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Evolutionary pattern of 5′-UTR of enteroviruses and primer update for the detection of enteroviral RNA in environmental samples

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ABSTRACT

Objective: To study the recombination events among enterovirus strains and the development of specific primers for the detection of enteroviruses in environmental samples. **Methods:** Nucleotide sequence analysis of enteroviruses deposited in the international database GenBank (www.ncbi.nlm.nih.gov/Genbank) was conducted to develop specific primers for the detection of these viruses. The specificity and sensitivity of the method were tested using coxackievirus B3 strain Nancy, environmental isolate of human hepatitis A virus and human rotavirus strain WA. Seventy sewage samples were analyzed. **Results:** Enterovirus genome was detected in all positive samples. The genome of enterovirus was not detected in negative samples. The level of detection of these viruses was 10^2 $TCID_{50}$/mL. **Conclusions:** The development of new primers is an important issue for the detection of enteroviruses in the environment and the assessment of risk factors to human health.

1. Introduction

Various enterovirus serotypes are spreading globally by temporal and regional factors. Widely distributed throughout the year in tropical and semitropical regions, they are prominent in summer and fall, and far less detected in winter and spring months[1].

Several studies have revealed peaks in both human enteric virus infections and excretion in summer and early fall in temperate climates, which also coincide with increased water recreational activities and human-water contact[2-5]. In tropical climates, human enteric viruses, especially enteroviruses, are isolated throughout the year and in some cases are more prevalent during rainy seasons[6].

The family of Picornaviridae is consisted of 12 genera: *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus*, and 5 additional genera as approved by the International Comity of the Taxonomy of Viruses in 2009 (-ICTV-): *Teschovirus*, *Sapelovirus*, *Senecavirus*, *Tremovirus* et *Avihepatovirus*. The genus *Enterovirus* consists of 10 species: *Human enterovirus* A (22 serotypes), *Human enterovirus* B (60 serotypes), *Human enterovirus* C (20 serotypes), *Human enterovirus* D (4 serotypes), *Simian enterovirus* A (1 serotype)[7-12], *Bovine enterovirus* (at least 2 serotypes)[13], *Porcine enterovirus* B (2 serotypes), *Human rhinovirus* A (75 serotypes), *Human rhinovirus* B (25 serotypes) and *Human rhinovirus* C (48 types)[14,15].

However, the presented classification is far to be final and other propositions are under discussion to re-class these serotypes as well as the new detected types (http://www.ictvonline.org).

Several environmental factors are known to influence the epidemiology of enteroviruses. In tropical climates, the circulation of enteroviruses is more or less constant around the year, whereas in temperate climates, infections are typical for late summer and fall[16].

Picornavirus RNA-dependent RNA polymerases are highly error prone and lack proofreading ability, resulting in a misincorporation frequency of 1 per 10^3 to 10^4 nucleotides[17]. The relative infidelity of these polymerases is believed to enable rapid adaptability under selective pressure. Large-

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impact evolutionary events, such as recombination within and between enterovirus serotypes, also contribute to their evolution and genetic diversity[18-21] and may lead to changes in disease associations with human enterovirus infections.

The introduction of newly developed molecular techniques significantly improved the procedure to detect enteroviruses in clinical and environmental samples. Reverse transcription followed by PCR has several advantages for the detection of viruses and the establishment of a viral monitoring program if compared to the cell culture method. The advantages include: rapid results, viral detection without cultivation, it is less labour intense and less expensive than cell culture propagation. The use of PCR to detect enteroviruses has been simplified by the existence of highly conserved areas of nucleotide sequences within the 5′-UTR of the genome. This has allowed the design of primers capable of detecting the majority of human enteroviruses[22-26].

However, several findings have described enterovirus genomes with a coding region that clusters with one species and a 5′-untranslated region (5′-UTR) that clusters with a different species, suggesting possible interspecies recombination events[27,28]. In addition, several studies have reported the detection of new enterovirus EV-109 with recombination in the 5′-UTR[29] and the detection of a new enterovirus genotype EV-104, undetectable by conventional methods, that has a divergent 5′-UTR[30]. Hence, the development of new primers taking into account these phenomena of recombination within the 5′-UTR of enterovirus genomes is crucial to cover all viruses belonging to genus enterovirus.

The main objective of the present study is to carry out nucleotide sequence analysis of enteroviruses available in the international GenBank in order to design new primers for the detection of enteroviruses in environmental samples.

2. Materials and methods

2.1. Viruses

Five strains were used in this study: coxakievirus B3 Nancy strain, echovirus 6, and echovirus 11. In addition, group A rotavirus WA strain (kindly provided by Pr Buesa Javier, Department of Microbiology, University of Valencia, Spain) was used in this study.

2.2. Cell culture

Vero cells (African green monkey kidney cell line) were used for culturing enterovirus strains. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco, Tunisia), 2 mM L-glutamin, and 1% of antibiotics PCS (10 000 IU Penicillin mL^{-1}, 25 000 IU colimycin mL^{-1}, 10 mg Streptomycin mL^{-1}, Sigma). MA104 cells were used for culturing rotavirus WA strain. The cells were grown in DMEM supplemented with 5% FBS, 2 mM L-glutamin, and 1% of antibiotics PS (10 000 U penicillin mL^{-1}, 10 mg streptomycin mL^{-1}, Sigma, Tunisia). All the cells were cultured at 37 ℃ in a humidified atmosphere supplied with 5% CO_2.

At confluence, Vero cell lines were infected with enteroviral strains at 37 ℃ for one hour. For MA104 cell lines, rotavirus WA strain was incubated with trypsin (10 μg/mL) at 37 ℃ for 30 min prior to infection. All cell lines were reincubated at 37 ℃ in a humidified atmosphere supplied with 5% CO_2. Cells were screened daily for the appearance of cytopathic effect (CPE). After the appearance of CPE, flasks were subject of three freezing/thawing rounds.

The virus titer was estimated from cytopatogenicity according to the Reed and Muench dilution method and expressed as 50% infectious doses per millilitre ($TICD_{50}\,mL^{-1}$). To evaluate the virus purification method, virus titration was carried out before and after the extraction/concentration steps.

2.3. Environmental samples

In this study, 50 of sewage enterovirus-positive samples were investigated. Negative samples were also included the present study (n=20).

2.4. Virus extraction/concentration

Two litres of waste water were filtered using filter paper (Whatman no.1) using vacuum pump. The filter was mixed with 100 mL of the resultant filtrate and homogenized.

The resultant filtrate (100 mL) was added to 1% (v/v) of 0.05 M aluminum chloride and adjusted to pH 3.5 using HCl. The mixture was homogenized for 30 min then centrifuged at 6 000 tr/min for 15 min at 4 ℃. The pellet was resuspended in 100 mL of 10% beef extract and pH was adjusted to 7.0. The mixture was again homogenized and centrifuged at 6 000 g for 30 min at 4 ℃. The supernatant was used for virus extraction.

Virus extraction was carried out by precipitation with 10% polyethylene glycol 6000 (PEG-6000) as described previously by[31]. After an overnight incubation at 4 ℃, the extract was centrifuged at 6 000 g for 60 min, and the pellet was resuspended in 3 mL phosphate buffer, pH 7.2 (100 mM) and 600 μL of antibiotics. This suspension was collected as virus concentrate and stored at 4 ℃ for 24 h. The viral RNA was used immediately or stored at -20 ℃ until use.

2.5. Viral RNA extraction

Prior to reverse transcription and amplification of the enteroviral RNA genome, the genome must first be extracted from the protein capsid. Four hundred microliter of viral suspension was used for extracting RNA using TRIZOL reagent (GIBCO BRL, Tunis, Tunisia) according to the manufacturer's instructions. Viral RNA was eluted in 50 μL of RNase-free water and the extracted viral RNA was used for RT-PCR.

2.6. Primer design

The nucleotide sequences of the complete genome

of the reference strains and field isolates of viruses belonging to genus *Enterovirus* under accession numbers EF174469 (coxsackievirus B2 strain KOR 04-279), AJ420884 (coxsackievirus B3 strain Nancy), GU109481 (coxsackievirus B3 strain SSM-CVB3), AF311939 (coxsackievirus B4 strain E2), DQ530409 (Human coxsackievirus B5 isolate CB5/2005/Seoul-13), AF114383 (coxsackievirus B5 strain Faulkner), AF105342 (coxsackievirus B6 strain Schmitt), DQ995647 (coxsackievirus A22 strain ban99-10427), GU366191 (enterovirus 71 strain Henan10-08-China), AY843300 (enterovirus 82 strain USA/CA64-10390), FJ859064 (poliovirus 1 strain 10097c), HM107835 (poliovirus 2 strain CHN8316), GU256222 (poliovirus 3 strain P3/Jinan/1/09), AF083069 (echovirus 5), EU870490 (echovirus 6), EU870492 (echovirus 7), AF524866 (echovirus 9 strain Barty), EU870487 (echovirus 33), EU840737 (enterovirus 104 CL-1232386 strain), EU840735 (enterovirus 104 CL-1227499 strain), EU840733 (enterovirus 104 CL-12310945 strain), EU840738 (enterovirus 104 CL-1234691 strain), EU840736 (enterovirus 104 CL-1231100 strain), EU840734 (enterovirus 104 CL-C22 strain), and GQ865517 (enterovirus 109 isolate NICA08-4327) were used in this study. The analysis, alignment of nucleotide sequences, and the choice of probes were conducted using the program package BioEdit version 7.0.5.2[32], ClustalW[33], SVARAP[34] and Simplot, version 3.5.1[35]. Plot similarity was carried out using Simplot, version 3.5.1[35].

3. Results

3.1. Evolutionary pattern of enteroviruses

Simplot analysis showed some characteristics of enterovirus serotypes. A cross point at position 207 showed a recombination event between coxsackievirus A22 strain ban99-10427 (DQ995647) and enterovirus 109 isolate NICA08-4327 (GQ865517) (Figure 1). Furthermore, coxsackievirus B4 strain E2 (AF311939) and enterovirus 82 strain USA/CA64-10390 (AY843300) showed cross events between serotypes in positions 208 and 230, respectively. In addition, the cross events in positions 182 and 205 were also detected between coxsackievirus B3 strain Nancy (AJ420884) and enterovirus 71 strain Henan10-08-China (GU366191). This latter serotype showed cross events with Human coxsackievirus B5 isolate CB5/2005/Seoul-13 (DQ530409) and coxsackievirus B6 strain Schmitt (AF105342) in positions 298 and 310 (Figure 1). The same events were shown for coxsackievirus B6 strain Schmitt (AF105342) with echovirus 5 (AF083069), coxsackievirus B3 strain Nancy (AJ420884), and coxsackievirus B5 isolate CB5/2005/Seoul-13 (DQ530409) at positions 201, 249, and 285, respectively. The same events were shown between echovirus 5 (AF083069) and coxsackievirus B5 isolate CB5/2005/Seoul-13 (DQ530409) at position 121. Finally, coxsackievirus B5 isolate CB5/2005/Seoul-13 (DQ530409) showed cross events with coxsackievirus B3 strain Nancy (AJ420884) and echovirus 5 (AF083069) at positions 256 and 277, respectively (Figure 1).

3.2. Development of virus-specific probes and RT-PCR

The sequence design of probes is the factor governing the sensitivity and specificity of RT-PCR assays. The 5′ end of the enteroviral genome as a suitable target for the development of specific probes for the detection of enteroviruses. Based on the sequence data generated, and the results of BLAST analysis, two probes were designed and synthesized. The primary structure of probe and the expected size of the amplified DNA fragment are shown in

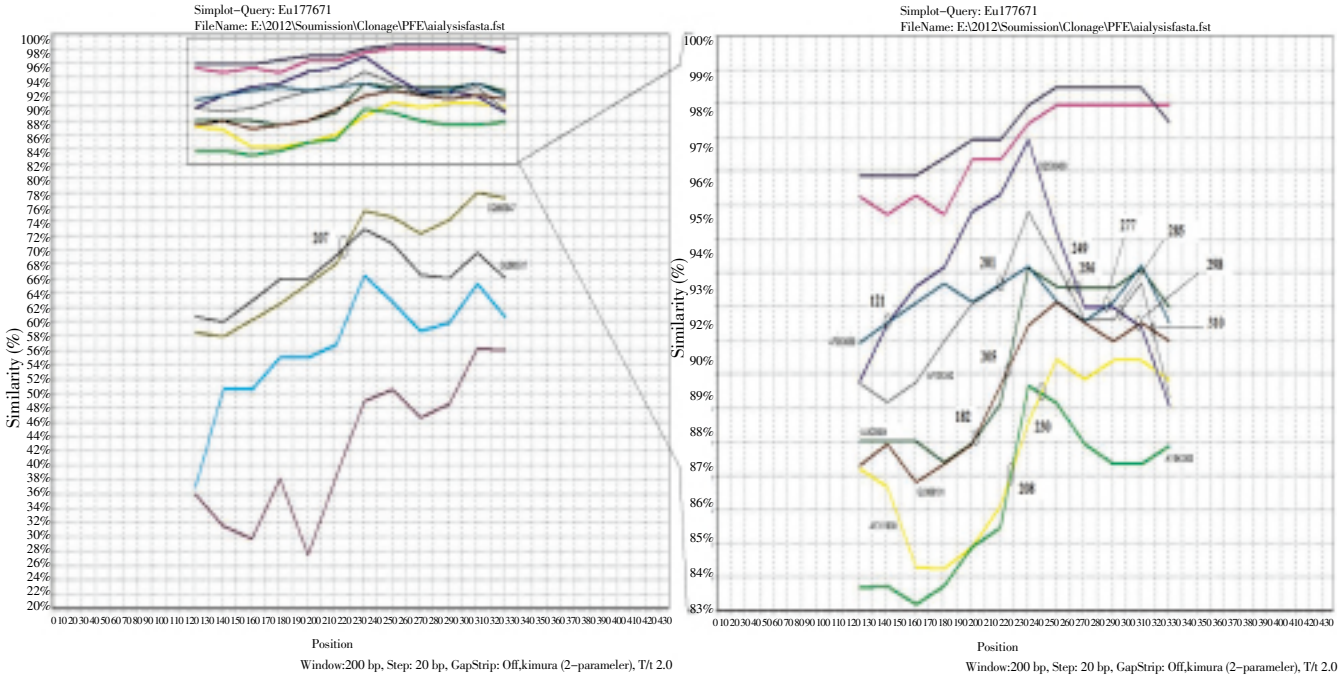
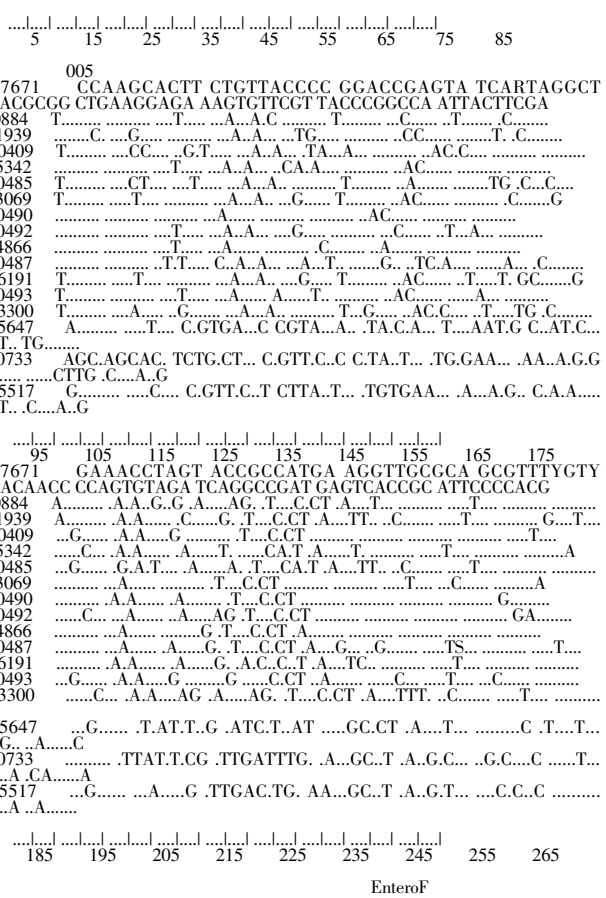


Figure 1. Plot of nucleotide similarity (generated by SimPlot) of a set of enterovirus 5′-UTR. Each point plotted is the percent identity within a sliding window of 200 bp, with a step size between points of 20 bp. Positions containing gaps were excluded from the comparison. The cross positions between strains are shown by circles and numbers. The accession numbers were also shown.

Table 1.

The thermal conditions, $MgCl_2$ concentration, and the annealing temperatures were optimized. The optimal results (maximum band intensity and minimal background non specific staining) were obtained using the following RT-PCR procedures: for reverse transcription, 5 μL of the extracted RNA was added to a mixture of reagents consisting of 5 μL of 5× M-MLV (Fermentas, Tunisie); 1 mM of each deoxyribonucleotide triphosphate (dNTP) (Fermentas, Tunisie); 10 μM of probe EnteroF (BioBasic Inc., Canada); 1.25 U of M-MLV reverse transcriptase (Promega, Washington, USA); and bidistilled water was added to make up a total volume of 25 μL. The mixture was incubated at 42 ℃ for 1 h. The cDNA (DNA copy) was either stored at -20 ℃ or amplified immediately. The amplification of DNA was carried out using probes EnteroF and EnteroR. The reaction contained 5 μL cDNA, 2.5 μL of 10× Green Go-Taq DNA polymerase buffer (Promega, Washington, USA); 1.5 mM $MgCl_2$ (25 mM) (Promega, Etats Unis); 200 μM each of dGTP, dATP, dTTP, and dCTP; 5 μM of EnteroF and EnteroR probes; and 1.25 μL of Go-Taq DNA polymerase (Promega, Washington, USA). Deionized water was added to make up a total volume of 25 μL. The amplification step was carried out in Thermocycler (MJ Research MiniCyclerTM PTC-150, USA) using the following thermal cycles: the denaturation step at 94 ℃ for 5 min was followed by 40 cycles of the amplification step (denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s, and extension at 72 ℃ for 1 min), a final extension step at 72 ℃ for 5 min, and then the tubes were held at 4 ℃. The expected size of the amplified product was 154 bp (Figure 3).



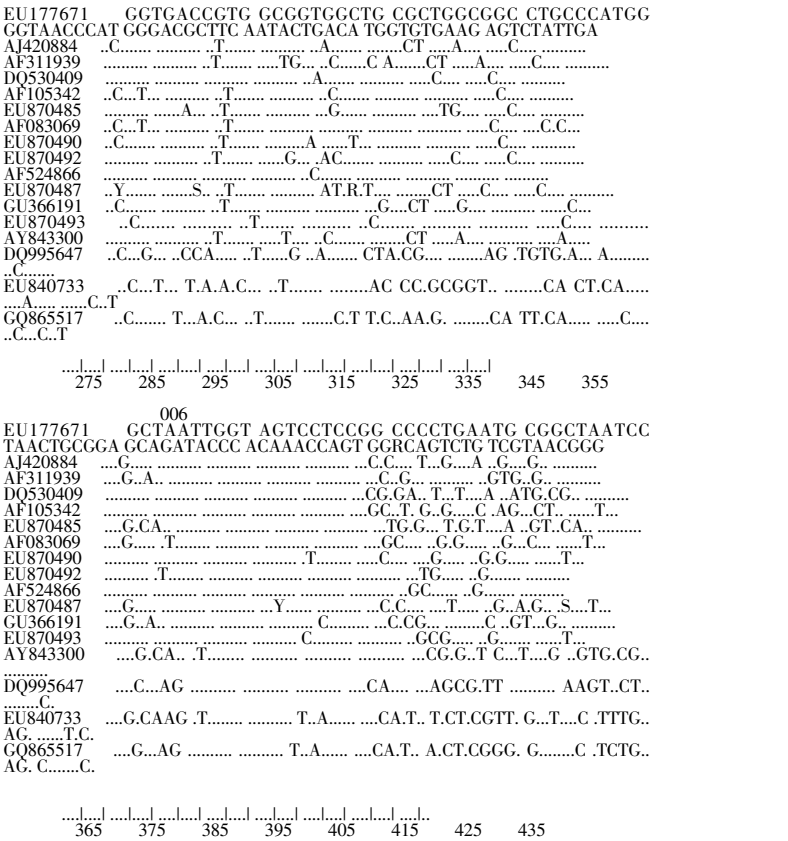


Figure 2. Alignment of the nucleotide sequences of 5′-UTR of enteroviruses. The positions of primers are underlined.

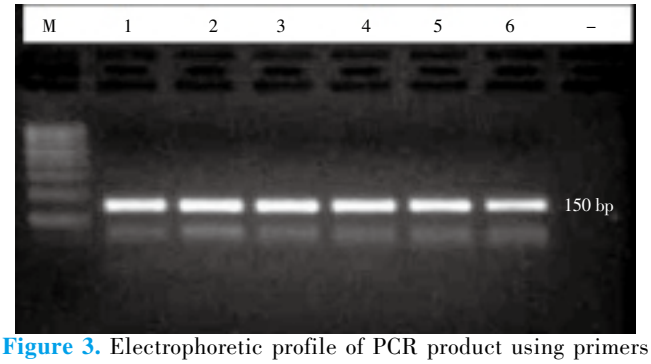


Figure 3. Electrophoretic profile of PCR product using primers EnteroF et EnteroR. M: Molecular weight 100 bp, 1-sewage sample; 2-sewage sample; 3-sewage sample; 4-sewage sample; 5-echovirus 6; 6-echovirus 11, 6-coxakievirus B3, (-) HAV isolate.

3.3. Sensitivity and specificity of RT-PCR

The RT-PCR assay was standardized by testing the viral positive controls. All RT-PCR products were identified by

Table 1
Primary structure of primers used in this study.

Primers	Structure primaire (5′ – 3′)	Position*	References
EnteroF (sens)	5′ – ACA–TGG–TGC–GAA–GAG–TCT–ATT–GA – 3′	324–342	This study
EnteroR (anti–sens)	5′ – AAA–CAC–GGA–CAC–CCA–AAG–TA – 3′	477–458	This study
007 (anti–sens)	5′ – TGG–CTG–CTT–ATG–GTG–ACA–AT – 3′	513–494	Zoll *et al*[36]
005 (sens)	5′ – CAA–GCA–CTT–CTG–TTA–CCC–CGG – 3′	78–98	
006 (sens)	5′ – TCC–TCC–GGC–CCC–TGA–ATG–CG – 3′	359–378	

*According to the complete genome of coxsackievirus B3 (accession number AJ420884).

molecular sequencing (data not shown). In addition, the specificity of the RT–PCR was tested using HAV isolate. To compare the analytical sensitivity of the RT–PCR, 10–fold serial dilutions of cell culture derived viruses were carried out. The developed RT–PCR was able to detect enteroviruses at a concentration up to 10^2 $DICT_{50}$/mL. The RT–PCR was not able to detect the genome of HAV isolate. The comparison of primer sequences developed in the present study with primers developed and used previously[36] showed that the new primers are more specific (Table 1 & Figure 2). The developed method was used to test the enterovirus–positive and negative sewage samples. The developed primers were able to detect the genome of enterovirus from all positive samples.

4. Discussion

The use of molecular methods for the detection of enteroviruses continues to be a priority in many laboratories all over the world. Enterovirus surveillance remains crucial, before and during the implementation of any vaccination strategy. However, the accumulation of point mutations and recombinations events at the primer–specific binding sites may result in failures to detect the genome of enteroviruses. For many years, the 5′–UTR of the enterovirus genome was believed to be the most conserved region of the genome and several primers targeting this region were developed.

Recently, it becomes evident that this region undergoes genetic variability which may influence the specificity and the sensitivity of primers[27–30]. When these mutations occur at primer–specific regions of the 5′–UTR of the genome can lead to the loss of complementarity with the primers and a failure to detect the circulating strains. Indeed, modification of the primer sequence to take account of these changes may result in the successful detection of the genome of enterovirus.

Conflict of interest statement

The authors declare no conflict of interest.

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