

Detection of Enteroviruses

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Summary

Enteroviruses are members of the Picornaviridae family and represent one of the most important water-transmitted pathogens. Detection of enteroviruses in water sources, or water-contaminated food, is a very valuable tool not only to prevent waterborne diseases but also to track down animal or human environmental viral pollution. Nowadays, molecular biology techniques allow the use of very sensitive and specific reverse-transcription polymerase chain reaction (RT-PCR) procedures to detect enteroviruses. In this chapter, using bovine enterovirus as a model, we describe procedures for enterovirus detection. Detailed descriptions of proper sample collection, storage, and processing, including methods for water concentration and solid sample extraction to obtain viral RNA, are outlined. Next, we describe methods for enterovirus detection based on virus isolation in appropriate cell culture. Finally, protocols for molecular detection of enterovirus are described, including procedures for conventional, nested, and real-time RT-PCR.

Key Words: Environmental contamination, water concentration, RNA extraction, cell culture, virus isolation, molecular detection, reverse transcription (RT), polymerase chain reaction (PCR), real-time RT-PCR.

1. Introduction

More than 100 virus species have been identified so far as contaminants of water, although not all of them cause illness in humans or animals. Significant pathogens, such as poliovirus, hepatitis A and E viruses, coxsackieviruses, and coronaviruses, may be detected in sewage-polluted water and food (especially in shellfish), making them a very important water-related health problem worldwide (*I*).

Enteric viruses are shed in high concentrations in feces of infected individuals (10^5 to 10^{11} particles/g of stool) and are potential contaminants of water in its different uses: water supply, irrigation, and recreation (*I*). Therefore, detection of

viruses in water sources, or water-contaminated food, is a valuable tool to prevent waterborne diseases; it can also be useful to indicate animal or human environmental viral contamination.

Enteroviruses are one of the most important water-transmitted viruses. They are very stable and may remain infectious for long periods of time under a wide range of environmental conditions. Enteroviruses belong to the Picornaviridae family (<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/>), thus being small RNA viruses, and include the most common virus infecting mammals.

Advances in molecular biology techniques have provided highly sensitive and specific reverse-transcription polymerase chain reaction (RT-PCR) procedures to detect enteroviruses. Here we describe our experience in the detection of bovine enteroviruses (BEV) as a model of water-contaminant enteroviruses (2). We have successfully applied a similar approach for other enteric viruses, such as porcine teschoviruses (3), as did others for detection of human enteroviruses, such as poliovirus, echovirus, and coxsackievirus (4–19).

2. Materials

1. Filters and filtration system: electropositive filters (Virosorb filters, 1 MDS, size 47 mm, CUNO Inc, Meriden, CT), prefilters (AMF CUNO, size 47 mm), Whatman filter paper no. 1 (Merck, Whatman 3MM, Darmstadt, Germany), peristaltic pump (Watson-Marlow, model IP55, Falmouth, England), silicone tubing, filter holders (AMF CUNO, size 47 mm, 60 PSI MAX), and 0.20- and 0.45- μ m syringe filters (Pall Corp., Ann Arbor, MI)
2. Centrifuge Heraeus Megafuge 1.0 R (Kendro Laboratory Products GmbH, Hanau, Germany), microfuge (Hermle Z 160 M, Wehingen, Germany), ultracentrifuge Optima L-90K ultracentrifuge, (Beckman Coulter Fullerton, CA) with rotors SW28, SW41, or equivalent), high-speed centrifuge (Beckman Avanti J25 I with rotor JA-14 or equivalent). Ultracentrifuge and high-speed centrifuge tubes for various volumes.
3. Elution buffer: 0.1 M glycine, pH 9.5, with 3% beef extract (Sigma, St. Louis, MO)
4. Extraction buffer: phosphate-buffered saline (PBS) with antibiotics (penicillin 100 U/mL and streptomycin 100 μ g/mL, Biowhittaker, Verviers, Belgium).
5. RNA extraction reagents (phenol-chloroform-isoamylalcohol) and equipment and/or commercial kits (QIAmp Viral RNA, Qiagen, Valencia, CA).
6. Cell and tissue culture equipment: laminar flow hood, water bath, phase contrast microscope, CO₂ incubator, plastic or glass ware, micropipets and tips, syringes.
7. Culture medium: Eagle's minimal essential medium (EMEM) (Biowhittaker), or similar medium, e.g., minimal essential medium (MEM), fetal calf serum (FCS) (Biowhittaker) and antibiotics (penicillin 10,000 U/mL, streptomycin 10,000 μ g/mL, Biowhittaker).
8. RT-PCR 10X loading buffer: 0.025% Orange G (Sigma), 20% Ficoll 400 (Calbiochem Inc., La Jolla, CA) 0.1 M EDTA, pH 8.0.
9. Agarose (Promega, Madison, WI) and electrophoresis equipment.

10. Vortex, shaker, pH meter and storage equipment (refrigerator, -20°C and -70°C)
11. Thermocycler (Perkin Elmer Applied Biosystems, PEAB, Branchburg, NJ), optionally, real-time equipment (ABI Prism 7700, PEAB) and 0.2-mL optical PCR tubes and optical caps (PEAB).
12. Superscript one-step RT-PCR (Gibco BRL, Life Technol., Grand Island, NY)
13. 100-bp ladder (Roche Molecular Biochemicals, Mannheim, Germany).
14. RT-PCR reagents: primers, probes, enzymes, dNTPs.

3. Methods

3.1. Samples

Collection of samples is always the initial and crucial point to investigate a suspected case of water- and food-borne viral disease. Samples should be taken from the affected individuals and their contacts (serum, swabs, spinal fluid, tissue, etc.) and from the environment (water, feces, food [especially seafood and fish], etc.). Implementation of systematic procedures and databases, including integration of sample labeling, registration of essential information (such as date and place of collection, nature of the sample, and any other relevant data), conservation, and storage are always critical points to track back every result obtained during the investigation (20). Samples should ideally be split into aliquots and immediately processed as soon as they are received at the laboratory, but adequate means for conservation and storage of samples should be available in case further analysis will be required. In this regard, it should be noted that enteric viruses are usually relatively stable and persist in normal environmental conditions for long periods; therefore, liquid samples are adequately stored at 4°C for several days and at -70°C for years, but freeze-thaw of samples should be avoided. Solid samples (feces, seafood, etc.) can be stored at -70°C for years (*see Note 1*).

3.2. Sample Processing

3.2.1. Water Concentration

Viruses are usually at low concentration in water samples; therefore, it is necessary to concentrate the samples for proper virus detection. Several concentration methods have been described in the literature aimed at this purpose. These methods are based on organic flocculation, filtration-elution, ultrafiltration, lyophilization, ultracentrifugation, and combinations of two or more of these systems (8,12,21–24). Among them, the filtration-elution method using electronegative (23) or, more commonly, electropositive filters has gained acceptance, the latter one being the most used either alone or combined with other methods (8,12). As a consequence, the American Public Health Association has chosen it as the standard method for water-virus examination (24). Our experience is that this simple concentration method provides enough

concentration power (up to 100 times) for most water samples tested but if further concentration is needed, an additional ultracentrifugation step may be used (*see Subheading 3.2.1.2.*).

3.2.1.1. CONCENTRATION OF WATER SAMPLES BY FILTRATION AND ELUTION THROUGHOUT ELECTROPOSITIVE FILTERS

This method assumes that the net electrostatic charge of most viruses at neutral pH is negative and thus, filters with positive charges in their surface can retain them. Viral particles are then eluted from the filters by simply changing pH conditions. Here we provide a simple protocol using membrane electropositive filters for volumes up to 5 L. For higher volumes, cartridges that filtrate up to 1000 L of water are also commercially available (CUNO).

1. Before filtration, adjust pH of sample to 6.0 to 7.0.
2. Clarification: Environmental water samples contain variable amounts of materials in suspension; thus, to avoid filter clogging, a clarifying step is often required before filtration (*see step 3*). Coarse material might be decanted for at least 2 h at room temperature or, preferably, overnight at 4°C. If the decanted water still contains too many fine particles in suspension, it can be prefiltered through a Whatman filter paper, or further cleaned up by centrifugation at 9800g for 20 min in a Beckman (Avanti J25 I) centrifuge using a JA-14 rotor. This procedure yields a clarified supernatant already suitable for filtration.
3. Filtration: We use the filtration system outlined in **Fig. 1**. Basically, a peristaltic pump drives the liquid sample through a prefilter and an electropositive filter (*see Section 2*) placed consecutively down the flow. Optimal flow rate is dependent on the size of the filter. For CUNO 47-mm filters, the maximum flow rate is 70 mL/min.
4. Elution: Once the sample has been filtered, the filter must be removed from its cassette and incubated for 5 to 10 min, with shaking, in 10 mL elution buffer (0.1 M glycine, pH 9.5, with 3% beef extract [*see Notes 2 and 3*]).
5. Neutralization: Because excessive exposure to alkaline pH may produce loss of virus viability, the pH of the eluate must be neutralized by the addition of 0.1 M HCl immediately after incubation (*see Note 4*). After neutralization, and before storage, we find that filter sterilization of eluates through 0.2- μ m pore-diameter syringe filters is useful to have them ready for further analysis that require sterile conditions, such as virus isolation in cell culture.

3.2.1.2. VIRUS CONCENTRATION BY ULTRACENTRIFUGATION

This concentration method may be useful in two instances:

1. When no virus is detected in the sample after a first filtration-elution step, this second concentration step may be applied before it is convincingly concluded that the sample is free of virus.

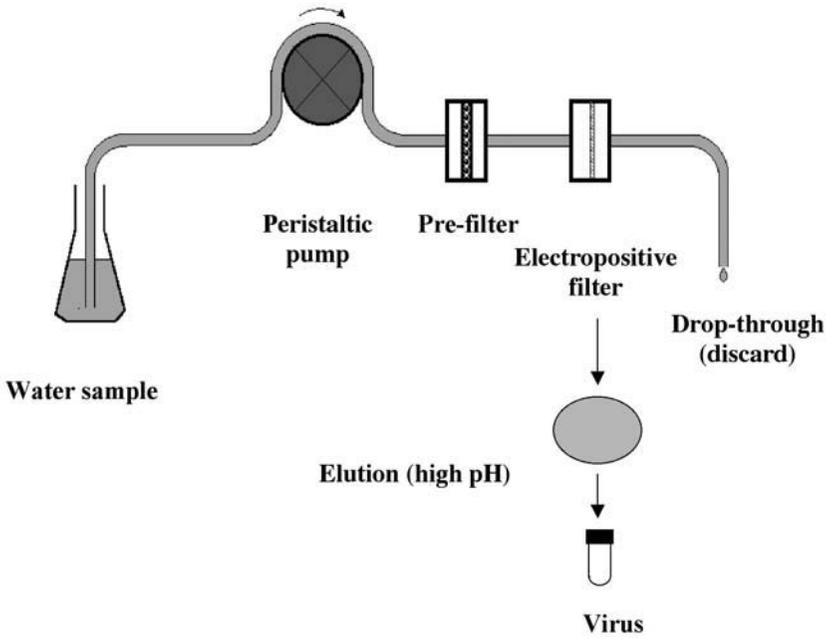


Fig. 1. Schematic representation of the devices for concentration of water samples by filtration and elution through electropositive filters.

2. When the filtration-elution method used could presumably prevent virus detectability, e.g., in infectivity tests (*see Note 4*).

In these cases the following protocol is used:

- a. Clarify the samples by centrifugation at 9800g 20 min at 4 to 8°C and discard the pellet or, alternatively, filter the samples through 0.2- μ m filters.
- b. Ultracentrifuge the supernatant at 120,000g using a SW-28 rotor (Optima L-90K ultracentrifuge, Beckman Coulter) for 3 h at 4 to 8°C (*see Note 5*).
- c. Discard the supernatant and resuspend the pellet in 0.5 to 1 mL of RNase-free water (*see Note 6*).

3.2.2. Feces Extraction

1. Mix fecal samples (1–15 g) with extraction buffer (PBS with antibiotics, penicillin 100 U/mL, streptomycin 100 μ g/mL) at a 1:2 ratio (w/v).
2. Homogenize the mixture by vortex and/or other means (*see Note 7*).
3. Centrifuge the mixture at 1200g for 10 min and transfer the supernatant to a clean centrifuge tube. Repeat this step once more and, finally, transfer the supernatant to clean microfuge tubes and microfuge at maximum speed (around 16,000g) for 10 to 15 min.

4. Take the clarified supernatant and filter it through a 0.2- μm pore-diameter sterile syringe filter. Alternatively, treat it with chloroform to eliminate bacteria and enveloped viruses. Carefully, add chloroform to a final concentration of 10% and vortex vigorously for 1 min, then microfuge at maximum speed and transfer the aqueous phase (sample) to a clean tube. The clarified supernatant, either filtered or chloroform-treated, is suitable for both cell culture procedures and molecular detection methods (after RNA extraction; see **Subheading 3.3**) (see **Note 8**).

3.2.3. Solid Food Sample Extraction

In addition to the protocols describing the extraction of clinical specimens, such as serum and cerebrospinal fluid (**16,18,19**), several procedures have been described for solid sample extraction, including food (**7,11,15**).

Here we describe an example of a simple method for RNA extraction from oyster (*Crassostrea virginica*) tissue.

1. At the place of sampling, using a syringe, aspirate the hemolymph from the adductor muscle and place it in clean tubes. Aspirates from 10 oysters can be mixed at this point or later on, just before extraction. Dissect the stomach and gills and place them in separate clean tubes. Store at 4°C for transportation.
2. Within 24 h of collection, cut individual tissues in small pieces, place them in clean tubes, and suspend them in 5 mL of MEM (1 mL/g of tissue) for 1 h with occasional vortexing.
3. Centrifuge at 1200g for 10 min.
4. Filter supernatants through a 0.4- μm filter into clean tubes, add antibiotics (penicillin 100U/mL, streptomycin 100 μg /mL) to the filtrates, and store at -20°C until use.

3.3. RNA Extraction

Enteroviruses are RNA viruses; therefore, as RNA is highly labile, during nucleic acid extraction and handling special care is needed afterward to maintain its integrity (use of sterile gloves; filter tips; specific pipets for RNA handling only; RNase-free tubes, buffers, and media; disposable labware). To avoid possible contamination during further PCR amplification, strict adherence to guidelines should be maintained (**25**), including the availability of a separate ('clean') area to perform RNA manipulations (ideally under a laminar flow hood) physically apart from the place where amplified cDNA is handled.

RNA extraction can be manual or automated. Manual methods are home-made or supplied as commercial kits. The former are based mostly in guanidinium isothiocyanate denaturation followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation (**26**), while the latter are usually based on virus denaturation and RNA adsorption to RNA-binding matrices, followed by a final elution step. Kits are convenient, easy to use, efficient, and fast, and avoid the use of harmful and environmentally hazardous reagents (such as

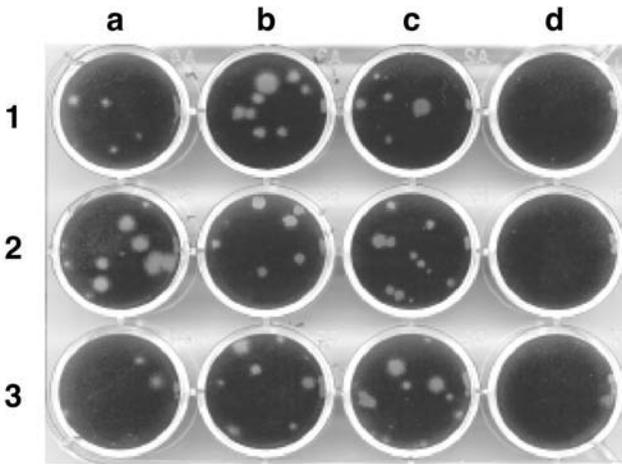


Fig. 2. Representative example of BEV CPE on MDBK cell monolayers. Calves (white spots) on the crystal violet stained cell monolayers are individual plaque-forming units (PFU) of BEV. Lanes 1–3 are triplicates (rows a, b, and c) of 3 BEV field samples. Wells of row d are control mock-infected cells.

phenol), but they are expensive and present important drawbacks, particularly when a high number of samples is to be processed (*see Note 9*). In this case, diverse automated systems for nucleic acid extraction are commercially available (such as ABI 6100 Nucleic Acid PrepStation, Applied Biosystems, www.appliedbiosystems.com; Biorobot 9604, Qiagen, www.qiagen.com).

3.4. Detection

3.4.1. Virus Isolation in Cell Culture

Detection of viral infectivity in cell culture or animal models is the only way to determine the presence of infectious viral particles, as molecular techniques do not establish whether the pathogen is active. Virus infection in cell culture is the 'classic' method for virus detection, and it is still considered the 'gold standard' in virus detection techniques. In addition, its combination with serological methods (virus neutralization with specific antisera) and/or molecular techniques (*see Subheading 3.5*) can lead to the characterization of the isolated virus.

Isolation of viruses in cell culture relies on the ability to detect a particular effect caused by *in vitro* virus propagation in the target cells. Cytopathic viruses, as most of the enteroviruses, cause a characteristic cell lysis known as cytopathic effect (CPE) (**Fig. 2**). For noncytopathic viruses, detection of virus propagation in cell culture is achieved by means of more sophisticated methodologies,

such as immunomicroscopy (immunofluorescence or immunohistochemistry) or *in situ* hybridization techniques, provided that virus-specific molecular probes and/or antibodies are available.

3.4.1.1. CELL LINES FOR ENTEROVIRUS ISOLATION

A wide variety of cell lines of different origin, available at the American Type Culture Collection, (ATCC, www.atcc.org), are commonly used for enterovirus isolation: HeLa, CaCo2, rhabdomyosarcoma, buffalo green monkey (BGM), baby hamster kidney (BHK), bovine epithelial cells (MDBK), porcine cell lines (IB-RS-2 [not available at ATCC] and PK15). In any case, it is usually better to use cell lines from the species where the virus was originally isolated.

3.4.1.2. PROTOCOL FOR VIRUS ISOLATION

1. Inoculation: Carefully remove supernatant from semiconfluent (70–80%) cell monolayers in 25-cm² cell culture flasks (with screw cap) and overlay the cells with the inocula, consisting of a filter-sterilized virus suspension in physiologic media, i.e., neutral pH and isotonic salts (*see Note 10*).
2. Adsorption: Gently swing the flask to ensure spread of the inoculum over all the cell monolayer and close the cap of the flask. Incubate at 37°C in a CO₂ incubator for 30 to 60 min, swinging the flask gently every 10 to 15 min to prevent the monolayer from drying, as well as to allow interaction between remaining free virus and cells.
3. Incubation: Remove the remaining inocula and add fresh cell culture medium to the flasks (*see Note 11*). Incubate at 37°C in a CO₂ incubator and observe the cells daily for CPE (*see Note 12*), under the microscope if needed. Usually, 2 to 3 d are enough to detect CPE, but sometimes incubation for up to 5 to 6 d is recommended to assess infectivity signs. To conclude absence of cytopathic effect due to virus infection in a given sample, at least three 'blind' passages are required, that is, absence of CPE after three successive rounds of infection using the supernatant of the former round of infection to infect a new monolayer.
4. Virus recovery: Clarify the supernatant of infection by centrifugation at 1200g for 10 min to remove cell debris (*see Note 13*).

This supernatant of infection constitutes the 'isolate' and is the source of virus for further characterization, including biological, antigenic, and molecular analyses (*see Subheading 3.6*). Virus isolates are best conserved in aliquots frozen at -70°C, and freeze-thaw cycles should be avoided. Given that enteroviruses are highly stable, once thawed, each aliquot is better maintained at 4°C up to several weeks. Stored viruses must be labeled and registered in a way that allows easy identification. Registration data should include at least: name of sample, origin, date, and cells used for isolation and number of passages. Care must be taken to maintain virus isolates within a low number of passages, as RNA viruses are highly variable and can drift to cell-culture adapta-

tions as the number of passages grow, making cell-adapted viruses often quite different from those originally isolated.

3.5. Molecular Detection: RT-PCR Methods

RNA extracted from water concentrates, fecal extracts, sera, spinal fluid, supernatants of infection, and so on is assayed for the presence of enterovirus sequences by RT-PCR. Based on increasing sensitivity criteria, RT-PCR methods are classified as *conventional*, *nested*, and *real-time* modes. We find it convenient to use conventional RT-PCR methods when assaying samples with an expected high concentration of viral RNA, such as feces, gills, and supernatants of infection, whereas samples with an expected low viral RNA content, such as waters and water concentrates, require much more sensitive methods, such as nested or real-time RT-PCR. On the other hand, RT-PCR methods can be of *wide* or *narrow* range specificity, that is, respectively, those aimed at detecting 'as many enterovirus types as possible' (generic methods) and those aimed at detecting a particular enterovirus species or strain (specific methods). The latter can be combined in the so-called multiplex methods to detect several different enterovirus species in a single determination (5,9). Technically, the main difference between generic and specific methods relies on the relative evolutionary conservation of the target viral RNA sequence selected for amplification (<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/>). The 5'-noncoding and polymerase regions of enterovirus genomes are highly conserved and thus are frequently chosen for generic methods (2,7,12,27), whereas specific methods rely on a primer design targeted at sequences found only in a particular enterovirus type, and that do not cross-react with other types of enterovirus. For this purpose, appropriate sequences are often found in genomic regions containing the highest variability, such as the domains encoding for the structural proteins (10,11,14,17,27-31). Primer design must thus take into account both the mode (conventional, nested, or real-time) and the range of specificity of the RT-PCR to be applied (30). Quantitative real-time RT-PCR (32) requires additional probe design, as those for the diverse available commercial devices (TaqMan, LightCycler, iCycler), but this issue is beyond the scope of this chapter.

Variation among the different procedures relies mainly on the primers chosen and therefore in the adjusted annealing temperature better fitted for them. Here we provide the protocols for conventional, nested, and *real-time* RT-PCR for an enterovirus model, the bovine enterovirus (BEV). A similar approach was applied for us by other enteric viruses, such as teschovirus, after setting the protocol conditions to those better fitted for the porcine teschovirus specific designed primers. By this approach we were able to track down a pig slurry spillage, and demonstrate that the developed methodology is similar to current

methods for determining unspecific organic matter and better than many other conventional chemical analyses applied to evaluate water contamination (3).

3.5.1. Protocol for Bovine Enterovirus Conventional RT-PCR

Viral RNA is extracted from 140 μL of sample (water, water eluates, oyster washes, fecal extracts, or culture supernatant) with a commercial kit (QIAamp viral RNA kit) following manufacturer's instructions, and eluted in 60 μL of the kit's elution buffer. Due to the ability of the PCR to amplify a single molecule, special care should be taken to avoid RNA contamination and false-positive results owing to trace amounts of DNA contaminants, particularly if positive controls are included in the extraction or RT-PCR procedures. To check for possible contamination, negative control (tubes with virus-free water, instead of sample) must be included in the reactions.

Six μL of RNA (1/10 of the total eluted volume) is used to carry out the RT-PCR with a commercial kit (such as Superscript one-step RT-PCR, Gibco BRL), or the like, following the kit's protocol. Additionally, RT and Taq polymerase can be purchased separately. In this case, manufacturer-recommended buffers should be used and the appropriate amount of dNTPs (around 200 mM) must be added to the mixture. RT-PCR conditions must be adapted to the specific target, so that selection of primers and best-fitted annealing temperature are critical points.

For bovine enterovirus, amplification with outer forward (5' GGG GAG TAG TCC GAC TCC GC, nt 124 to 143) and reverse (5' CGA GCC CCA TCT TCC AGA G, nt 391 to 409) primers give rise to a 272-bp amplified fragment. The working concentration of primers is 0.2 to 0.5 μM in a 25- μL final reaction volume. Positions of the primers correspond to the 5' noncoding region of bovine enterovirus genome (PS87, GenBank accession no. X79368).

1. Briefly centrifuge all reagents before beginning the procedure.
2. Add 6 μL of eluted RNA (sample) in 0.2 mL sterilized PCR tubes (on ice).
3. Add 19 μL of the following premix to each tube (volumes are for 1 reaction/tube):
 - a. 15 μL 2X kit buffer.
 - b. 0.6 μL RT/Taq.
 - c. 1.2 μL each primer.
 - d. 4 μL RNase-free water.

Mix gently to produce a homogeneous mixture and centrifuge briefly to collect the sample at the bottom of the tube. Place tubes in the thermal cycler (*see Note 14*) and proceed with the amplification using the following conditions:

1. RT: 30 min at 48°C
2. PCR (hot start): 2 min at 92°C
3. Cycles:

Denaturation:	30s at 94°C
Annealing:	60s at 57°C
Elongation:	60s at 72°C

Proceed for 40 cycles with a final elongation step for 10 min at 72°C. Keep tubes at 4°C until analysis.

Assess correct size of amplified products by electrophoresis of 5 to 10 µL of the RT-PCR mixed with 1 µL of 10X loading buffer, through a 1.2% agarose gel stained with ethidium bromide (0.5 µg/mL) (*see Note 15*). Run the gel at 100 mA for 2 h. Include molecular weight markers of appropriate size for the amplified products, i.e., 5 µL (200 ng, 20 ng/band) of a 100-bp ladder, 0.2 mg/mL (Roche Molecular Biochemicals, Mannheim, Germany).

3.5.2. Protocol for Bovine Enterovirus "Nested" RT-PCR

Nested RT-PCR is usually applied when RNA sample concentration is low; it is based on the use of the first PCR product as starting material for the second PCR round. In some instances, a hemi-nested RT-PCR is used, where one of the second-round primers, usually the forward one, is the same as in the first-round RT-PCR. For the bovine enterovirus, first step of the nested RT-PCR is carried out as described above. The second step (nested) is carried out with internal forward (5' ACT GGT ACG CTA GTA CCT TT, nt 166 to 185) and reverse (5' CAG AGC TAC CAC TGG GGT TGT GG, nt 373 to 395) primers, yielding a 230-bp amplified fragment (**Fig. 3**).

1. Prepare the following mixture (volumes are for 1 reaction/tube) and add 15 µL mix/tube:
 - a. 0.6 µL *Taq* polymerase (Perkin-Elmer).
 - b. 1.2 µL forward inner primer.
 - c. 1.2 µL reverse inner primer.
 - d. 12.5 µL 2X PCR buffer: for 5 mL of 2X buffer, mix 1 mL de (10X) PCR Buffer II (Perkin-Elmer), 40 µL of each dNTP (stocks at 50 mM), 0.6 mL of 25 mM MgCl₂, and 3.2 mL RNase-free water.
2. Add 8.5 µL RNase-free water/tube.
3. Add 1.5 µL of first-round RT-PCR product.

Mix gently, centrifuge briefly, place tubes on thermal cycler, proceed under the same conditions described above for the conventional PCR without the RT step, and assess correct size of amplified products by electrophoresis through agarose gels.

3.5.3. Protocol for Bovine Enterovirus Real-Time RT-PCR

In recent years several commercially available methodologies have been developed to carry out real-time PCR procedures. Real-time is a fluorescence-based RT-PCR that is easy to perform, capable of high throughput, and can combine high sensitivity with reliable specificity. Although real-time RT-PCR is a rapidly evolving methodology, it also engenders associated problems, however, these should be resolved in the coming years (**32**).

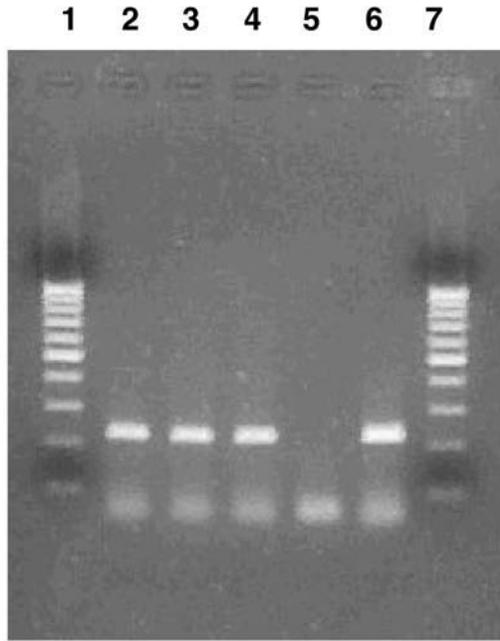


Fig. 3. Representative example of RT-nested-PCR amplified products of BEV resolved by electrophoresis through an ethidium bromide-stained 1.5% agarose gel. Lanes 1 and 7, molecular weight markers (100-bp ladder); lanes 2–4, BEV-positive field samples; lane 5, BEV-negative field sample; and lane 6, BEV-positive control.

At present a variety of real-time procedures has already been applied for enterovirus detection (3,16,17). The following procedure has been optimized for bovine enterovirus detection by the TaqMan technology, using the TaqMan One-step RT-PCR Master Mix Reagents kit (P-E AB). Viral RNA extraction is carried out as for conventional RT-PCR.

1. First, bovine enterovirus specific primers for the TaqMan procedure are diluted to a stock solution of 500 μM (working final concentration 0.5 μM). Primers are BEV5fl (5' GCC GTG AAT GCT GCT AAT CC, nt 533 to 552) and BEV3fl (5' GTA GTC TGT TCC GCC CCT GAC T, nt 604 to 625). Working concentration of the probe is 25 μM (BEVprobe-FAM 5' CGC ACA ATC CAG TGT TGC TAC GTC GTA AC, nt. 570 to 598). Nucleotide positions correspond to that of PS87 strain (GenBank accession no. X79368).
2. Prepare clean, RNase-free 0.2-mL optical PCR tubes for a 25- μL final volume of reaction.
3. Add 37 μL /tube of the following mixture (volumes are for 1 reaction):
 - a. 28 μL reaction buffer (2X).
 - b. 1.4 μL MS 40x (enzymes mix).

- c. 5.6 μL each primer (5 μM).
- d. 0.56 μL probe (BEVprobe-FAM) 25 μM .
4. Add up to 13 μL sample RNA/tube (complete with DEPC-treated water up to 13 μL , if needed). Close tubes with optical caps, place them on real-time thermal cycler, and proceed under the following conditions:
 - a. RT: 30 min at 48°C
 - b. PCR (hot start) 10 min at 95°C
 - c. 50 Cycles:

Denaturation	15s at 95°C
Annealing/Elongation	60 s at 60°C

3.6. Virus Characterization

3.6.1. Antigenic Characterization

Enteroviruses are members of the *Picornaviridae* family and are characterized by their capacity to multiply in the gastrointestinal tract (33). Enteroviruses had been classically grouped by serological criteria based on neutralization of viral infectivity in cell culture, complement fixation, immunoprecipitation, and hemagglutinating activity (34). Later on, panels of antisera against different enteroviruses were made available to the scientific community to facilitate enterovirus identification (33). However, sometimes significant cross-reaction of serotype specific antibodies led to ambiguous serotyping (29). Nowadays, molecular techniques, particularly nucleotide sequence determination, are frequently applied for viral classification, so that under certain circumstances, molecular characterization is overtaking old serological procedures for enterovirus classification.

3.6.2. Molecular Characterization

Advances in molecular biology techniques have allowed the classification of enteroviruses on the basis of their nucleotide sequences and phylogenetic analyses and, as a consequence, in some instances, classical classification has been modified (<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/>). RT-PCR-amplified fragments are sequenced with commercial kits (such as the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 2.0, PEAB) following manufacturer's instructions and sequence reactions are further run in an automated sequencer (such as the ABI Prism 3100 Genetic Analyzer, PEAB). Due to the cost of sequencing equipment, not too many laboratories are equipped with such apparatus, but a variety of worldwide companies offer sequencing services. Today RT-PCR amplification and further sequencing of detected enteroviruses can be easily applied to molecular epidemiological studies, allowing surveillance, control, and eradication of waterborne disease outbreaks and tracking of viral contaminants (2,10,14,17,27–29). However, phylogenetic analyses need a skilled worker able to apply the appropriate methodology to

analyze the results. In any case, a variety of software for sequence analysis and phylogenetic and evolutionary studies is available (<http://evolution.genetics.washington.edu/phylip/software.html>), including some that can be obtained free of charge.

4. Notes

1. We find it convenient to store water samples after a first concentration step, as this greatly reduces the need for storage space. Similarly, solid samples (food, feces) are better stored after the extraction step. In the case of seafood, filtering organs (gills) accumulate virus filtered from the water; thus, a minimal processing step consisting of dissection and separation of gills will facilitate their storage.
2. The volume of elution buffer necessary for an adequate virus recovery is determined by the surface of the filter.
3. To prevent microbial growth, it is convenient to filter-sterilize the elution buffer and store it at 4°C, being careful to open it under sterile (laminar flow hood) conditions.
4. Some viruses completely lose their viability upon alkaline treatment during filtration through electropositive filters. On the other hand, at high concentrations, beef extract is directly toxic for most cell cultures, thus being necessary to dilute the concentrates to avoid this effect (this drawback can be partially overcome by reducing the beef extract content of the elution buffer to 1%). Consequently, the filtration-elution method is less effective when virus infectivity is to be tested. However, detection of virus by molecular methods is not affected by these drawbacks.
5. For this purpose we use ultracentrifuge rotor SW28 (Beckman). The capacity of the tubes for this rotor is approximately 38 mL. For higher volumes of sample one should fill as many tubes as needed, whereas for volumes lower than 38 mL, one should dilute it with distilled water up to 38 mL, or, alternatively, use tubes and rotors suited for lower volumes, i.e., SW41 (12 mL).
6. Virus recovery is increased when, before resuspension, pellets are kept overnight at 4°C with a RNase-free water overlay.
7. The homogenization is more efficient after an overnight incubation at 4°C with extraction buffer.
8. One aliquot can be treated with chloroform, and another one can be filter-sterilized. Comparison of the results of infectivity in cell culture obtained in each case indicates whether the cytopathic effect is due to enveloped or nonenveloped viruses.
9. Many commercial kits for RNA extraction are optimized for tissue or cell extractions, and thus are not well suited for liquid samples. We have found that those labeled as 'viral RNA extraction kit' are better suited for the purposes discussed in this chapter.
10. The volume of the inoculum should ideally be high enough to overlay all the surface of the cell monolayer, but as low as possible to increase virus concentration to facilitate virus-cell contact. As a general rule, 15 to 20% of the volume of medium used for cell growth is adequate for inoculation.
11. Low fetal calf serum (FCS) concentrations during the infection (1–2%) are recommended in most cases, as the growth of many enteroviruses is prevented by FCS components, and remarkably by bovine serum albumin.

12. It is helpful to mock-infect one flask in parallel as a control for null CPE.
13. To increase virus content in the supernatant, before clarification, freeze–thaw the centrifuge tubes containing the infection supernatant three times successively.
14. When the thermal cycler does not have a top heater, then overlay the reaction mixture with 30 μ L of mineral oil.
15. Extreme care should be taken when manipulating ethidium bromide, as it is a powerful mutagen. Gloves should be worn when working with solutions containing the mutagen and all reactive and gels in contact with it should be carefully discharged in appropriate containers.

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