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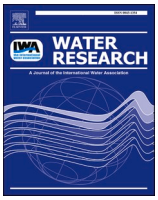

Human intestinal enteroids platform to assess the infectivity of gastroenteritis viruses in wastewater

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ABSTRACT

Fecal-orally transmitted gastroenteritis viruses, particularly human noroviruses (HuNoVs), are a public health concern. Viral transmission risk through contaminated water results underexplored as they have remained largely unculturable until recently and the robust measuring of gastroenteritis viruses infectivity in a single cell line is challenging.

This study primarily aimed to test the feasibility of the human intestinal enteroids (HIE) model to demonstrate the infectivity of multiple gastroenteritis viruses in wastewater. Initially, key factors affecting viral replication in HIE model were assessed, and results demonstrated that the reagent-assisted disruption of 3D HIE represents an efficient alternative to syringe pass-through, and the filtering of HuNoV stool suspensions could be avoided. Moreover, comparable replication yields of clinical strains of HuNoV genogroup I (GI), HuNoV GII, rotavirus (RV), astrovirus (HAstV), and adenoviruses (HAdV) were obtained in single and multiple co-infections. Then, the optimized HIE model was used to demonstrate the infectivity of multiple naturally occurring gastroenteritis viruses from wastewater. Thus, a total of 28 wastewater samples were subjected to (RT)-qPCR for each virus, with subsequent testing on HIE. Among these, 16 samples (57 %) showed replication of HuNoVs ($n = 3$), RV ($n = 5$), HAstV ($n = 8$), and/or HAdV ($n = 5$). Three samples showed HuNoV replication, and sequences assigned to HuNoV GI.3[P13] and HuNoV GII.4[P16] genotypes. Concurrent replication of multiple gastroenteritis viruses occurred in 4 wastewater samples. By comparing wastewater concentrate and HIE supernatant sequences, diverse HAstV and HAdV genotypes were identified in 4 samples.

In summary, we successfully employed HIE to demonstrate the presence of multiple infectious human gastroenteritis viruses, including HuNoV, in naturally contaminated wastewater samples.

1. Introduction

Human gastroenteritis viruses are an important cause of morbidity and mortality in humans of all ages with acute gastroenteritis worldwide. They include rotavirus (RV), human norovirus (HuNoV), human astrovirus (HAstV), sapovirus and human adenovirus (HAdV) (Chatterjee et al., 2004; GBD 2015, 2016; GBD 2016, 2018; Qi et al., 2018; Valcarce et al., 2021). These viruses are excreted at high concentrations (up to 10^{12} viruses/g feces) by infected individuals and transmitted to other humans via the fecal-oral route (Haramoto et al., 2018). Given the high persistence of these viruses in wastewater and their high transmissibility, their presence in drinking water or irrigated foods could pose substantial public health risks. Many foodborne virus outbreaks have been linked to produce or bivalve molluscs contaminated by fecally impacted waters (EFSA and ECDC, 2022). Traditionally, detection of gastroenteritis viruses in environmental samples relies on molecular methods and realtime polymerase chain reaction (PCR) detection methods are widely used. However, for viruses such as HuNoV, the lack

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of a robust cell culture method to determine viral infectivity hampered assessing public health risks (Condit, 2013; Gerba et al., 2018; Hamza et al., 2011). A major breakthrough was the successful replication of HuNoV in non-transformed stem cell-derived human intestinal enteroids (HIEs) (Costantini et al., 2018; Desdouits et al., 2022; Ettayebi et al., 2021; Ettayebi et al., 2016; Randazzo et al., 2020). In addition to HuNoV, the HIE model has also been successfully employed to demonstrate replication of sapovirus, HAstV, HAdV, and RV (Euller-Nicolas et al., 2023; Holly and Smith, 2018; Kolawole et al., 2019; Saxena et al., 2016).

As recently estimated with the HIE model, HuNoVs retain their infectivity for 7 to 28 days in tap, surface and seawater (Desdouits et al., 2022; Kennedy et al., 2023; Shaffer et al., 2022), which is a relevant data to implement prevention and control strategies for viral infections transmitted through contaminated waters (Pandiscia et al., 2024). However, experimental evidence for HuNoV infectivity and simultaneous detection of different gastroenteritis viruses in wastewater samples using the HIE model has not been reported.

This study aimed to assess the feasibility of the HIE model to detect replication of co-infections of different human gastroenteritis viruses in naturally contaminated wastewater samples. To this end, a protocol for passaging HIE and differentiate HIE monolayers was optimized, single and concurrent replication of diverse gastroenteritis virus in optimized HIE model assessed, and finally it was applied to evaluate viral infectivity in wastewater. Thus, this HIE platform could contribute to a deeper comprehension of viral behaviour in wastewater, with relevant implications for public health and environmental safety.

2. Material and methods

2.1. Wastewater sampling and concentration

A total of twenty-eight wastewater grab samples were collected between June and August 2022 from eleven urban wastewater treatment plants across Spain with equivalent inhabitant values ranging from 60,600 to 1900,800. The samples were transferred on ice to the laboratory, and processed within 24 h. Briefly, 200 mL of each wastewater sample was centrifuged at 4654 × *g* for 30 min to remove larger particles. The viral particles were then concentrated from the supernatants using Centricon Plus-70 centrifugal filter devices with a cut-off of 10 kDa

Table 1
Human gastroenteritis viruses tested for replication on jejunal HIEs.

Sample ID	Virus	Genogroup / Genotype / Strain	Collection Date[a]	Patient	Mean RNA/DNA copies/ µL	Virus RNA/DNA increase (log_{10})[b]
S1	Human norovirus	GI.4	2011		2.20×10^6	
S2		GI [P2]	2018		3.13×10^5	
S3		GI [P7]	2018		1.74×10^7	
S4		GI.3[P13]	2022 May	Pediatric, 2y 4 m, female	1.44×10^7	1.03
S5		GI	2019		1.13×10^6	
S6		GI	2017		2.73×10^5	
S7		GI	2016		4.88×10^7	
S8		GI	2011		3.11×10^5	
S9		GI	2013		1.17×10^7	
S10		GI	2011		2.14×10^5	
S11		GI	2014		2.47×10^6	
S12		GI			1.73×10^7	
S13		GI			1.21×10^7	
S14		GI			1.28×10^7	
S15		GI			2.59×10^8	
S16		GI			3.03×10^7	
S17		GI.3	2018	Pediatric, 1 m 6 d, female	4.99×10^6	
S18		GI.3	2019	Pediatric, 2y 3 m, male	9.35×10^5	
S19		GI.3	2020	Adult, 45y, male	4.40×10^7	
S20		GI.4			1.07×10^7	
S21		GI.4	2018	Pediatric, 1y 3 m, male	3.33×10^5	
S22		GI.4	2019	Pediatric, 1y 8 m, female	9.66×10^7	
S23		GI.7	2020	Pediatric, 1y 9 m, male	3.09×10^7	
S24		GI.1		Adult, 62y	9.44×10^6	
S25		GI.1		Adult, 96y, male	2.24×10^7	
S26		GI.1	2020	Pediatric, 4y 5 m, female	7.22×10^5	
S27		GI.1	2020	Adult, 63y, female	6.05×10^6	
S28		GII.3 [P12]	2021 Oct	Pediatric, 1y 7 m, female	1.16×10^8	3.44
S29		GII.3 [P12]	2021 Sept	Pediatric, 1y 9 m, female	1.44×10^7	2.12
S30		GII.4 [P31]	2021 Sept	Pediatric, 2y, male	1.37×10^7	4.63
S31		GII.4 [P31]	2021 Sept	Pediatric, 1y 28d, male	2.65×10^7	3.90
S32		GII.4 Sydney			2.51×10^7	3.29
S33		GII.4 Sydney			3.56×10^8	2.79
S34		GII.4 Sydney			2.24×10^7	3.28
S35		GII.4 Sydney [P16]	2021 Oct	Pediatric, 10 m, male	1.07×10^8	3.33
S36	Human rotavirus	G9 [P8]	2019 May		3.14×10^8	1.00
S37		G9 [P8]	2019 June	Pediatric, 6y 6 m, female	1.47×10^8	1.24
S38		G9 G12 [P8]	2019 June	Pediatric, 5y, male	2.15×10^7	2.05
S39		G1 [P8]		Wa, cell culture adapted strain	3.23×10^8	2.26
S40		G3 [P8]		Ito, cell culture adapted strain	8.88×10^8	2.85
S41	Human adenovirus		2022 May	Pediatric, 1 year, male	7.94×10^{10}	3.69
S42	Human astrovirus		2022 May	Pediatric, 4 years, male	2.79×10^9	3.24

[a] When month was not available, only year of collection is reported.
[b] Virus RNA/DNA increase at 48 hpi expressed as log_{10} fold increase. For each fecal filtrate, data represent highest mean viral RNA/DNA increase among 1:10, 1:100, 1:10,00 and 1:10,000 dilutions tested in tree replicates each.

(Millipore, the Netherlands) and repeated centrifugation steps at 1500 × *g* for 15 min. The concentrate (1–2 mL) was serially filtered through 5 μm, 1.2 μm, 0.8 μm, and 0.45 μm polyvinylidene fluoride (PVDF) filters, and diluted 1:10 in infection media for immediate infection of the HIE monolayers, while remaining aliquots were stored at −80 °C.

2.2. Gastroenteritis viruses used in the study

Forty-two gastroenteritis virus-positive stool specimens (IDs: S1-S42) were tested on HIE, including 35 HuNoV-positive specimens, 3 human rotavirus-positive specimens, 2 cell-adapted rotavirus strains (Ito and Wa), a HAdV-positive specimen, and a HAstV-positive specimen (Table 1). Fecal specimens were stored at –80 °C until testing.

2.3. Optimization of the human intestinal enteroid model for viral replication

Three-dimensional (3D) HIE derived from human jejunal biopsy (J2 cell line) were provided by Dr. Mary K. Estes (Baylor College of Medicine, Houston, TX).

The impact of three key experimental variables on viral replication was investigated to optimize the HIE for environmental studies: (i) mechanical vs reagent-assisted disruption of 3D HIE cells prior to passaging and single cell seeding; (ii) preparation of viral suspensions with or without filtering steps, and (iii) single vs multiple viral replication. The experiments to assess (i) and (ii) were conducted using HuNoV in a round robin test involving two different laboratories.

Two alternative procedures for disrupting and passaging 3D HIE were compared. The originally reported passage of 3D undifferentiated HIE using 2× pipetting steps with a 25Gx5/8 syringe (Ettayebi et al., 2016; Zou et al., 2019) was compared to incubation with Gentle Cell Dissociation Reagent (STEMCELL Technologies, BC, Canada) (Carmona and Randazzo, 2023).

Three HuNoV-positive stool samples from hospitalized children with acute gastroenteritis (ID:S34 for HuNoV GII.4 Sydney; ID:S28 for HuNoV GII.3 [P12]; ID:S35 for HuNoV GII.4 Sydney [P16]) were diluted to 10 % in sterile phosphate-buffered saline and divided into portions used to compare two alternative procedures for preparing viral suspensions. An aliquot of each sample was vortexed for 30 s, and the supernatant was recovered after centrifugation at 1500 × *g* for 1 min (unfiltered samples). Alternatively, 10 % fecal suspensions were serially filtered through PVDF filters with pore sizes of 5 μm, 1.2 μm, 0.8 μm, and 0.45 μm, as originally described (Ettayebi et al., 2016).

HuNoV GI.3[P13] (Sample ID: S4) and GII.4 Sydney [P16] (Sample ID: S35), human RV G9 G12 [P8] (Sample ID: S38), HAdV (Sample ID: S41), and HAstV (Sample ID: S42) were inoculated into HIE monolayers alone (single infections) or concomitantly (multiple infections including all the five pathogens) and the rate of replication was determined after 48-hour post-infection (hpi).

2.4. HIE infection

Undifferentiated 3D HIEs and differentiated monolayers were routinely maintained and produced using commercial IntestiCult™ Organoid Growth Medium (Human) as described (Carmona and Randazzo, 2023). To determine the infectivity of human gastroenteritis viruses, (RT)-qPCR was used to quantify viral RNA/DNA from HIE monolayers at 1 and 48 hpi (Costantini et al., 2018; Ettayebi et al., 2016). For this purpose, two sets of 96-well plates with 100 % confluent 7–10 day-old-differentiated HIE monolayers were inoculated in triplicate with 100 μl of fecal suspension (diluted at 1:10, 1:100, 1:1000, and 1:10,000) or wastewater concentrate diluted at 1:10 in infection media supplemented with 500 μM sodium glycochenodeoxycholate. Infections were performed in triplicate (technical replicates), and experiments repeated at least twice. For each set of infections, one 96-well plate was immediately frozen at −80 °C at 1 hpi and the second plate was incubated at 37 °C and 5 % CO_2 for 48 h and then frozen (48 hpi). Cytotoxicity was monitored for all infections by visual/microscopic inspection of HIE monolayers.

2.5. Extraction, detection, and quantification of gastroenteritis viruses

Total nucleic acid was extracted from wastewater concentrates, and from supernatants and cell lysates of infected HIEs using the Maxwell® RSC Instrument (Promega, Spain) and the Maxwell RSC Pure Food GMO and authentication kit (Promega) (Pérez-Cataluña et al., 2021). The viral genomes were detected and quantified using TaqMan RT-qPCR with the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen, MA, USA) for RNA viruses, and the Premix Ex TaqTM kit (Takara Bio Inc., Japan) for HAdV on a LightCycler 480 instrument (Roche Diagnostics, Germany) (see Supplemental section S1 and Table S1 for details).

2.6. Gastroenteritis virus genome amplification, and sequence analyses

Sixteen wastewater samples that demonstrated replication on HIE at 48 hpi for at least one human gastroenteritis virus were chosen for sequence analysis. Briefly, viral genomes extracted from the wastewater inocula used for HIE infections and the supernatants of infected HIE at 48 hpi were used as templates to generate viral amplicons.

Complementary DNAs were synthesized, amplified and semi-nested PCR assays were performed to obtain PCR products for Sanger sequencing (Aladin et al., 2010; Belliot et al., 2001, 1997; Beuret et al., 2002; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gómara et al., 2004, 2001, 2000; Kojima et al., 2002; Mena and Gerba, 2009; Pina et al., 1998). All sequences that passed the quality and control checks were deposited into GenBank with accession numbers OR252328 for HuNoV GI, OR252346 and OR252347 for HuNoV GII, from OR260001 to OR260015 for RV, from OR260023 to OR260032 for HAstV, and from OR260016 to OR260022 for HAdV.

Phylogenetic trees were built using viral sequences obtained from previous wastewater (Santiso-Bellón et al., 2020) and clinical (Navarro-Lleó et al., 2022) surveillance studies in Spain, as well as reference clinical sequences retrieved from GenBank (see Supplemental Section S2 and Table S2 for details).

3. Results

3.1. Human gastroenteritis virus replication in optimized human intestinal enteroid model

Based on the integration of data from multiple experiments, we observed that reagent-assisted disruption of 3D HIE using GCDR was an efficient alternative to syringe pass-through. The optimized protocol allowed (i) efficient passage of the J2 HIE line from P17 to >P43, (ii) induced differentiation without affecting cell differentiation or monolayer formation, and (iii) supported replication of various genetically distinct gastroenteritis viruses. The concentration of the number of single cells for monolayer set up was reduced to 10^4 cells/mL from the initial 10^6 cells/mL, resulting in a 3D dome:differentiated monolayer ratio of 1:6 and improved overall efficiency. However, at HIE passage higher than 30, a ratio of 1:4 was required to obtain monolayers.

In a round robin test, six samples were tested in two different laboratories. Results demonstrated that HuNoV positive stool suspensions showed similar levels of viral replication in either GCDR or syringe pass-through derived monolayers (Fig. 1). There were no statistical differences between the two monolayers preparation methods based on 1 hpi data, while two out of six samples (Filtered ID:S34 diluted 1:100, and unfiltered ID:S35 diluted 1:1000) showed statistically significant differences in viral replication after 48 hpi (Fig. 1). Moreover, replication rates of unfiltered and filtered HuNoV GII.4 Sydney [P16] fecal suspensions showed no statistical differences (Fig. 1).

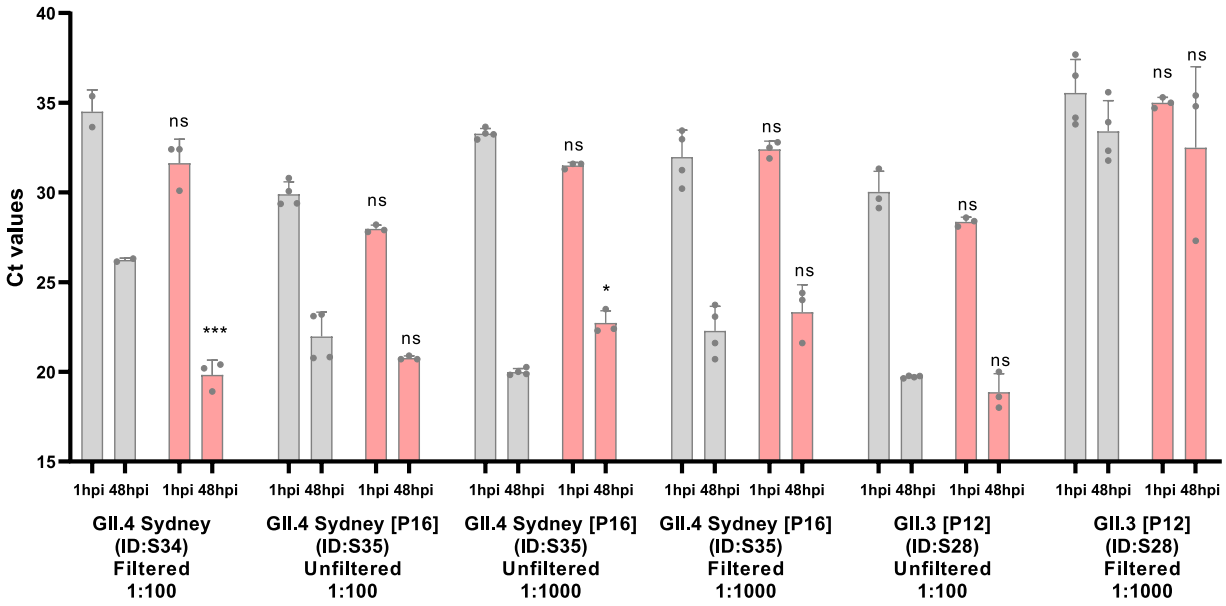

Fig. 1. Replication of human norovirus from either filtered or unfiltered fecal suspensions in human intestinal enteroids maintained and differentiated according to reagent-assisted disruption (grey bars, Lab 1) or syringe pass-through (red bars, Lab 2). Data represent Ct values of 3 replicates for each experiment. Specimens ID according to Table 1. For each sample and each infection time point, 1-way analysis of variance followed by Dunnett's test was performed. P-values of 1 hpi and 48 hpi infections in Lab2 are compared with those of Lab1: ns, not significant, $*p < 0.05$, $***p < 0.001$. hpi, hours postinfection.

A total of 40 stool suspensions and two rotavirus cell-adapted strains were tested for viral infectivity in HIE (Table 1). Only one HuNoV GI sample (ID:S4) replicated on HIE exhibiting a 1.03 $\log_{10}$ RNA increase. All HuNoV GII samples successfully replicated on HIE showing an average of 3.35 $\log_{10}$ RNA increase (Table 1). Among different HuNoV GII variants, GII.4 showed 3.54 $\log_{10}$ RNA increase on average, which resulted higher than GII.3 [P12] (2.78 $\log_{10}$ RNA). HIEs did not support virus replication of the majority of HuNoV GI positive stool samples, and the increase in yields for HuNoV GI (ID:S4) was significantly lower than that observed for GII HuNoVs. All three rotavirus-positive stool suspensions replicated on HIE with a mean 1.43 $\log_{10}$ RNA increase. Wa and Ito cell-culture adapted strains showed higher replication (2.26 and 2.85 $\log_{10}$ RNA increase, respectively) than rotavirus-positive specimen (1.00–2.05 $\log_{10}$ RNA increase). Furthermore, both HAstV and HAdV samples successfully replicated, showing 3.24 and 3.69 $\log_{10}$ DNA/RNA increase, respectively.

Single and multiple viral (co)-infections on HIEs were performed using HuNoV GI, HuNoV GII.4, rotavirus, HAdV, and HAstV (Fig. 2). The results demonstrated the successful and concurrent replication of multiple gastroenteritis viruses present in the same sample on HIE monolayers. At 1 hpi, viral attachment was not affected when single or multiple viruses were inoculated. The titer of attached virus (1 hpi) ranged between 2.32 (HuNoV GII) and 3.49 (rotavirus) $\log_{10}$ genome copies (GC)/well. Moreover, the replication yields at 48 hpi for HuNoV GI, HuNoV GII, RV, and HAdV showed no statistical differences between single and multiple (co)-infections. Replication in single and multiple infections was deemed as statistically significant for HAstV ($p = 0.004$), even though the differences in titer resulted in 0.16 $\log_{10}$. HuNoV GII showed the highest replication rate (3.48 $\log_{10}$), followed by HAdV (2.96 $\log_{10}$) and HAstV (2.77 $\log_{10}$), while HuNoV GI showed the lowest (0.65 $\log_{10}$) (Fig. 2).

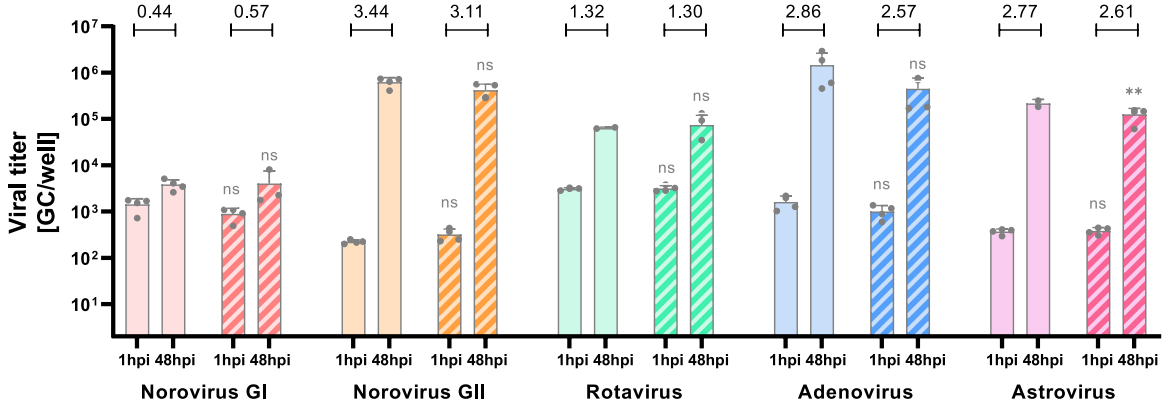

Fig. 2. Comparison of human gastroenteritis virus replication in human intestinal enteroids (HIEs) infected by single (plain bars) versus multiple (striped bars) viral pathogens. Multiple co-infections included HuNoV GI.3[P13] (ID: S4) and GII.4 Sydney [P16] (ID: S35), human RV G9 G12 [P8] (ID: S38), HAdV (ID: S41), and HAstV (ID: S42). The $\log_{10}$ increase of each virus in single and multiple infections is provided on top of each set of bars.
For each virus, a 1-way analysis of variance followed by Dunnett's test was performed. The P-values obtained from multiple infections were compared with those from single infections: ns, not significant, $**p = 0.04$. hpi, hours postinfection.

3.2. Replication of multiple gastroenteritis viruses from wastewater samples on HIE

Initially, a subset of 6 samples was used to validate the concentration procedure of virus from wastewater samples in terms of viral recovery efficiency and cytotoxicity on HIE monolayers. Centricon concentration with or without additional PVDF filters increased HuNoV GI, HuNoV GII and RV RNA titers by up to 2 $\log_{10}$ GC/L compared to direct extraction from unconcentrated wastewater (data not shown). Furthermore, the Centricon concentration coupled with PVDF filtering procedure showed the lowest cytotoxicity and thus selected for concentrating 28 wastewater samples.

Among the 28 wastewater samples analysed by (RT)-qPCR, 24 (86 %) tested positive for all five targeted gastroenteritis viruses. Two samples did not contain HuNoV GI or HAdV (Fig. 3A). The mean viral load in the wastewater samples was 5.08±1.49 $\log_{10}$ GC/L for HuNoV GI, 8.07±0.42 $\log_{10}$ GC/L for HuNoV GII, 8.28±0.66 $\log_{10}$ GC/L for RV, 6.88±0.76 $\log_{10}$ GC/L for HAstV, and 7.64±2.23 $\log_{10}$ GC/L for HAdV.

HIE monolayers were infected with each of the 28 concentrated wastewater samples. Specific cut-off criteria were adopted to designate a wastewater sample as infectious: (i) no cytotoxic effect on monolayers at 48 hpi, (ii) at least a 3-fold RNA/DNA increase in HIEs culture (Desdouits et al., 2022), and (iii) quantifiable viral load at both 1 hpi and 48 hpi with statistically significant difference between time points. Sixteen of 28 wastewater samples (57 %) showed viral replication that met these criteria (Fig. 3B). Single infectious viruses were detected in 12 wastewater samples. Infectious RV, HAstV, and HAdV were detected in Sample 2, while Samples 8, 11 and 22 showed replication of two different gastroenteritis viruses (HuNoV GI/HAstV, HuNoV GI/RV, HAstV/HAdV, respectively) (Fig. 3B). Sample 2 had the highest replication yields with increases of 4.37 for HAstV and 3.42 $\log_{10}$ for HAdV. In contrast, 1.01 to 1.33 $\log_{10}$ increases were detected for HuNoV GI and GII in Sample 7, 8, or 11. RV increased from 1.02 (Sample 11) to 1.40 $\log_{10}$ (Sample 2), HAstV from 1.04 (Sample 19) to 4.37 $\log_{10}$ (Sample 11), and HAdV from 1.03 (Sample 8) to 3.42 $\log_{10}$ (Sample 2).

No significant correlation could be established between the viral load detected by RT-qPCR in wastewater samples and successful viral replication on HIEs. For example, wastewater samples with lower HAstV titers (5.74 $\log_{10}$ GC/L) showed successful replication in HIEs, while samples with higher titers (8.85 $\log_{10}$ GC/L) did not. Similarly, HuNoV GI detected at levels of 5.86±0.16 (Sample 8) and 6.10±0.03 $\log_{10}$ GC/L (Sample 11) successful replicated in HIEs with 1.33 and 1.27 $\log_{10}$ increases, respectively. However, other samples did not exhibit replication in HIEs despite having comparable viral titers. Likewise, HuNoV GII viruses detected at 8.59±0.01 $\log_{10}$ GC/L in wastewater (Sample 7) yielded a replication rate of 1.01 $\log_{10}$ in HIEs, while samples at higher concentrations did not. Upon ranking all wastewater samples according to initial viral load, successful replication of either HuNoV GI or GII in HIEs occurred in samples within the first quartile of the ranked list (>5.08 $\log_{10}$ GC/L for HuNoV GI, and >8.32 $\log_{10}$ GC/L for HuNoV GII). In other words, those wastewater samples with higher HuNoV loads tended to result in successful replication in the HIE system. Unlike HuNoV, the successful replication of RV, HAstV, and HAdV in the HIE system did not exhibit a consistent pattern based on their viral load in wastewater samples.

3.3. Genotypes of gastroenteritis viruses replicating in HIE from wastewater

Confirmation of viral replication in HIE infected with wastewater samples was achieved by comparing viral sequences from wastewater nucleic acid and supernatants and cell lysates from HIEs (Table 2). However, in some positive samples the genotype could not be determined.

A HuNoV GI.3[P13] was detected from Sample 8 infected HIEs, while HuNoV GII.4 Sydney [P16] was detected from both wastewater and HIEs from Sample 7 (Figures S1, S2). The capsid sequences from wastewater and HIEs in Sample 7 were identical while the polymerase sequences showed 99.5 % identity.

RV G3 was detected in wastewater and HIEs from Sample 11 and

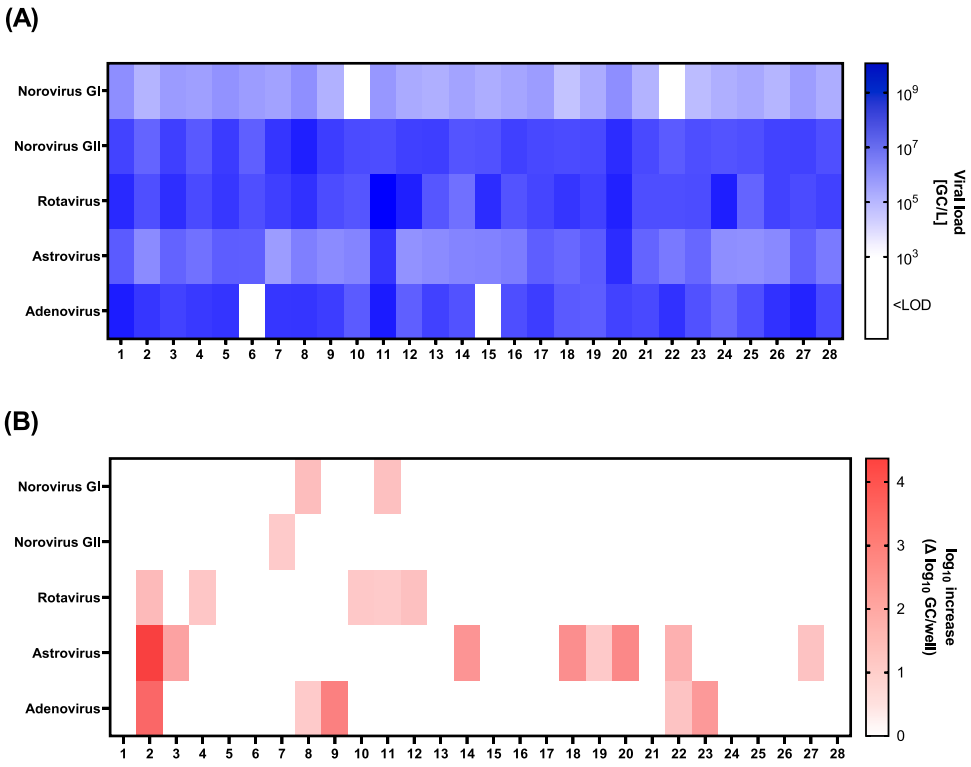

Fig. 3. Heat map of gastroenteritis virus concentrations in influent wastewater samples (A) and viral replication from these samples on human intestinal enteroids (B).

Table 2
Genotypes of gastroenteritis viruses in wastewater samples replicating in HIE.

Sample		HuNoV	RV		HAstV	HAdV
#	Type		VP7	VP4		
2	WW			P[8]	HAstV-4	ND
	HIEs			P[8]	HAstV-4	HAdV-41
3	WW				HAstV-4	
	HIEs				HAstV-4	
4	WW		ND	P[8]		
	HIEs		G9	P[8]		
7	WW	GII.4[P16]				
	HIEs	GII.4[P16]				
8	WW	ND				HAdV-41
	HIEs	GI.3[P13]				ND
9	WW					HAdV-40
	HIEs					HAdV-41
10	WW		G9+G12	P[8]		
	HIEs		ND	P[8]		
11	WW	ND	G3			
	HIEs	ND	G3			
12	WW		G3	P[8]		
	HIEs		G3	P[8]		
14	WW				ND	
	HIEs				ND	
18	WW				HAstV-8	
	HIEs				HAstV-1	
19	WW				ND	
	HIEs				ND	
20	WW				HAstV-8	
	HIEs				HAstV-8	
22	WW				HAstV-8	HAdV-41
	HIEs				HAstV-1	ND
23	WW					HAdV-5
	HIEs					HAdV-41
27	WW				ND	
	HIEs				ND	

Abbreviations: WW, wastewater; HIEs, human intestinal enteroids; ND, not determined (either the sequence was not identified or the genotype assigned based upon available sequence).

Sample 12, while RV G9 was found in HIEs from Sample 4 and in wastewater coupled with G12 from Sample 10 (Figure S3). Notably, G12 sequence was 100 % similar with a Spanish clinical sequence (ESPv148) and 96 % similar with sequences from a previous Spanish wastewater surveillance study (R5 and R15). While G9 genotypes clustered with previous sequences from Spanish wastewater samples, G3 did not. For the VP4 gene, P[8] was the only genotype obtained from all RV positive samples and all sequences clustered together (Figure S3D). All HAstV sequences belonged to group A of the MAstV-1 genus (Figure S4). HAstV-4 was identified in wastewater and HIEs from Sample 2 and Sample 3, while HAstV-8 was found in wastewater and HIEs from Sample 20. Intriguingly, both Sample 18 and Sample 22 displayed HAstV-8 sequences in wastewater, while HAstV-1 sequences were obtained from the corresponding HIE supernatants. Four sequences from wastewater and three from HIE samples were successfully assigned to human mastadenovirus F or C species (Figure S5). Specifically, sequences from wastewater showed 100 % similarity with HAdV-41 (Sample 8, Sample 22) or 96 % similarity with HAdV-40 (Sample 9). The sequence from wastewater Sample 23 shared 99 % identity with HAdV-5 belonging to Human mastadenovirus C species. All three sequences from HIE supernatants clustered with HAdV-41 (Sample 2, Sample 9, and Sample 23).

4. Discussion

The detection of infectious viruses in wastewater holds crucial public health implications as understanding their dynamics and persistence is imperative for assessing potential risks and refining reclamation procedures to safely discharge and reuse water resources. Our data demonstrate the successful replication of multiple gastroenteritis viruses in HIE monolayers from urban wastewater samples. By using the HIE model it was demonstrated that HuNoV remained infectious for between 7 and 35 days in artificially spiked tap water, creek and seawater samples (Desdouits et al., 2022; Kennedy et al., 2023; Shaffer et al., 2022), but there is no information on wastewater. We showed replication of HuNoV GI.3[P13], GII.4 Sydney [P16], RV G3, G9 and P[8], HAstV-1, −4, and −8, and HAdV-41 genotypes, all of which are predominant among the Spanish population (Navarro-Lleó et al., 2022; Pérez-Ortín et al., 2019; Vu et al., 2019). Concomitant detection of infectious HuNoV GI and GII, RV, HAstV and HAdV from wastewater samples demonstrates that HIEs can support replication of a wide diversity of human infectious gastroenteritis viruses present in a single wastewater sample.

We used optimized methodologies for testing viral infectivity in wastewater samples including the use of GCDR to disrupt 3D HIE for passaging and setting up cell monolayers. Our data also suggest that the serial filtration steps for stool suspension preparation as in the original publication of Ettayebi may not be necessary (Ettayebi et al., 2016), as reported recently (Overbey et al., 2021).

The viral load of gastroenteritis viruses in our samples was comparable to those reported in previous studies (Farkas et al., 2018; Haramoto et al., 2018; Santiso-Bellón et al., 2020). This indicates that the Centricon-filtering procedure effectively recovers viral particles while maintaining their infectivity. These implemented methodologies are essential for matching the HIE model with the specific needs of food and environmental virology laboratories.

Another relevant aspect of our study is the establishment of a biobank of infectious gastroenteritis virus-positive fecal specimens that show successful replication on HIEs. As reported previously, HuNoV GII.4 viruses exhibit higher replication yields compared to other genotypes (Costantini et al., 2018; Ettayebi et al., 2021), while the majority of GI noroviruses failed to replicate (Costantini et al., 2018; Ettayebi et al., 2016). This discrepancy in replication success could be attributed to the lower mean concentrations of GI in stools compared to GII (Ettayebi et al., 2021), to the loss of infectious particles due to long period storage, and to other unknown factors in the stool or media supplements required for successful infection (Estes et al., 2019; Murakami et al., 2020).

Concurrent infections were also studied using the HIE model. Similar replication rates were observed in both single- and multiple- (co)infections (Fig. 2), strengthening the hypothesis that the HIE model can simultaneously show replication of multiple viruses from stool samples and contaminated wastewater. Cell culture systems have been used to detect infectious gastroenteritis viruses from environmental samples. However, a single cell culture system capable of replicating multiple viruses, including HuNoV, has been lacking. Continuous Caco-2 cell line has been used to isolate RV, HAdV, HAstV, coxsackieviruses, reovirus, and poliovirus, while Buffalo green monkey (BGM) cell line was selected by the United States Environmental Protection Agency for detecting human enteroviruses in water (Gerba and Betancourt, 2019). In contrast, HIEs, which maintain relevant *in vivo* features, are able to support the replication of a wider variety of gastroenteritis viruses (Euller-Nicolas et al., 2023; Giobbe et al., 2021; Peña-Gil et al., 2023; Zhao et al., 2021). In our study, not all viruses detected in wastewater samples replicated in the HIE model (Fig. 3). Importantly, the absence of replication does not necessarily indicate that a sample is non-infectious, as there may still be infectious viral particles present at levels below the limit of detection of the HIE assay (10^{3-4} GC/well). Additional factors, such as model preferences for certain viruses or disturbances caused by physical and chemical substances (e.g., unspecific binding, undetected cytotoxicity), may have also influenced the observed replication rates.

False-negative outcomes carry significant implications for public health responses. Therefore, it is imperative to ensure quality assurance and quality control procedures, representative sampling approaches, effective virus concentration, and clear guidelines for data interpretation before the HIE platform can be considered for wastewater monitoring and/or water reuse purposes. To this end, interlaboratory

comparisons studies utilizing standardized reference materials and protocols to achieve results' reliability should be prioritized.

Diverse fold increases for HuNoV GII, HAstV, and HAdV were observed in fecal compared to wastewater samples (Sample 7, 8, and 19 respectively), suggesting the presence of defective and non-infectious viral particles. However, the infectious particle-to-genomic copies ratio for each virus could not be estimated using the HIE model, as viral load in wastewater and viral replication did not correlate.

In addition, sequence analyses of HAstV and HAdV for paired samples demonstrated that genotypes that replicated in HIEs differed from those detected in wastewater (Samples 18 and 22 for HAstV, and Samples 9 and 23 for HAdV). This suggests that either the majority genotype present in wastewater may not be infectious, or that minority genotypes could replicate with higher efficiency in the HIE system.

The HIE was instrumental for enriching the Sample 8 for subsequent sequence analysis. Indeed, HuNoV GI was identified in wastewater by RT-qPCR (Fig. 3a), but typing was unsuccessful. However, it was possible to genotype the RNA from the supernatant as HuNoV GI.3[P13] following replication on HIEs (Table 2). HuNoV GII genotypes are more frequently detected in wastewater compared to GI genotypes, and they are usually found at higher titers (Ekundayo et al., 2021; Fumian et al., 2019; Huang et al., 2022; Mabasa et al., 2022; Tao et al., 2015). This lower prevalence and concentration of HuNoV GI compared to GII creates a bias in understanding the genotypes circulating among the population. Similar occurrence patterns were observed in a previous study conducted in the same Spanish region as the current study. In that study, GII.2 was present at 40 %, GII.6 at 8.6 %, and GII.17 at 5.7 %, alongside GI.2 (6.25 %) and GI.4 (6.25 %), while 87.5 % of GI could not be genotyped (Santiso-Bellón et al., 2020).

We intentionally did not spike wastewater samples with a recovery control to avoid saturating the binding sites of HIE monolayers. This limited the direct estimation of the efficiency of the concentration protocol. Another limitation is the occurrence of cytotoxicity attributed to wastewater in a considerable proportion of monolayers, estimated to be around 20 %, which resulted in the exclusion of those monolayers from the study.

Future experiments should benchmark the HIE model with continuous cell lines, such as Caco-2 or BGM, to provide a comprehensive assessment of the efficiency of the overall method for detecting infectious virus from environmental water samples, as recently demonstrated for nonculture-adapted RV clinical strains (Peña-Gil et al., 2023). Additionally, the HIE platform ideally needs testing with a broader range of gastroenteritis virus strains and combinations of genotypes.

Moreover, next-generation sequencing (NGS) technology should be coupled with viral infectivity data in the HIE system to study the relationship between viral detection and infectivity at the genotype/variant level. Additionally, it would be crucial to challenge the sensitivity of the HIE system with effluent wastewater. Understanding the behaviour and persistence of infectious viruses in effluent wastewater is essential for assessing potential risks and ensuring the safety of water reuse applications.

5. Conclusions

The HIE model has demonstrated its robustness for studying single and concurrent replication of naturally occurring viruses from wastewater. Therefore, the HIE model provides a unified platform for culturing human gastroenteritis viruses, potentially enabling the isolation of a greater diversity of viruses, that has to date been unattainable with standard cell lines. Besides the limitation of the HIE model, the advancements of this study will contribute significantly to our understanding of viral infectivity in wastewater and its implication for public health and environmental safety.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the CDC.

CRediT authorship contribution statement

Noelia Carmona-Vicente: Methodology, Investigation. **Annamaria Pandiscia:** Methodology, Investigation. **Cristina Santiso-Bellón:** Methodology, Investigation. **Alba Perez-Cataluña:** Writing – review & editing. **Jesús Rodríguez-Díaz:** Writing – review & editing, Investigation. **Veronica P. Costantini:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Javier Buesa:** Writing – review & editing, Supervision, Investigation. **Jan Vinjé:** Writing – review & editing, Validation, Investigation. **Gloria Sánchez:** Writing – review & editing, Resources, Conceptualization. **Walter Randazzo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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