 10× (Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3 at 25ºC), 1.5 mM of MgCl2, 25 µM of primer left for HAV (HAV1), and 20 µM of external primers for EV (EV1) and Ad (hexAA1913/ hexAA1885), and 2,5 U of Ampli Taq Gold polymerase (Applied Biosystems). The first cycle of denaturation was carried out for 10 min at 95ºC. The thermocycling conditions were: denaturing at 94ºC for 1 min, annealing at 51ºC for 1 min, and extension at 68ºC for 1 min (30 cycles).

For the nested PCR amplification, 1 µl of the amplified DNA from the first PCR reaction was added to a new batch of 50 µl of PCR reaction mix containing 9 µM of each inner primer (nHAV1 / nHAV2 for HAV, nEnt1 / nEnt2 for EV and nehexAA1893/ nehexAA1905 for Ad). After 10 min heating at 95ºC, the amplification 30 cycles were as follows: 94ºC 1 min, 53ºC 1 min and 68ºC 1 min. Thermal cycling was carried out in a programmable heat block (Gene Amp PCR System 2400, Applied Biosystems). The results were analyzed by gel electrophoresis on a 3% agarose gel and stained with ethidium bromide.

Standard precautions were applied in all the manipulations to reduce the probability of samples contamination. Separate areas were used for reagents, treatment of samples, and manipulation of amplified samples. Undiluted samples and 10-fold dilutions of nucleic acid extracts were analyzed twice in independent experiments.

2.7. Sequencing

Nested HAV, EV and Ad amplicons were sequenced to control the presence of potential false positive results and to evaluate the variability of the detected hepatitis A virus and enterovirus strains. Enterovirus typing was performed using the degenerate primers described by Casas et al. (2001) that allow the amplification of VP1 region.

Briefly, nested PCR-products were purified by using the Cleanmix Extraction Kit protocol according to the manufacturer's instructions (Genotek). Both strands of the purified DNA were sequenced with the ABI Prism Big Dye Terminator cycle sequencing kit 2.0 (Applied Biosystems) according to the manufacturer's instructions. The results were checked with the ABI Prism 3700 DNA analyzer (Applied Biosystems). The obtained sequences were compared with all sequences of the GenBank and EMBL with the PubMed NCBI BLAST program.

2.8. Evaluation of the sensitivity of detection

Ten-fold dilution series of viral standards were analyzed in limiting-dilution experiments to establish and to compare the sensitivity of the developed nested multiplex RT-PCR with that of each virus nested (RT)-PCR amplification previously described.

Ten grams of digestive gland from mussel commercial samples were supplemented with 10^4 genome equivalents (GE) of each viral specie in order to evaluate the sensitivity of the multiplex PCR applied to shellfish samples.

3. Results

3.1. Sensitivity

The sensitivity of each monoplex assay has been analyzed in previous studies (Allard et al., 1992; Puig et al., 1994; Pina et al., 1998; Formiga-Cruz et al., 2002). Briefly, the detection limit for human adenoviruses and enteroviruses goes from 1 to 10 viral particles, both in sewage and shellfish samples. For hepatitis A virus, a limit of 1 to 100 genomic equivalents has been estimated. Figure 1 shows the level of sensitivity obtained by the nested multiplex PCR assay developed in this study, which is within the limits of detection of the monoplex reactions for each virus. Thus, the multiplex PCR developed detects down to 1 genome equivalent (GE) of Ad, and 10 GE for both EV and HAV.

To asses if the sensitivity of the multiplex assay was maintained in presence of varying concentrations of each virus, we mixed different concentrations of each viral specie and performed the nested multiplex RT-PCR assay (Fig. 2). Detection of RNA viruses was affected when virus concentrations differed in 2 or more logarithms (Fig. 2a). In that case, some of the viral species present in lower levels were sometimes not detected. However, detection of Ad (a DNA virus) was not altered whatever their levels were. All viral species were detected when virus concentration only differed in 1 logarithm (Fig. 2b).

Figure 3 shows the sensitivity results obtained for inoculated samples. The detection limits of the multiplex assay were similar to the previous described sensitivities for monoplex PCR. Hence, whereas Ad and EV were detected down to 1-10 GE, HAV was detected to 1-100 GE.

3.2. Analysis of shellfish by Multiplex PCR

The results of the analysis of cockle samples associated to a HAV outbreak obtained by individual nested (RT)-PCR assays were the same as the results obtained by the nested multiplex method developed. Therefore, two of the samples were only positive for HAV, and the other one was positive for both EV and HAV. The sequence of the EV amplicon

Fig.1.
Limit-dilution experiments to assess the level of sensitivity of the multiplex assay developed. *M:* molecular weight standard marker X174 *Hae*III digest. *Lane 1:* 10^3 genome equivalents (GE) of Ad, 10^4 GE EV, and 10^4 GE HAV. *Lane 2:* 10^2 GE Ad, 10^3 GE EV, and 10^3 GE HAV. *Lane 3:* 10 GE Ad, 10^2 GE EV, and 10^2 GE HAV. *Lane 4:* 1 GE Ad, 10 GE EV, and 10 GE HAV. *Lane 5:* 1 GE EV and 1 GE HAV.

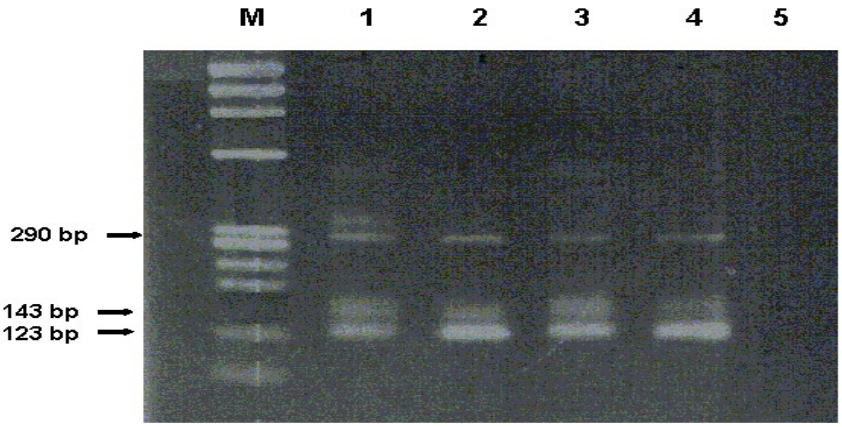


Fig.2.
Effect of varying concentrations of Ad, EV, and HAV in the level of sensitivity of the multiplex assay developed. 2a) Effect of concentrations varying 2 decimal logarithms. *M:* molecular weight standard marker X174 *Hae*III digest. *Lane 1:* 10^3 genome equivalents (GE) of Ad, 10 GE EV, 10 GE HAV. *Lane 2:* 10^3 GE Ad, 10 GE EV, 10^3 GE HAV. *Lane 3:* 10 GE Ad, 10 GE EV, 10^3 GE HAV. *Lane 4:* 10 GE Ad, 10^3 GE EV, 10^3 GE HAV. *Lane 5:* 10 GE Ad, 10^3 GE EV, 10 GE HAV. *Lane 6:* 10^3 GE Ad, 10^3 GE EV, 10 GE HAV. 2b) Effect of concentrations varying 1 decimal logarithm. *M:* molecular weight standard marker X174 *Hae*III digest. *Lane 1:* 10^3 GE Ad, 10^2 GE EV, and 10^2 GE HAV. *Lane 2:* 10^3 GE Ad, 10^2 GE EV, and 10^3 GE HAV. *Lane 3:* 10^2 GE Ad, 10^2 GE EV, and 10^3 GE HAV. *Lane 4:* 10^2 GE Ad, 10^3 GE EV and 10^3 GE HAV. *Lane 5:* 10^2 GE Ad, 10^3 GE EV, and 10^2 GE HAV. *Lane 6:* 10^3 GE Ad, 10^3 GE EV, and 10^2 GE HAV.

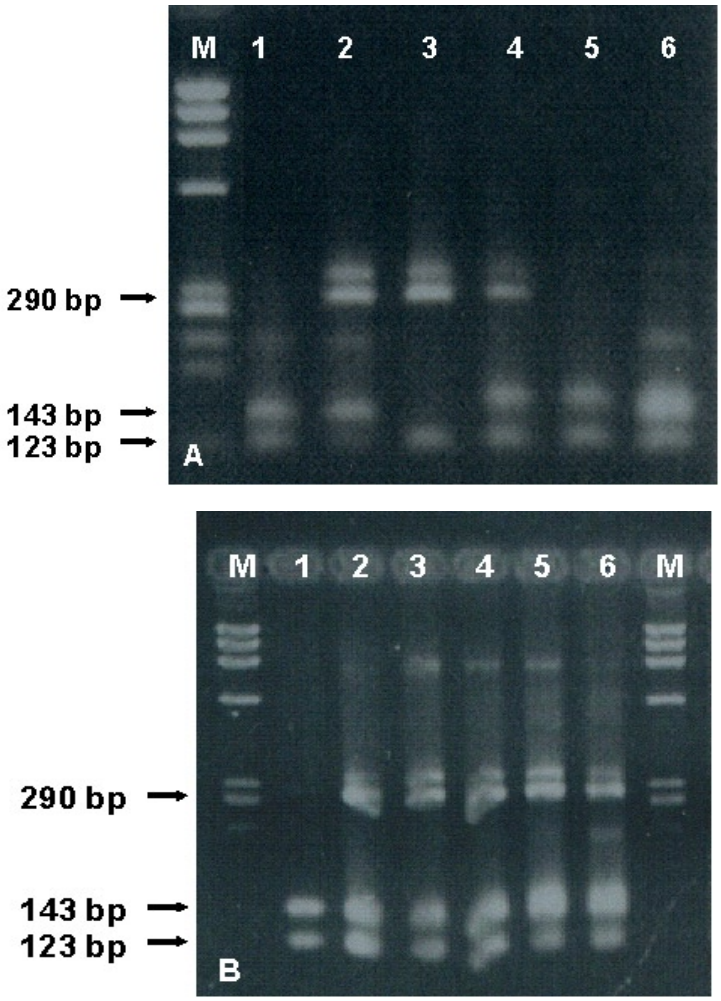
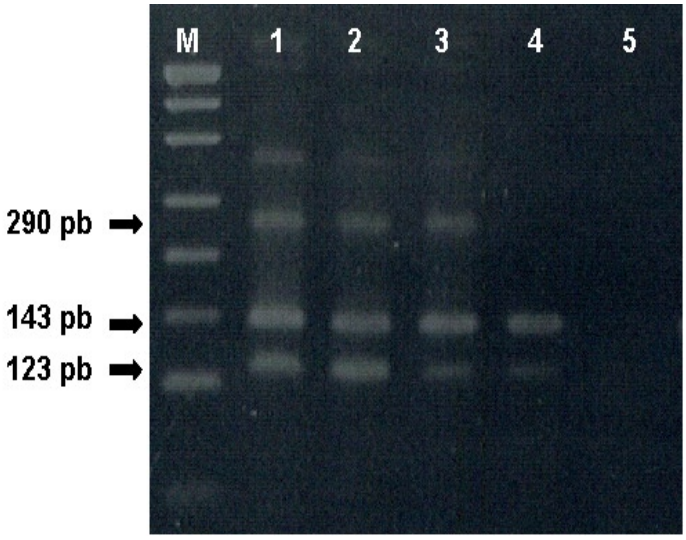


Fig.3.
Detection of inoculated viruses in mussel samples. *M:* molecular weight standard marker X174 *Hae*III digest. *Lane 1:* 10^4 genome equivalents (GE) of Ad, 10^4 GE EV, 10^4 GE HAV. *Lane 2:* 10^3 GE Ad, 10^3 GE EV, 10^3 GE HAV. *Lane 3:* 10^2 GE Ad, 10^2 GE EV, 10^2 GE HAV. *Lane 4:* 10 GE Ad, 10 GE EV, 10 GE HAV. *Lane 5:* 1 GE Ad, 1 GE EV, 1 GE HAV.



was found to be identical to Poliovirus 1 Sabin strain except for 1 nucleotide. Regarding the hepatitis A virus-positive cockle samples, sequence analysis of the nested-PCR amplicon from the 5'NTR showed a high degree of identity (100%) between them.

Concerning the commercial samples of shellfish, one of the mussel samples was positive for adenovirus and another one for enterovirus. Finally, all stripped venus samples were negative for Ad, EV, and HAV.

3.3. Analysis of urban sewage samples by Multiplex PCR

We summarized the results of the coamplification of sewage samples in Table 2. Briefly, 90% of the samples were positive for Ad (9/10), 40% for EV (4/10) and 20% for HAV (2/10). These percentages are very similar to that obtained in previous studies (Pina et al., 1998) when analysis of sewage samples was performed by monoplex PCR assays (88% of positive samples for Ad, 33% for EV, 22% for HAV). To confirm the obtained results, some amplicons were sequenced. In addition, the monoplex amplification of HAV of some samples confirmed the two positives detected by multiplex PCR. The two enterovirus strains detected were classified as echovirus 14 by typing the hipervariable region VP1.

Table 2
Detection od adenovirus, enterovirus, and hepatitis A virus in sewage samples by nested multiplex RT-PCR

	Virus analyzed by multiplex PCR		
Sample	Ad	EV	HAV
SA 110610	-	-	-
SA 160103	+	+	-
SA 140303	+	+	-
SA 030703	+	-	-
SA 010803	+	-	-
SA 210802	+	+	-
SA 270902	+	+	-
SA 051102	+	-	+
SA 300503	+	-	-
SA 300803	+	-	+

4. Discussion

Multiplex PCR is the first step towards PCR automatization in routine laboratory analysis, which will reduce time and cost without affecting the effectiveness of the assay. For that purpose, we have developed a nested multiplex RT-PCR for the detection of adenovirus, enterovirus, and hepatitis A virus in shellfish and sewage samples. This assay not only will save time and cost in the process for the detection of these enteric viruses, but also it will reduce the volume of sample tested, which can be a limiting factor in routine analysis. Additionally, our multiplex assay has been found to be highly sensitive in the several experiments performed. Thus, the limit of detection of this multiplex assay goes down to 1 genome equivalent for adenovirus and to 10 genome equivalents for enterovirus and hepatitis A virus. This multiplex PCR has been optimized for detecting all three viruses when present in levels equal or lower than 1,000 genome equivalents in shellfish and environmental samples, which from our experience are the most prevalent in the environment (Formiga-Cruz et al., 2002). After analyzing different

combinations of each virus concentration, the optimal detection of the three viruses was found when the levels of each viral specie do not differ more than 1 logarithm. However, the detection ratio of Ad, EV, and HAV obtained by multiplex PCR was very similar to previously described proportions between these three enteric viruses and that had been obtained by monoplex PCR assays.

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ANNEX I. Desenvolupament i optimització de sistemes de PCR multiplex

En el present annex es revisen les bases pel desenvolupament i l'optimització de sistemes de PCR multiplex:

Les primeres etapes dels cicles tèrmics tenen un efecte substancial en la sensibilitat i especificitat generals de la PCR. Si s'assumeix una desnaturalització eficient de la diana, l'èxit d'una amplificació específica depèn de la taxa a la qual els iniciadors un cop hibridats s'extenen al llarg de la seqüència desitjada durant els diferent cicles d'amplificació (Elnifro i col., 2000). Entre els factors que poden provocar que les taxes d'anellament no siguin òptimes trobem iniciadors mal dissenyats i constituents del tampó de reacció i temperatura d'anellament subòptims. La taxa d'elongació depèn de l'activitat de l'enzim, de la disponibilitat de components essencials com els deoxinucleòsids trifosfat (dNTPs) i de la naturalesa de l'ADN diana. Tot això provoca que la majoria de modificacions de cara a la millora d'una reacció de PCR multiplex vagin dirigits als factors que afectin les taxes d'hibridació i elongació.

L'optimització de les PCR multiplex pot presentar certes dificultats com baixes sensibilitat o especificitat i/o l'amplificació preferent de certes dianes en detriment d'altres. L'amplificació preferent d'una seqüència diana envers altres (biaix en la relació motlle/producte) és un fenomen conegut en les PCR multiplex. Hi ha dues classes principals de processos que indueixen aquests biaix, la deriva de la PCR (*PCR drift*) i la selecció de la PCR (*PCR selection*). La deriva de la PCR es produiria per la fluctuació estocàstica en les interaccions dels reactius de la PCR en els cicles primerencs, fet que s'incrementaria en presència de baixes concentracions de la cadena motlle; variacions en els perfils termals d'un termociclador que resultarien en temperatures de connexió desiguals; o per simple error experimental. La selecció de PCR es defineix com un mecanisme que afavoreix de forma inherent l'amplificació de certes cadenes motlle degut a certes característiques de la diana, les seqüències que la flanquegen o el genoma sencer que conté la diana. Aquestes característiques inclouen diferències en el contingut GC que comportaria una desnaturalització preferencial; major eficiència d'unió degut a iniciadors rics en GC; accessibilitat diferencial a les dianes dins els genomes a causa d'estructures secundàries; i al número de còpies del gen dins un genoma (Elnifro i col., 2000).

Una bona solució a aquests problemes en el desenvolupament de la PCR multiplex és l'utilització d'una PCR "hot start" i/o d'una PCR imbricada. L'ús d'un "hot start" (10 minuts a alta temperatura abans de l'inici de la PCR) serveix per a eliminar reaccions inespecífiques (concretament l'aparició de dímers entre els iniciadors) que es produeixen per l'anellament dels iniciadors a baixes temperatures (4-25ºC) abans de l'inici dels diferents cicles (Chou i col., 1992). Amb aquesta finalitat es pot utilitzar una polimerasa com l'*AmpliTaqGold* (Applied Biosystems) que s'activa quan se sotmet a una temperatura de 94ºC durant 10 minuts. La PCR imbricada (*nested-PCR*), tal i com s'ha comentat en la Introducció, incrementa la sensibilitat i l'especificitat del test. Possibles inconvenients inclouen l'augment de probabilitat de contaminació creuada i la dificultat d'automatització.

D'altra banda, cal tenir en compte que a mesura que s'amplifiquen més fragments en una PCR multiplex, la quantitat d'enzim i nucleòtids esdevé un factor limitant i cal més temps per tal que la polimerasa completi la síntesi de tots els productes. A la pràctica, això comporta que a l'augmentar el temps d'elongació s'incrementi la quantitat del producte més llarg.

Sovint cal optimitzar els diferents components de la reacció per obtenir un millor rendiment de la PCR múltiple (Henegariu i col., 1997; Elnifro i col., 2000; Markoulatos i col., 2002):

• Iniciadors: Les seqüències dels iniciadors han de presentar un contingut en GC del 30 al 60%, entre 18 i 24 parells de bases i eficiències d'amplificació similars.

• Nucleòtids: Els estocs de nucleòtids són sensibles als cicles de congelació/descongelació; després de 3 o 5 cicles, les PCRs multiplex no funcionen bé. La baixa estabilitat dels nucleòtids no és tan òbvia a les PCR monoplex o clàssiques.

• Clorur de magnesi: L'optimització del Mg^{2+} és crítica ja que la polimerasa és un enzim magnesi-depenent. A més a més, l'ADN i els dNTPs s'uneixen al Mg^{2+}. Així doncs, la concentració òptima de Mg^{2+} ve donada per la concentració de dNTPs, d'ADN motlle i de la composició del tampó. A la pràctica, només cal mantenir una proporció constant amb la concentració de dNTPs.

• Concentració del tampó de reacció: Per a incrementar l'eficàcia de la reacció s'acostuma a augmentar la concentració del tampó a 2x, no obstant s'ha de tenir en compte que els iniciadors amb productes d'amplificació llargs treballen millor a baixes concentracions de sals i que els iniciadors amb productes curts a altes concentracions.

• ADN motlle: Per baixes quantitats de motlle, la disminució de la temperatura d'anellament pot millorar l'amplificació.

• Polimerasa: Empíricament s'ha determinat que la concentració òptima és de 2,5 U de polimerasa en un volum final de 50 µl. Una quantitat excessiva d'enzim provoca una amplificació desequilibrada de les diverses dianes i l'augment del soroll de fons, segurament a causa de l'alta concentració de glicerol de l'estoc.

• Adjuvants: l'albúmina sèrica bovina (BSA) funciona millor que altres adjuvants com el DMSO o el glicerol.

En la Taula I.1 es recullen les concentracions òptimes de cadascun dels components de la reacció.

Taula I.1. Optimització dels components d'una reacció de PCR multiplex.

Components	**Quantitat**
Iniciadors	concentracions equimolars 0,1-0,5 µM, però si: - ADN de baix nº de còpies o alta complexitat: 0,3-0,5 µM - ADN d'alt nº de còpies o baixa complexitat: 0,04-0,4 µM
Nucleòtids	200-400 µM de cada nucleòtid
$MgCl_2$	1,5-2 mM de $MgCl_2$ per 200-400 µM de nucleòtids
Tampó	concentració òptima a 2x, però: - producte d'amplificació llarg: baixa concentració de sals - producte d'amplificació curt: alta concentració de sals
Polimerasa	2,5 U en un volum de reacció de 50 µl
ADN motlle	davant baixes quantitats d'ADN motlle: disminuir temperatura d'anellament
Adjuvants	0,8 µg/ µl de BSA