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Review Article

روش‌های نوین ارزیابی و پایش مولکولی انتروویروس‌ها به‌عنوان شاخص ویروسی در محیط‌های آبی

رضا صدیقی شیری[۱]، محمد کارگر[۲*]، فرشید کفیل‌زاده[۳]، نیلوفر نیسی[۴]

[۱] دانشجوی دکتری، گروه میکروبیولوژی، واحد جهرم، دانشگاه آزاد اسلامی، جهرم، ایران.
[۲] استاد، گروه میکروبیولوژی، موسسه آموزش عالی زند، شیراز، ایران. [۳] استاد، گروه میکروبیولوژی، واحد جهرم، دانشگاه آزاد اسلامی، جهرم، ایران.
[۴] دانشیار، مرکز تحقیقات بیماری‌های عفونی و گرمسیری، پژوهشکده سلامت، گروه ویروس‌شناسی پزشکی، دانشکده پزشکی، دانشگاه علوم پزشکی جندی‌شاپور، اهواز، ایران.

چکیده

پایش کیفیت آب محیطی یک مسئله جهانی است و تلاش‌های زیادی برای مدیریت صحیح آن انجام می‌شود. انتروویروس‌ها یکی از مهم‌ترین ویروس‌های منتقل شونده از راه آب هستند. این ویروس‌ها از طریق تخلیه فاضلاب، عملیات کشاورزی و آب‌های جاری از محل‌های دفن زباله با نفوذ به آب‌های سطحی و زیرزمینی، خطرات قابل توجهی را برای سلامت عمومی انسان‌ها، حیوانات و گونه‌های آبزی ایجاد می‌کنند. شناسایی زودهنگام، سریع و مؤثر انتروویروس‌های قابل کشت در سیستم‌های آبی برای اطمینان از سطح بهداشتی آب و اجرای راهبردهای مناسب تصفیه آب و فاضلاب ضروری هستند. پایش زیستی محیط‌های آبی با وجود هزینه کمتر سنجش شاخص‌های باکتریایی مانند کلی‌فرم های مدفوعی، *انتروکوکوس‌ها* و *کلاستریدیوم پرفرنجنس*، نمی‌تواند تضمین کننده عدم وجود ویروس‌های روده‌ای باشد زیرا به کلرزنی و تصفیه مرحله سوم مقاوم هستند. انتروویروس‌ها یکی از شاخص‌های ویروسی پایش کیفیت آب و تصفیه فاضلاب هستند، که هنوز به صورت گسترده و متداول مورد استفاده قرار نگرفته‌اند. در این مقاله، با ارزیابی پایگاه‌های معتبر علمی، کفایت کاربردی انواع روش‌های جدید تغلیظ و شناسایی مولکولی انتروویروس‌ها در آب و فاضلاب مورد ارزیابی قرار گرفته است.

کلمات کلیدی: ویروس‌های منتقل شونده آب، *انتروویروس‌ها*، شاخص‌های ویروسی، پایش زیستی، روش‌های تغلیظ.

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*) آدرس برای مکاتبه: گروه میکروبیولوژی، موسسه آموزش عالی زند، شیراز، ایران
تلفن: ۰۹۱۷۳۱۴۹۲۰۳ پست الکترونیک: m.kargar@zand.ac.ir

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Review Article

New methods for molecular assessment and surveillance of *Enteroviruses* as viral indicators in aquatic environments

Reza Sedighi Shiri[1], Mohammad Kargar[2], Farshid Kafilzadeh[3], Niloofar Neisi[4]

[1] PhD Candidate, Department of Microbiology, Ja.C, Islamic Azad University, Jahrom, Iran.
[2] Professor, Department of Microbiology, Zand Institute of Higher Education, Shiraz, Iran.
[3] Professor, Department of Microbiology, Ja.C, Islamic Azad University, Jahrom, Iran.
[4] Associate Professor, Infectious and Tropical Diseases Research Center, Health Research Institute, Department of Medical Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Abstract

Environmental water quality monitoring is a global issue, and many efforts are being made to manage it properly. *Enteroviruses* are one of the most significant waterborne viruses. These viruses pose significant risks to the public health of humans, animals, and aquatic species through the discharge of sewage, agricultural operations, and runoff from landfills, penetrating into surface and groundwater. Early, rapid, and effective detection of culturable EVs in aquatic systems is essential to ensure water hygiene levels and implement appropriate water and wastewater treatment strategies. Biological monitoring of aquatic environments, despite the lower cost of measuring bacterial indicators such as fecal coliforms, *Enterococci*, and *Clostridium perfringens*, does not guarantee the absence of enteric viruses due to their resistance to chlorination and tertiary treatment. *Enteroviruses* are one of the viral indicators for monitoring of water quality and wastewater treatment systems, which have not yet been widely and commonly used. In this article, the practical adequacy of various new methods for the concentration and molecular identification of *Enteroviruses* in water and wastewater is assessed by evaluating reputable scientific databases.

Keywords: Waterborne viruses, *Enteroviruses*, Virus indicators, Biomonitoring, Concentration methods.

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Introduction

Notwithstanding impressive global progress in improving access to drinking water, approx. 600 million people globally regularly consume untreated water; it leads to many deaths related to ingesting waterborne pathogens (1-3). According to the EPA (Environmental Protection Agency) and RWQC (Recreational Water Quality Criteria) recommendation, the microbial quality of freshwater and reused wastewater is evaluated by surveying fecal indicator bacteria (4,5), *Enterococci* and *Escherichia coli,* and viral

Correspondence to: Mohammad Kargar
Tel: +98 9173149203
E-mail: m.kargar@zand.ac.ir
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pathogens are not usually incorporated into water quality monitoring schedules (6,7). Adenovirus, *enteroviruses*, noroviruses, astroviruses, rotaviruses, and hepatitis A viruses are mammalian waterborne viruses (8-10). Human *enteroviruses* (11) are shed in infected individuals' feces (up to 10^{11} viruses/g-feces), and infectious sewage can contaminate waters reused in agriculture, drinking water, and recreational water (12-14). EVs are caused about 30-90 % of waterborne disease (15). However, they are not routinely monitored in water samples because of some limitations, including time-consuming techniques and the necessity of virus concentrations (16,17). *Enteroviruses* can survive up to 130 days in water and sludge. Even at low doses, their ingestion is responsible for various syndromes, including Hand, foot, and mouth diseases (HfMD) (18), respiratory infections, encephalitis, aseptic meningitis, paralytic illness (including AFP), myocarditis, and gastroenteritis (19). Enterovirus A71 (EV71) is the prevailing etiologic agents of HFMD disease (20). Therefore, in case of suspected viral infections due to water pollution, the surveillance activities to rapidly and effectively identify enterovirus infections and determine disease-related serotypes could enhance public health protection alongside environmental protection from fecal contamination (21). Viral detection methods should be sensitive, fast, resistant to false-positive results, and inexpensive for drinking water and sewage (22). Although there are no effective treatments for enterovirus infections, nevertheless, their identification can provide the cause of the outbreak and, more importantly, the conditions for prevention. In addition, it will prevent physicians and public health authorities from inappropriate treatments and lead to significant savings in the health sector.

A) Enterovirus structure: *Enteroviruses* belong to *Picornaviridae*, a family of non-enveloped viruses with a positive-stranded RNA genome (~7.4 KB)(23,24). *Enteroviruses* consist of a single open reading frame (25), at each end, ORF is surrounded by untranslated regions (UTRs) and encodes a precursor polyprotein. This polyprotein is then cleaved through two virus-encoded proteases, 2A (2Apro) and 3C (3Cpro)(26, 27), to yield four capsid proteins (VP1-VP4) and seven non-structural proteins (Figure 1) involved in the virus life cycle (28,29).

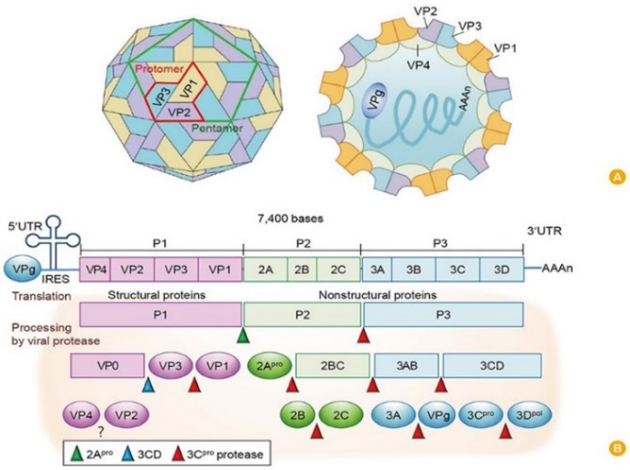

Fig 1. The genome structure of *enteroviruses* (30).

B) Global distribution and predominating genotypes: *Enteroviruses* are globally distributed and commonly cause asymptomatic infections. One of Europe's most significant outbreaks of enterovirus-related infections emerged in 2003 in Minsk, Belarus. The source of infection was water contaminated with ECHO 30, 6, and Coxackie B5 viruses. The number of patients referred to the hospital reached 1300 (31, 32). The next outbreak occurred in 2014 in Ontario, Canada, and EV-D68 infection associated with severe respiratory illness was laboratory confirmed among 16.9% of persons tested (33). 5'UTR has a high level of conservation

within the enterovirus genus, so it is primarily used to recognize all enterovirus genomes from other viral genera (34). The most increased variability is devoted to gene coding regions (especially the VP1sequences) which led to the subdivision of *enteroviruses* into different Species and serotypes (35). *Enteroviruses* are classified into five groups: poliovirus, coxsackievirus, echovirus, human *enteroviruses* (4 serotypes A-D and some non-human viruses (E-L) (Table 1) (36). Serological studies have identified 70 types of human enterovirus using their antibodies. Distinct enterovirus types can exhibit various biological properties related to virulence, transmissibility, and pathogenesis, and they cause different diseases. More than 100 human enterovirus types have been described (37,38). Given the nature of *enteroviruses*, many others with the capacity to make human disease will likely be discovered. Brouwer *et al.* have analyzed the global prevalence and genotypic distribution of *enteroviruses* in Africa, Asia, and Europe (39). Of the four types, enterovirus B was the most common all over. The rate of enterovirus A was exceptionally high in Asia. At the same time, Enterovirus C was the predominant species in Africa, and Enterovirus D was the second enormous species in Europe (39). Another study showed that the prevalence of different serotypes of EV-A varies considerably over time from place to place in Asia and Europe. Overall, EV-A71, CVA6, and CVA16 are a few of the foremost prevalent serotypes (40,41).

Table 1. Classification and taxonomy of *enteroviruses*.

Species			Serotypes
Coxsackievirus		A	CVA-2, CVA-3, CVA-4, CVA-5, CVA-6, CVA-7, CVA-8, CVA-10, CVA-12, CVA-14, and CVA-16.
		B	CVB-1, CVB-2, CVB-3, CVB-4, CVB-5, CVB-6, and CVA-9.
		C	CVA-1, CVA-11, CVA-13, CVA-17, CVA-19, CVA-20, CVA-21, CVA-22, and CVA-24.
Echovirus		B	E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-9, E-11 to E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E32, and E-33
Enterovirus	Human	A	EV-A71, EV-A76, EV-A89 to EV-A92, EV-A114, EV-A119, EV-A120, EV-A121, SV19, SV43, SV46, and BabEV-A13.
		B	EV-B69, EV-B73 to EV-B75, EV-B77 to EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 to EV-B113, and SA5.
		C	EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, and EV-C118.
		D	EV-D68, EV-D70, EV-D94, EV-D111, and EV-D120
	Non-human	E	EV-E1, EV-E2, EV-E3, EV-E4, and EV-E5
		F	EV-F1, EV-F2, EV-F3, EV-F4, EV-F5, EV-F6, and EV-F7.
		G	EV-G1 to EV-G20
		H	EV-H.
		I	EV-I1 and EV-I2.
		J	EV-J1, EV-J103, and EV-J108.
		K	EV-K1 and EV-K2
		L	EV-L1.
Rhinovirus		A	RV-A1, RV-A1B, RV-A2, RV-A7 through RV-A13, RV-A15, RV-A16, RV-A18 to RV-A25, RV-A28 to RV-A34, RV-A36, RV-A38 to RV-A41, RV-A43, RV-A45 to RV-A47, RV-A49 to RV-A51, RV-A53 to RV-A68, RV-A71, RV-A73 to RV-A78, RV-A80 to RV-A82, RV-A85, RV-A88 to RV-A90, RV-A94, RV-A96, and RV-A100 to RV-A108
		B	RV-B3 to RV-B6, RV-B14, RV-B17, RV-B26, RV-B27, RV-B35, RV-B37, RV-B42, RV-B48, RV-B52, RV-B69, RV-B70, RV-B72, RV-B79, RV-B83, RV-B84, RV-B86, RV-B91 to RV-B93, RV-B97, and RV-B99 to RV-B104
		C	RV-C1 to RV-C51, RV-C54, RV-C55, and RV-C56.
Poliovirus		C	PV-1, PV-2, and PV-3

C) Reduction and recovery of enteroviruses by treatment process: *Enteroviruses* are non-enveloped viruses with unique structures, making them highly tolerant to residual chlorine from sewage treatment and other viral-removal-water treatment strategies, such as UV irradiation, ozone, chlorine dioxide, peracetic acid, salinity, and temperature fluctuations (42). Simhon *et al.* have enumerated the *enteroviruses* in urban sewage effluent before and after disinfection by UV and chlorine at five wastewater treatment plants. They have revealed that the PCR-detected *enteroviruses* are still abundant in post-disinfection effluent (2.1×10^4 -7.2×10^5) gene copies (GC)/L) (43). Without appropriate disinfection, virus infectivity can be maintained for up to 60 days. These attributes significantly facilitate the *enteroviruses* survival in the aquatic environment (44). Therefore, advanced disinfection processes have been applied to maximize EV removal from wastewater and drinking water, such as combinations of ozone and UV radiation, hydrogen peroxide and ozone, hydrogen peroxide and UV radiation, titanium dioxide with UV radiation, and advanced membrane technologies (ultrafiltration 0.01-0.1 µm) (45, 46), However none of these technologies can completely eliminate EVs in treated water if water is supplied from inappropriate sources with high EV titers. Therefore, EVs are considered waterborne pathogens, and completely removing them in wastewater treatment plants (WWTPs) is difficult.

D) Enteroviruses concentration methods: The dose of enterovirus particles in aquatic environments is too tiny for direct detection, so relatively large volumes of sludge samples or wastewater (<100 mL) and large quantities of recreational and drinking waters (100–1,000 L) are often required, which must be condensed before any detection procedure (47,48). For this purpose, one or more in-series viral concentration methods have been developed. The ability to recover viral particles during the concentration process is one of the paramount which affect the efficiencies of downstream detection techniques (49). Virus concentration usually involves at least two steps. The initial concentration phase reduces the volume of water to 100- 500 ml, and the second phase reaches 2-10 ml (50). Adsorption/Elution, ultrafiltration (UF) (Dead-end ultrafiltration (DEUF) and Tangencial flow ultrafiltration (TFUF)), viral flocculation/precipitation with organic/inorganic flocculants, ultracentrifugation (UC), and centrifugal ultrafiltration (CeUF) are standard viral concentration methods of water samples (51). Brinkman *et al.* have suggested that celite (diatomaceous earth) concentration followed by nucleic acid extraction can result in 47–98% recovery of *enteroviruses* from wastewater (52). The virus adsorption/Elution method (VIRADEL) has a recovery rate of about 60-74%, which acts based on electrostatic interactions between viruses and electropositive/electronegative filters and elution by beef extract ,glycine, and polyethylene glycol (41). Eluates are then re-concentrated to reduce the volume sample and increase the efficiency of the detection methods (53). Electronegative filters are cost-effective and widely available with high recoveries for commonly tested *enteroviruses* (48). According to the EPA rules, using 1MDS electropositive filters for concentrating enteric viruses from water is required (54,55). However, these filters are not cost-effective for frequent virus monitoring. Karim et al. evaluated a cheap electropositive filter, NanoCeram. They have reported that NanoCeram can trap 84% of the poliovirus of tap water samples (100L).

Likewise, the recovery efficiency of echovirus7, coxsackievirus B5, and poliovirus was reported to be 32%, 27%, and 54%, respectively. Finally, they pointed out that *enteroviruses* recovery using NanoCeram is similar to or higher than the 1 MDS (56). Prata *et al.* has shown that the average efficiency of viral recovery by ultracentrifuge is 76% and 69% for recreational water and wastewater samples, respectively. The organic flocculation method (skimmed milk flocculation) included only 38% and 22% of the viral recovery, respectively. However, ultracentrifuge also has some difficulties, including the high cost and minimal sample volumes (10 mL to 1 L) (57). Hmaïed *et al.* have compared polyethylene glycol and ultracentrifugation (110,000×g) concentration methods for detecting *enteroviruses* in raw and treated sewage. Their results showed that the PEG-based method provided higher genome copies of *enteroviruses* (5.9 $\log_{10}$ genome copies/ 100 ml) from raw sewage samples. In contrast, the ultracentrifuge method reduced the number of genomic copies to 4.5 $\log_{10}$ genomic copies/ 100 ml. In this way, they stated that the PEG-based method is more precise for samples with high organic matter load (58). Hollow-fiber ultrafiltration (HFUF) as a primary concentration method can also effectively recovers (80%) poliovirus, echovirus 7, enterovirus 70, and coxsackievirus B4 from the large tap and river water volumes (59). In briefly, there are two concentration procedures: Pellet and Two-phase. The Pellet process, for the first time, is recommended by the authors of this study. To concentrate by this method, the supernatant was transferred to a sterile flask. Then from the remainder of sewage, 75 ml was transferred to 5 sterile centrifuge tubes and it was centrifuged for 10 min with 5000 rmp at 5° C and the tubes were kept at 4°C. The Two-phase process was performed by using the recommended technique of Hovi in 2001(60). For destroying the bacteria and fungus 1 ml of chloroform were added to 4 ml of the Straight, Pellet and Two-phase samples and were shake for 20 min whit 200 rpm. The containers of the tubes were centrifuged in 2000 rpm at 5°C and supernatant was collected in 1.8 ml sterile cryotube (Figure 2).

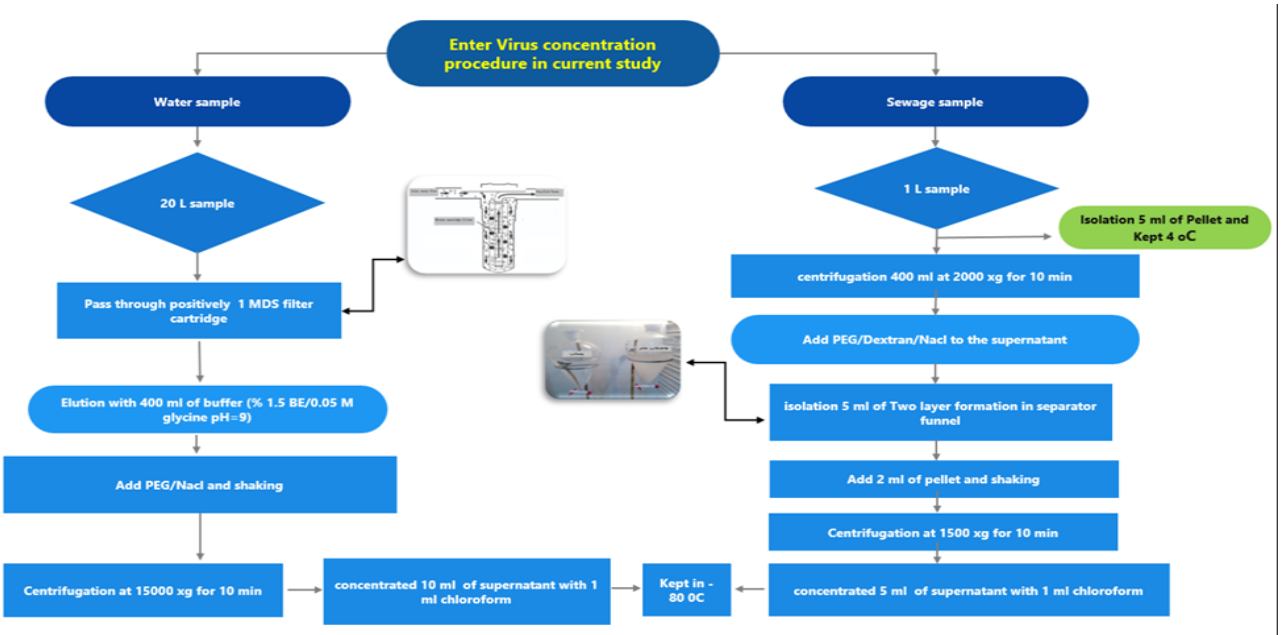

Fig 2. Flowchart depicting *enteroviruses* concentration in water and sewage: electropositive filter (Left) and two-phase (Right) methods.

E) Detection methods for enteroviruses in water and wastewater: The potential and capability of virus proliferation in cell culture indicate the pathogenicity of the virus (61). In this way, it is necessary to provide methods to identification virus in water and wastewater to assess the risks associated with its exposure. In this regard, this review has investigated the current methods to evaluate the infectivity of water-borne *enteroviruses*.

E.1. Cell culture: The cell-culture-based assay is deemed a gold standard for virus detection. The cell cultures are monitored daily for the appearance of cytopathic effects (CPE) by light microscopy (62, 63). Plaque assay, Most Probable Number (MPN), and 50% Tissue Culture Infectious Dose (TCID50) are used to quantify CPE-producing infectious viruses (64). These techniques are also used to identify enteric viruses in wastewater; however, the main challenge of *enteroviruses* cultivation is choosing a suitable eukaryotic host cell (65). Buffalo green monkey (BGM) continuous cell line is the most sensitive cell line for detecting *enteroviruses* in water and wastewater (66). However, a single cell line could not detect all *enteroviruses*, even those of the same genus (Table 2). As well as not all enteric viruses will produce plaque. In this way, the high cost of analysis, time-consuming, susceptibility to bacterial and fungal contamination, and problems related to non-cultivable enteric viruses (low sensitivity) are among the disadvantages of cell culture-based methods (67).

E.2. Biosensors: Biosensors are transportable bioanalytical devices with ultra-sensitivity and ultra-specificity, which for the most part, comprises an analyte, bio-receptor, signal transducer, and signal reader to detect biochemical interplays (75). Based on the transducers and bioreceptors, biosensors are organized into optical, electrochemical, mass-based, calorimetric, and thermometric (76,77). The development of biosensors dramatically helps to detect waterborne viruses and can be substituted for time-consuming conventional methods (78). Chauhan *et al.* developed a multimodal gold–aptamer nanoconstruct-based biosensor that detects conserved nucleic acid sequences amongst 96% of all known *enteroviruses* (79). Specific binding of aptamers to enterovirus RNA leads to converting the purple aggregated gold nanoparticles into the red disaggregated structure. It creates a signal transduction pathway that can be identified by spectroscopic, colorimetric, or lateral flow assays (80). In addition, the immunosensor based on thiol-modified gold nanoelectrodes also enabled the detection of *enteroviruses*. This biosensing method immobilizes specific monoclonal enterovirus antibodies on a gold electrode. Then the electrical properties of antibody-virus interaction are analyzed by electrochemical impedance spectroscopy (EIS) (81).

Table 2. Recommended cell culture systems for isolation and detection of waterborne *enteroviruses*.

Virus	Cell Line	Origin	Ref.
Coxsackievirus A	RD	Human skeletal muscle	(68)
	MRC5	Human fetal lung fibroblast cell	(69)
	HEK293A	Human embryonic kidney 293	(70)
Coxsackievirus B	BGM	Buffalo Green Monkey	(71)
	HeLa	Human cervical cancer cell	(72)
	HEp2	Human papillomavirus type 18	(73)
Enterovirus	RD	Human skeletal muscle	(68, 74)
Echovirus	MRC5	Human fetal lung fibroblast cell	(73)
Poliovirus	BGM	Buffalo Green Monkey	(71)
	MRC5	Human fetal lung fibroblast cell	(73)

F) Enterovirus nucleic acids detection for aquatic Biomonitoring

F.1. Reverse transcription polymerase chain

reaction (RT-PCR): The advent of molecular techniques facilitated the development of diagnostic tools with intensive sensitivity for detecting human pathogenic viruses with low concentration. Reverse transcription polymerase chain reaction (RT-PCR), multiplex RT-PCR, microarray, real-time or q-PCR, and gold nanoparticle-improved immuno-PCR have been widely developed to detect EVs (82-85). These have high specificity, sensitivity, and throughput; thus, they can detect the virus quickly and with higher reliability than traditional laboratory methods. However, PCR-based methods are susceptible to inhibition by environmental water matrices. They are strongly affected by environmental compounds such as humic and fulvic acids, heavy metal ions, and nucleases (86). These compounds can degrade the viral genome or interfere with polymerase and reverse transcriptase. Besides, because of the low concentration of enterovirus in environmental samples, inhibitors can lead to false negative results, and the risk of exposure is underestimated (87). In this way, removing inhibitors of environmental waters is one of the necessary steps to detect *enteroviruses* (88). Optimizing the sample concentration or nucleic acid extraction process by adding genome amplification enhancers (dimethyl sulfoxide, bovine serum albumin, or DNA carriers) or using optimized polymerases can be limited the effects of inhibitors (89,90). As well as in order to eliminate free viral nucleic acids and remove their adverse effects in detecting infectious viruses, various treatment methods can be done prior to PCR-based quantification (RT-qPCR and qPCR) (91-93). Enzymatic treatments, RNase or DNase coupled with proteinase K treatment, have been shown to eliminate free nucleic acids somewhat (94). Another approach is viability treatment using intercalating dyes such as propidium monoazide (PMA)/sodium lauroyl sarcosinate (93), ethidium monoazide (EMA), cis-dichlorodiammineplatinum (CDDP), and platinum chloride (PtCl4) (95). These covalently bind to nucleic acids preventing PCR amplification (95,96). Immunomagnetic separation (12) is also used to purify enterovirus particles from environmental samples and prevents PCR inhibition. Magnetic beads (Dynabeads M-280 sheep anti-mouse immunoglobulin G) are coated with mouse anti-enterovirus monoclonal antibody in this method (97).

F.2. Isothermal nucleic acid amplification: Isothermal amplification of nucleic acids (Recombinase polymerase amplification) is a rapid and efficient amplification performed under simple conditions (constant temperature without thermocycling) (91). Recently, isothermal amplification methods have been extended to detect a wide range of viral targets based on microfluidic chips and capillary platforms. Recombinase polymerase amplification (38) is an isothermal amplification method with high specificity and sensitivity to detect pathogens and viruses. RPA products can be analyzed using agarose gel electrophoresis, probe-based fluorescence monitoring, and lateral flow strips (98, 99). Recently, Xiaohan Yang et al. developed an RPA-LFS assay for rapid, specific, sensitive, and accurate assay detection of EV. They have shown that EV-RPA-LFS is an ideal diagnostic tool for detecting EVs, and its results were entirely consistent with the q-PCR assay's clinical performance (100).

F.3. Integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR): The fundamental constraint of molecular-based approaches is the incapability to assess the infectivity of detected viral particles (101). Only the virus propagating in a cellular model

can indicate this aspect (102), so to overcome this drawback, an integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) was developed for rapid and specific detection of both infectious and noninfectious *enteroviruses* from sewage, marine water, and surface drinking water sources (103, 104). EPA developed an enterovirus detection strategy from reagent-grade and ground waters known as Method 1615 based on cell Culture and RT-qPCR. The results showed that, in groundwater samples the recovery rate of poliovirus is 58% and 111% in reagent-grade water (105). Mayer et al. developed the target-specific ICC-RTqPCR technique for simultaneously detecting three types of *enteroviruses* (coxsackievirus B6, poliovirus 1, and echovirus 12) (106). Further on, Ryu *et al.* simultaneously detected four *enteroviruses* relevant to human health (coxsackievirus A10, echovirus 30, enterovirus 70, and poliovirus 1) using a developed ICC-RTqPCR in one test (107).

F.4. Next Generation Sequencing: Since mutation and frequent genetic recombination can be the origin of phenotypic and genotypic diversity in the *Enteroviruses* genome, whole genome sequencing can be very contributory and effective for surveillance, public health purposes, and basic research such as investigating the viral diversity in various geographic areas and populations (108,109). The traditional Sanger sequencing technique is capable of identifying the whole genome. However, it is time-consuming and cannot simultaneously sequence a mixture of viruses (110). Next generation sequencing (111) is an optimum alternative to the previous molecular methods based on ultra-high throughput, scalability, and speed. NGS could concomitantly detect the virus types and subtypes in a complex biological matrix (112). 454 Pyrosequencing, Ion Torrent, Illumina/Solexa, MinION, and ABI/Solid are various NGS platforms enabled for high-throughput sequencing of known and new -emerging viruses and could facilitate the detection of these viruses in water (42,113). Analyzing NGS genomic data by bioinformatics software provides an excellent platform for biomonitoring known and novel waterborne viruses in aquatic environments and wastewater (114). Nevertheless, NGS-based studies mainly face three significant challenges: the high cost of sample preparation, contamination, and the need for a strong and specialized computational infrastructure to analyze the results. High output of sequencing reads, assembly of millions of viral genomic reads, and identification and interpretation of these assembled genomes are some of the challenges of NGS data analysis (115). Joffret *et al.* have designed a rapid, sensitive sequencing approach to detect and genetically categorize all human *enteroviruses* (EV-A to -D) in a mixture of sewage concentrates using Illumina NextSeq HiSeq, and nearly 90% of the genome was sequenced (109). Bessaud *et al.* have developed a set of EV-C-specific primers for synthesizing RT-PCR products that cover the whole genome of EV-C, and have sequenced the RT-PCR products by Illumina sequencing technology (116). Before that, Baronti *et al.* also reported sequencing DNA amplicons covering the enterovirus A71 whole genome on an Ion Torrent Personal Genomic Machine System (117). They suggested that these techniques will serve as valuable tools for sequencing large panels of EVs during environmental surveillance.

Conclusion

The World Health Organization (WHO) emphasizes the quality of water (regardless of the type of consumption, such as drinking,

irrigation, or recreation) and the need for clean water free of various viruses, including enteric viruses that are easily spread among the society through the fecal-oral transmission route. Therefore, to ensure the sanitary safety of drinking water/wastewater, diagnostic methods, and analytical techniques must be sensitive, resistant to false-positive and false-negative results, and allow full automation. Although conventional methods are greatly applicable for the routine detection of viruses in clinical samples, they have never served alone as a preventive method to detect viral pathogens in contaminated resources such as aquatic environments. Considering the mentioned obstacles, there is an urgent need for new alternative methods that not only have the advantages of conventional methods and compensate for their deficiencies, but also are able to identify different serotypes of viral pathogens in contaminated water, and be able to identify enteric viruses in amounts much lower than conventional methods as well. Thus, due to the global concern for worldwide public health and waterborne outbreaks, we will always need to improve and identify the most effective diagnostic strategies to reduce health risks and improve water quality. Both viral and bacterial indicators are important in assessing water and wastewater quality, but their applications and meanings are different. Bacterial indicators (such as total coliforms and *Escherichia coli*) have long been used to assess fecal and sanitary contamination of water. These bacteria are usually easy to culture and count, and their presence indicates the possibility of the presence of enteric pathogens (including viruses). However, they also have disadvantages: Different persistence: Indicator bacteria may have different persistence in water and wastewater environments than pathogenic viruses. Uncertain correlation: the presence of indicator bacteria does not necessarily mean the presence of pathogenic viruses, and vice versa. Less sensitivity to treatment: some viruses (such as *Noroviruses* and *Adenoviruses*) may be more resistant to treatment processes than bacteria. In contrast, viral indicators can provide more accurate and relevant information, especially for the assessment of *Enteroviruses* in water and wastewater. *Enteroviruses* are a large group of viruses that can be transmitted through water and wastewater and cause various diseases (including polio, meningitis, and gastrointestinal diseases). The importance of viral indicators compared to bacterial indicators is due to the following reasons, first, direct relevance which means the use of viral indicators (such as non-pathogenic animal viruses or phages) can more directly indicate the presence of pathogenic viruses. Second, similar behavior in the environment which means indicator viruses can exhibit similar behavior to pathogenic enteroviruses in the environment (such as resistance to purification). Third, more accurate detection, the development of molecular techniques (such as PCR and qPCR), the detection and identification of viruses even at low concentrations has become possible recently.

Suggestions for molecular monitoring of *Enteroviruses* in water and wastewater:

A) Use of complementary viral indicators: In addition to bacterial indicators, viral indicators (such as somatic phages, RNA phages of the F genus, or non-pathogenic animal viruses) should be used as complementary indicators. These indicators can provide a more comprehensive view of viral contamination

B) Use of advanced molecular techniques: for the identification and quantification of *Enteroviruses*, molecular methods such as qPCR should be used. These methods have high speed, sensitivity, and specificity and also

allow the detection of non-culturable viruses.

C) Regular and targeted monitoring: regular and targeted monitoring programs should be developed to identify enteroviruses at key points in water and wastewater systems (such as the inlet and outlet of treatment plants, and drinking water distribution points).

D) Standardization of methods: sampling, virus concentration, and RNA/DNA extraction methods should be standardized so that the results are comparable and reliable

E) Genomic surveillance: where possible, next-generation sequencing (NGS) methods should be used to identify different strains of *Enteroviruses* and trace the source of infection. This will help to better understand the epidemiology and control the spread of diseases.

F) Data integration: results of viral and bacterial surveillance should be integrated with epidemiological data on waterborne diseases to enable a more comprehensive risk assessment and more effective preventive measures.

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Conflict of Interest

The authors declare no financial or non-financial competing interests that could affect the objectivity, integrity, or reporting of this study.

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