DETECTION AND TRACKING OF EMERGING VIRUSES OF PUBLIC HEALTH INTEREST IN WATERS THROUGH MOLECULAR AND METAGENOMIC PROCEDURES

Ph.D. Thesis

Enric Cuevas Ferrando

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GLORIA SÁNCHEZ MORAGAS
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ALBA PÉREZ CATALUÑA

Al meu germà.

"Ojos que no ven, caguerà que xafes"

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The front and back covers have been created by visual artist Víctor Rubio (@FoscaCinetica).

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Resumen

El objetivo inicial de esta tesis era detectar y rastrear virus entéricos (en especial el virus de la hepatitis E) en diferentes matrices acuáticas mediante la combinación de protocolos moleculares y metagenómicos. Para ello, se establecieron como objetivos principales el desarrollo de procedimientos para la concentración de virus en muestras de aguas residuales, el análisis de virus indicadores de contaminación fecal y la caracterización del viroma de estas muestras.

Los virus de transmisión alimentaria, o virus entéricos, se encuentran entre los principales riesgos sanitarios asociados al consumo de alimentos y son responsables de diversas patologías: desde gastroenteritis, normalmente leves, hasta patologías más graves como hepatitis agudas, miocarditis o incluso meningitis o encefalitis aséptica. Son transmitidos principalmente por la vía fecal-oral y, por tanto, pueden estar potencialmente presentes en alimentos que hayan sufrido contaminación directa con materia fecal o a través de aguas contaminadas. De esta manera, la correcta higienización del agua es crucial para la salud del consumidor, puesto que esta se ingiere como parte de la dieta, se utiliza para el riego de cultivos, para el lavado y limpieza de los alimentos durante su preparación y, además, es parte esencial en muchos productos alimentarios.

En cuanto al desarrollo de procedimientos para la concentración de virus entéricos emergentes en muestras de aguas residuales, el protocolo de adsorción-precipitación de hidróxido de aluminio, utilizado habitualmente en el grupo para la concentración de otros virus entéricos, resultó muy eficaz para el virus de la Hepatitis E, permitiendo su detección en muestras de aguas de entrada y salida de estaciones depuradoras de aguas residuales (EDAR). Por lo que respecta al seguimiento de la prevalencia de otros virus entéricos e indicadores virales en muestras de entrada y salida de EDARs, esta tesis proporciona información cuantitativa sobre la presencia del indicador crAssphage y otros virus entéricos (el virus de la hepatitis A, norovirus, rotavirus y astrovirus) de cápside intacta en aguas de diferentes EDARs ubicadas en la región de Valencia. Además, los resultados de la correlación muestran que crAssphage podría no ser un indicador óptimo de la presencia de virus entéricos infecciosos en las aguas residuales regeneradas.

En relación a la caracterización del viroma de las muestras de agua de las EDARs analizadas, en la presente tesis se describe un procedimiento de

referencia que permite la detección y caracterización de las poblaciones virales en las muestras de aguas residuales recogidas a la entrada y salida de la planta depuradora. Este trabajo también muestra el sesgo existente en los perfiles del viroma que se obtienen según las librerías de secuenciación que se empleen. En este sentido, esta investigación arroja luz sobre la diversidad de las comunidades virales en influentes y efluentes de aguas residuales, proporcionando información valiosa también en términos de indicadores fecales virales.

Con la llegada de la pandemia de COVID-19 a principios de 2020, se incluyó el SARS-CoV-2 como objeto de estudio, pasando a ser el protagonista de la segunda parte de la tesis. En este aspecto, se marcaron como objetivos implementar un sistema de monitorización de SARS-CoV-2 en aguas residuales y desarrollar y optimizar métodos moleculares rápidos para inferir la infectividad del SARS-CoV-2.

Los resultados de esta tesis han demostrado que la aplicación de la epidemiología basada en aguas residuales (WBE) es eficiente para estimar la presencia e incluso la prevalencia de COVID-19 en comunidades y puede servir de herramienta para la salud pública como alerta temprana ante situaciones pandémicas. Asimismo, esta tesis incluye el primer estudio publicado en España que realizó un análisis metagenómico de la diversidad del SARS-CoV-2 presente en las aguas residuales en las tres primeras oleadas epidemiológicas que se produjeron entre el año 2020 y 2021. Paralelamente, estos resultados confirmaron el potencial de la secuenciación masiva de aguas residuales para detectar nuevas mutaciones y linajes del SARS-CoV-2. Además, en esta tesis también se han comparado y optimizado los protocolos de concentración, extracción y detección de ácidos nucleicos de coronavirus a partir de muestras de aguas residuales, superficiales y de mar. Así, este trabajo amplía el conocimiento sobre los procedimientos analíticos y sus eficiencias para la detección del SARS-CoV-2 en aguas residuales constituyendo un paso adelante para la implementación global del COVID-19 WBE.

En cuanto al desarrollo y la optimización de métodos moleculares rápidos para inferir la infectividad viral del SARS-CoV-2, esta tesis ha implementado un protocolo de RT-qPCR de integridad de la cápside basado en el cloruro de platino que actúa como marcador de viabilidad para evitar la amplificación por RT-qPCR del ARN del SARS-CoV-2 no infeccioso. Además, se ha validado con éxito en muestras de aguas residuales contaminadas de forma natural. Así, los resultados de esta tesis apoyan la idea de que el SARS-CoV-2 presente en las aguas residuales no es infeccioso. En general, en el marco de esta tesis

doctoral se ha desarrollado una herramienta analítica rápida basada en la RT-qPCR de viabilidad para inferir la infectividad del SARS-CoV-2 con potencial aplicación en la evaluación de riesgos, la prevención y el control en los programas de salud pública.

Resum

L'objectiu inicial d'esta tesi era detectar i rastrejar virus entèrics (en especial el virus de l'hepatitis E) en diferents matrius aquàtiques mitjançant la combinació de protocols moleculars i metagenòmics. Així, es van establir com objectius principals el desenvolupament de procediments per a la concentració de virus en mostres d'aigües residuals, la anàlisi de virus indicadors de contaminació fecal i la caracterització del viroma d'estes mostres.

Els virus de transmissió alimentària, o virus entèrics, es troben entre els principals riscos sanitaris associats al consum d'aliments i són responsables de diverses patologies: des de gastroenteritis, normalment lleus, fins a patologies més greus com hepatitis agudes, miocarditis o fins i tot meningitis o encefalitis asèptica. Són transmesos principalment per la via fecal-oral i, per tant, poden estar potencialment presents en aliments que hagen patit contaminació directa amb matèria fecal o mitjançant aigües contaminades. D'aquesta manera, la correcta higienització de l'aigua és crucial per a la salut del consumidor, ja que aquesta s'ingereix com a part de la dieta, s'utilitza per al reg de cultius, per al rentat i la neteja dels aliments durant la preparació i, a més, és part essencial en molts productes alimentaris.

En referència al desenvolupament de procediments per a la concentració de virus entèrics emergents en mostres d'aigües residuals, el protocol d'adsorció-precipitació d'hidròxid d'alumini, utilitzat habitualment al grup per a la concentració d'altres virus entèrics, va resultar molt eficaç per al virus de l'Hepatitis E, permetent-ne la detecció en mostres d'aigües d'entrada i de sortida d'estacions depuradores d'aigües residuals (EDAR). Pel que fa al seguiment de la prevalença d'altres virus entèrics i indicadors virals en mostres d'entrada i sortida d'EDARs, aquesta tesi proporciona informació quantitativa sobre la presència de l'indicador crAssphage i altres virus entèrics (virus de l'hepatitis A, norovirus, rotavirus i astrovirus) de càpside intacta en aigües de diferents EDARs ubicades a la regió de València. A més, els resultats de la correlació mostren que crAssphage podria no ser un indicador òptim de la presència de virus entèrics infecciosos a les aigües residuals regenerades.

Quant a la caracterització del viroma de les mostres d'aigua de les EDARs analitzades, la present tesi descriu un procediment de referència que permet la detecció i caracterització de les poblacions virals a les mostres d'aigües residuals recollides a l'entrada i eixida de la planta depuradora. Este treball també mostra el biaix que presenten els diferents perfils del viroma obtinguts

segons la llibreria de seqüenciació que s'utilitze. En aquest sentit, esta investigació ha incrementat el coneixement sobre la diversitat de les comunitats virals en influents i efluents d'aigües residuals, proporcionant informació valuosa també en termes d'indicadors fecals virals.

Amb l'arribada de la pandèmia de COVID-19 a principis del 2020, es va incloure el SARS-CoV-2 com a objecte d'estudi, passant a ser el protagonista de la segona part de la tesi. En este aspecte, es van marcar com a objectius implementar un sistema de monitorització de SARS-CoV-2 en aigües residuals i desenvolupar i optimitzar mètodes moleculars ràpids per inferir la infectivitat del SARS-CoV-2.

Els resultats d'esta tesi han demostrat que l'epidemiologia basada en aigües residuals (WBE) és una ferramenta eficient per estimar la presència i fins i tot la prevalença de COVID-19 a nivell de comunitat i que serveix com a eina d'alerta primerenca de salut pública davant de situacions pandèmiques. Així mateix, esta tesi inclou el primer estudi realitzat a Espanya en fer una anàlisi metagenòmica de la diversitat del SARS-CoV-2 present a les aigües residuals a les tres primeres onades epidemiològiques que es van produir entre l'any 2020 i 2021. Paral·lelament, estos resultats han confirmat el potencial de la seqüenciació massiva d'aigües residuals per detectar noves mutacions i llinatges del SARS-CoV-2. A més, en esta tesi també es comparen i optimitzen els protocols de concentració, extracció i detecció d'àcids nucleics de coronavirus a partir de mostres d'aigües residuals, superficials i de mar. Així, este treball ha ampliat el coneixement sobre els procediments analítics i les seves eficiències per a la detecció del SARS-CoV-2 en aigües residuals i ha constituït un pas endavant per a la implementació global de l'epidemiologia basada en aigües residuals de la COVID-19.

Pel que fa al desenvolupament i l'optimització de mètodes moleculars ràpids per inferir la infectivitat viral del SARS-CoV-2, en esta tesi s'ha implementat un protocol de RT-qPCR d'integritat de la càpside basat en el clorur de platí que actua com a marcador de viabilitat per evitar l'amplificació per RT-qPCR de l'ARN del SARS-CoV-2 no infecciós. A més, es va validar amb èxit en mostres d'aigües residuals contaminades de manera natural. Així, els resultats d'esta tesi donen suport a la idea que el SARS-CoV-2 present a les aigües residuals no és infecciós. En general, en el marc d'aquesta tesi doctoral s'ha desenvolupat una eina analítica ràpida basada en la RT-qPCR de viabilitat per inferir la infectivitat del SARS-CoV-2 amb aplicació potencial en l'avaluació de riscos, la prevenció i el control en els programes de salut pública.

Abstract

The initial aim of this thesis was to detect and track enteric viruses (especially hepatitis E virus) in different aquatic matrices using a combination of molecular and metagenomic protocols. The main objectives were the development of procedures for the concentration of viruses in wastewater samples, the analysis of viral indicators of faecal contamination, and the characterisation of the virome in these samples.

Food-borne viruses, or enteric viruses, are among the main health risks associated with food consumption, and thus have an impact on food safety. They are responsible for a variety of pathologies: from gastroenteritis, usually mild, to more serious pathologies such as acute hepatitis, myocarditis, or even meningitis or aseptic encephalitis. They are mainly transmitted by the faecal-oral route and can therefore potentially be present in food that has been directly contaminated with faecal matter, or through contaminated water. Water is ingested as part of the diet, used for irrigation of crops, for washing and cleaning of food during food preparation, and is also an essential part of many food products. The presence of human enteric viruses in water is also well documented and may represent a major threat to consumer health.

Regarding the development of procedures for the concentration of emerging enteric viruses in wastewater samples, the aluminium hydroxide adsorption-precipitation protocol, commonly used in the group for the concentration of other enteric viruses, proved to be very effective for Hepatitis E virus, allowing its detection in water samples from inlet and outlet of wastewater treatment plants (WWTP). Regarding the monitoring of the prevalence of other enteric viruses and viral indicators in incoming and outgoing samples from WWTPs, this thesis provided quantitative information on the presence of the indicator crAssphage and other enteric viruses (hepatitis A virus, norovirus, rotavirus, and astrovirus) with intact capsid in water from different WWTPs located in the region of Valencia. Furthermore, the correlation results showed that crAssphage might not be an optimal indicator of the presence of infectious enteric viruses in reclaimed wastewater.

Regarding the virome characterisation of the WWTP water samples analysed, the present thesis described a reference procedure that allows the detection and characterisation of viral populations in wastewater samples collected at the inlet and outlet of the WWTP. This work also showed the bias in the virome profiles due to the use of different sequencing libraries. In this sense, this research shed light on the diversity of viral communities in wastewater

influent and effluent, providing valuable information also in terms of faecal viral indicators.

With the arrival of the COVID-19 pandemic in early 2020, SARS-CoV-2 was included as an object of study, becoming the focus of the second part of the thesis. In this aspect, the objectives were to implement a monitoring system for SARS-CoV-2 in wastewater and to develop and optimise rapid molecular methods to infer SARS-CoV-2 infectivity.

The results of this thesis demonstrated that wastewater-based epidemiology (WBE) is an efficient application to estimate the presence and even prevalence of COVID-19 in communities and serves as a public health early warning tool for pandemic situations. Furthermore, this thesis includes the first study conducted in Spain that made a metagenomic analysis of the SARS-CoV-2 diversity present in wastewater in the first three epidemiological waves occurring between 2020 and 2021. In parallel, these results confirmed the potential of mass sequencing of sewage to detect new SARS-CoV-2 mutations and lineages. In addition, this thesis also compared and optimised protocols for the concentration, extraction, and detection of coronavirus nucleic acids from sewage, surface, and seawater samples. Thus, this work expanded the knowledge of analytical procedures and their efficiencies for the detection of SARS-CoV-2 in wastewater and constituted a step forward for the global implementation of the COVID-19 WBE.

Concerning the development and optimisation of rapid molecular methods to infer SARS-CoV-2 viral infectivity, this thesis implemented a platinum chloride-based capsid integrity RT-qPCR protocol that acts as a viability marker to prevent RT-qPCR amplification of non-infectious SARS-CoV-2 RNA and was successfully validated in naturally contaminated sewage samples. Thus, the results of this thesis support the idea that SARS-CoV-2 present in wastewater is not infectious. Overall, a rapid analytical tool based on feasibility RT-qPCR to infer SARS-CoV-2 infectivity with potential application in risk assessment, prevention, and control in public health programmes has been developed in the framework of this Ph.D. thesis.

Content

ABBREVIATIONS

WWTP	Wastewater Treatment Plant
DWTP	Drinking water Treatment Plant
WBE	Wastewater-based epidemiology
COVID-19	Coronavirus disease 2019
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
ssDNA	Single-stranded DNA
dsDNA	Double-stranded DNA
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PMA	Propidium monoazide
EMA	Ethidium monoazide
$PtCl_4$	Platinum chloride
CDDP	Cisplatin
PEG	Polyethylene glycol
PGM	Porcine gastric mucin
RT-qPCR	Reverse transcription quantitative real-time PCR
TMA	Transcription-mediated amplification
LAMP	Loop-mediated isothermal amplification
UC	Ultracentrifugation
NGS	Next Generation Sequencing
Kbp	Kilobase pair

WHO	World Health Organisation
EC	European Comission
UN	United Nations
CDC	Centers for Disease Control and Prevention
EPA	United States Environmental Protection Agency
EFSA	European Food Safety Authority
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HuNoV	Human norovirus
HAdV	Human adenovirus
HAstV	Human Astrovirus
HPeV	Parechovirus
MHV	Murine hepatitis virus
MNV	Murine norovirus
MgV	Mengovirus
AiV	Aichivirus
PV	Poliovirus
AIV	Avian Influenza virus
RV	Rotavirus
CV	Coxsackievirus
EV	Enterovirus
TV	Tulane virus
SMV	Snow Mountain virus
PMMoV	Pepper mild mottle virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
PEDV	Porcine epidemic diarrhoea virus

INTRODUCTION

I. INTRODUCTION

1. Water and viruses

More than 150 different enteric viruses have been identified thus far as causes of waterborne diseases in humans (Sinclair, Jones, and Gerba 2009). Both freshwater consumption and wastewater output are expected to rise as the world's population grows and the climate changes. Virus analysis in water is widely utilized when assessing the potential of waterborne disease transmission (Wyn-Jones and Sellwood 2001). In both developed and developing regions, exposure to waterborne pathogens via food intake, irrigation water, and recreational activities (as well as related jobs) constitutes a risk to public health (Efstratiou, Ongerth, and Karanis 2017; Gibson 2014). Waterborne diarrheal illnesses cause around 4 billion cases and 2 million deaths each year, with the majority of them occurring in children under the age of five (WHO 2014). Enteric viral infections are responsible for the major share of these diseases (Ramani and Kang 2009). Human enteric viruses, especially norovirus and hepatitis A virus (HAV), cause foodborne outbreaks which are a threat to public health worldwide, along with other non-enveloped viruses human enteric viruses such as enterovirus (EV), Aichi virus (AiV), parechovirus (HPeV), hepatitis E virus (HEV), astrovirus (hAstV), rotavirus (RV), and human adenovirus (HAdV) (Table 1). These viruses may cause epidemics or rare occurrences of gastroenteritis, meningitis, respiratory sickness, conjunctivitis, paralysis, or hepatitis (only HAV and HEV), among other symptoms (Bellou, Kokkinos, and Vantarakis 2013; Jiang 2006; Parshionikar et al. 2003) (Table 1). Nonetheless, human enteric viruses have received much less attention than other foodborne pathogens despite being a significant source of foodborne outbreaks in high-income nations (WHO 2015). As enteric viral pathogens are mostly transmitted by the faecal-oral route, any item in the food chain may be contaminated by viruses, but green vegetables, berries, ready-to-eat foods, and shellfish are the products most commonly linked to viral outbreaks. Most human enteric viruses are extremely infectious and transmissible due to their excretion at high concentrations in the faeces of infected individuals (up to 10^{11} viruses/faeces-gram), their low infectious dose and their high environmental stability (Atmar et al. 2014; Bosch 1998; Prüss et al. 2002; Teunis et al. 2008; Wyn-Jones and Sellwood 2001).

The majority of enteric viruses survive in water bodies contaminated by residential wastewater discharge and are often linked to waterborne epidemics (Gibson 2014; Kauppinen, Pitkänen, and Miettinen 2018; Sekwadi

et al. 2018). Although typical wastewater treatment procedures may not completely eliminate enteric viruses, wastewater is often treated before being released into the environment (Kitajima et al. 2014; Qiu et al. 2015; Sidhu et al. 2017). The use of effective wastewater treatment systems may minimize the risk of viral infection, caused by having contact with recreational waters, intake of contaminated-potable water, or consumption of virus-contaminated shellfish or fresh produce. However, total virus elimination using traditional wastewater treatment procedures is very challenging (Sano et al. 2016). This and the lack of suitable sanitary infrastructure and wastewater treatment facilities themselves exacerbate faecal matter contamination of the environment and drinking water sources in many developing nations (Bain et al. 2014). Also, large quantities of untreated wastewater may also be released due to combined sewer overflows (CSOs) during significant rain events, as well as dry water overflows, which can occur during snowmelt, tidal infiltration, or system failures and obstructions (Ahmed, Payyappat, et al. 2020). These occurrences allow enteric pathogens to directly reach the environment (Fong et al. 2010), putting people who come into direct or indirect contact with such polluted water at risk of contracting viral illnesses (Sinclair et al. 2009). Also, the capability of waterborne viruses to adhere to solid materials in the water column or to build up in sediments boosts their transmissibility as they are easily transported in environmental fluids (Hassard et al. 2016). As a result, filter-feeding aquatic species, such as bivalve mussels and oysters harvested for human consumption, may take them up (Landry et al. 1983; Lowther et al. 2012). Furthermore, in regions where freshwater is scarce, wastewater is often utilized for irrigation; as a consequence, enteric viruses may directly contaminate fruit and leafy green vegetables, resulting in foodborne outbreaks (Bosch, Pintó, and Guix 2016). Thus, both filter-feeding organisms and fruit or leafy green vegetables, which are often eaten raw, represent a high risk of infection by enteric viruses for the consumers.

Human norovirus

Human norovirus, a positive-sense single-stranded RNA virus, is one of the most common causes of gastroenteritis and has become a growing public health concern across the globe. It is a member of the *Caliciviridae* family and is divided into ten genogroups (GI to GX), with the GI, GII, and GIV genogroups infecting humans (Chhabra et al. 2019). HuNoVs are responsible for a large share of gastroenteritis infections worldwide, with 685 million cases and 200,000 fatalities (Katayama and Vinjé, 2017). While HuNoV GII is the most common cause of reported outbreaks, HuNoV GI is also broadly detected in environmental water sources (Fuentes et al. 2014; Nenonen et al. 2008).

Hepatitis A virus, Aichi virus, poliovirus and parechovirus

Picornaviridae family is a large family of vertebrate viruses that include more than 30 genera and 75 species of small naked RNA-positive single-stranded viruses, some of the most important fecal-oral transmitted viruses. The Aichi virus affects both children and adults, causing gastroenteritis. Human parechoviruses primarily infect babies, producing gastroenteritis, meningitis, encephalitis, and paralysis (Boivin et al., 2005; Stanway et al., n.d.). Poliovirus is the causing agent of poliomyelitis, which historically is the most significant disease caused by an enterovirus. Nonetheless, the only human enteric virus that is almost completely eradicated is the poliovirus, and water has been linked to its spread (Leclerc, Schwartzbrod, and Dei-Cas 2008; Lodder et al. 2012). Only 22 cases of wild poliovirus and 96 cases of vaccine-derived poliovirus were reported worldwide in 2017 (Kew et al. 2004; WHO 2018). Wastewater-based epidemiology can provide crucial data on viral dissemination in the absence of clinical infections because the majority of infected individuals are asymptomatic (Hovi et al. 1986; Minor 2016). HAV can cause both sporadic and massive epidemics of hepatitis. HAV is divided into six genotypes (I to VI), each having its geographic distribution, with types IA and IB being the most frequent in Europe (Vaughan et al. 2014). HAV is geographically distributed in areas having high, intermediate, or low levels of hepatitis A virus infection (depending on the availability of efficient sanitation resources) and results in 1.5 million cases of clinical hepatitis annually. Also, WHO estimates that in 2016, 7134 persons died from hepatitis A worldwide (accounting for 0.5% of the mortality due to viral hepatitis) (WHO 2022a). This virus is one of the greatest concerns regarding food safety, as it is responsible for numerous outbreaks in the world. In underdeveloped nations, almost all adults have serological evidence of HAV infection. HAV transmission mostly happens via the faecal-oral pathway, which is closely linked to unhygienic settings (Nainan et al. 2006). HAV can spread through blood, but it is much more uncommon (Lemon 1994).

Human astrovirus

Astroviruses belong to the *Astroviridae* family. They are named after their characteristic icosahedral morphology with a five- or six-pointed star-like surface structure seen under the electron microscope. Like *Picornaviridae* and *Caliciviridae*, HAstVs are non-enveloped with a plus-sense, single-stranded RNA genome. Gastroenteritis is the most common symptom in children under the age of two. Elderly and immunocompromised people account for up to 20% of clinical cases and 0.5 to 15% of outbreaks (De Benedictis et al. 2011).

Rotavirus

Rotavirus is a member of the *Reoviridae* family and its genome is made up of 11 double-stranded RNA segments (Coluchi et al. 2002). Before the implementation of the RV vaccine, virtually every child under the age of five in the world (95%) would get infected by RV, which was also the most frequent cause of severe diarrhoea in new-borns (Lanata et al. 2013). Furthermore, it can also induce diarrhoea in the elderly. Even though RV infection is common globally, the majority of RV-related fatalities occur in developing nations, mostly owing to dehydration (Parashar, Nelson, and Kang 2013).

Human adenovirus

Human adenoviruses are categorized into seven groups, A through G, within the *Adenoviridae* family. Each species is then divided into several serotypes (Matsushima et al. 2013; Robinson et al. 2011). The genome is a non-segmented dsDNA with a length of 26 to 45 kbp. Ad-F types 40 and 41, as well as Ad-G type 52, are the most common causes of gastroenteritis. They may cause respiratory problems, conjunctivitis, pancreatitis, and encephalitis in rare cases. Group F mastadenoviruses suppose one of the principal causes of gastroenteritis in new-borns and young children along with rotaviruses (Desselberger and Gray 2009; Jiang 2006).

Hepatitis E virus

HEV is a positive-sense single-stranded RNA virus that belongs to the *Hepeviridae* family. HEV is an under-appreciated viral danger with case occurrence increasing annually, and the European Food Safety Authority (EFSA) has listed HEV infection as an emerging disease (Ricci et al. 2017). The virus is now divided into eight genotypes, with G1–4 and G7 capable of infecting humans. Yet, the different genotypes infect a wide spectrum of species, with G1 and G2 infecting mostly humans and nonhuman primates, while genotypes 3–8 mostly infect pigs, deer, camels, rabbits, and dolphins. Zoonotic genotypes infecting both humans and animals, have been isolated in some of these species, particularly in pigs (Van Der Poel 2014; Sooryanarain and Meng 2019). Only humans are infected with G1 and G2. In 2005, G1 and G2 HEV together caused an estimated 20 million infections globally, with 44,000 reported deaths owing to the virus (Rein et al. 2012). These two varieties have the potential to create large-scale waterborne epidemics (Aggarwal 2013). Since the virus spreads by a faecal-oral pathway, it is readily transmitted via faecally contaminated water (Doceul et al. 2016). Thus, human and farm sewage play an important role in HEV transmission, such as farm

runoff from animal slurry depots, application of animal slurry to crops, and pollution of surface waterways used for irrigation and shellfish farms.

Family	Genus associated with faecal transmission	Species found in water	Structure			Symptoms	Zoonotic	Reference
			Capsid	Genome	Size			
Adenoviridae	*Mastadenovirus*	Mastadenovirus A-F	Icosahedral	dsDNA	70–90 nm	Gastroenteritis, respiratory illness, ear infection, conjunctivitis	No	(King et al. 2011)
Anelloviridae	*Alphatorquevirus*	Torque teno virus	Icosahedral	ssDNA	30 nm	Unknown	Yes	(King et al. 2011)
Astroviridae	*Mamastrovirus*	Astrovirus	Icosahedral	ssRNA+	28–30 nm	Gastroenteritis	Potentially	(De Benedictis et al. 2011; King et al. 2011)
Caliciviridae	*Norovirus*	Norovirus GI, GII, GIV	Icosahedral	ssRNA+	35–40 nm	Gastroenteritis	No	(King et al. 2011)
	Sapovirus	Sapovirus GI, GII				Gastroenteritis	No	(King et al. 2011)
Circoviridae	*Circovirus*	Human-associated circovirus	Icosahedral	ssDNA	15–25 nm	Unknown	No	(Breitbart et al. 2017)
Hepeviridae	*Orthohepevirus*	Hepatitis E virus type 1-4	Icosahedral	ssRNA+	27–34 nm	Acute hepatitis	Yes	(Purdy et al. 2017)
Papillomaviridae	various	assorted papillomaviruses	Icosahedral	dsDNA	55 nm	Genital tract infection, cancer	No	(Van Doorslaer et al. 2018)
Parvoviridae	*Bocavirus*	Human bocavirus type 1-4	Icosahedral	ssDNA	22 nm	Gastroenteritis and respiratory disease	No	(King et al. 2011)
Picornaviridae	*Kobuvirus*	Aichivirus A-B	Icosahedral	ssRNA+	30–32 nm	Gastroenteritis	No	(Zell et al. 2017)
	Cosavirus	Cosavirus A						
	Enterovirus	Coxsackievirus B				Gastroenteritis, mild meningitis, encephalitis, myelitis, myocarditis, conjunctivitis	No	
		Enterovirus A-D						
		Poliovirus type 1-3						
	Hepatovirus	Hepatitis A virus				Acute hepatitis	No	
Polyomaviridae	*Alpha-polyomavirus*	MC polyomavirus	Icosahedral	dsDNA	40–45 nm	Cancer	No	(Moens et al. 2017)
	Beta-polyomavirus	BK polyomavirus	Icosahedral	dsDNA	40–45 nm	Respiratory, urinary tract and skin infection, cancer	No	(Moens et al. 2017)
		JC polyomavirus						
Reoviridae	*Reovirus*	Rotavirus A	Icosahedral	dsRNA	60–80 nm	Gastroenteritis	Potentially	(Cook et al. 2004; King et al. 2011)

Table 1. Main waterborne human pathogenic viruses. Adapted from (Farkas et al. 2020)**.**

2. Water safety

2.1. Urban Water Cycle

The Urban Water Cycle (UWC) is defined as the spatiotemporal connection between water and hydrological activities, as well as the usage, sanitation, provision, collection, distribution, and reuse that occurs in urban or large urban settings, according to (Peña-Guzmán et al. 2017) (Figure 1). The presence of viral particles in wastewater is linked to the emission of fluids or faeces from infected individuals. Wastewaters are released directly into receiving water bodies (e.g., surface waters) or routed to Waste Water Treatment Plants (WWTPs), depending on the city's sanitation infrastructure. Depending on the treatment technique used, the efficiency of virus removal may vary among different WWTPs.

Human pathogenic viruses have been found in almost every kind of water, including surface freshwaters like lakes and rivers, groundwater, estuarine and marine waters. In the United States, 72% of groundwater locations have been reported to be contaminated with enteric viruses (Okoh, Sibanda, and Gusha 2010). In (Sorensen et al. 2021) it is reported that seven out of eight British aquifers and 31% of samples tested positive for viral nucleic acid. The most prevalent viral nucleic acid targets were HAV (17% samples, 63% locations), human norovirus GI (14% samples, 38% locations), and HEV (7% samples, 25% locations). Also, in (De Giglio et al. 2017) Human norovirus, RVs, and EVs were detected in 15.6% of 147 wells with water considered appropriate for irrigation, with 58 of them (31.9%) posing a potential infectious risk for irrigation use. Contamination occurs via a variety of mechanisms, the majority of which are connected to human activities, such as the discharge of untreated sewage, the reuse of improperly treated effluent, and the use of animal waste as fertiliser. After that, exposure to polluted water may occur in a variety of ways, all of which are tied to one of the multiple uses of water, such as drinking, aquaculture, irrigation, and recreational activities among others. UWC is not optimally established globally, with hundreds of millions of people worldwide still lacking basic access to water, sanitation, and hygiene (WASH), and this contributes to diarrheal illnesses in developing nations (WHO 2016; WHO/UNICEF 2015). These diseases are one of the primary causes of under-

five mortality worldwide, and WASH insecurity affects the urban and rural poor disproportionately (Ford et al. 2017).

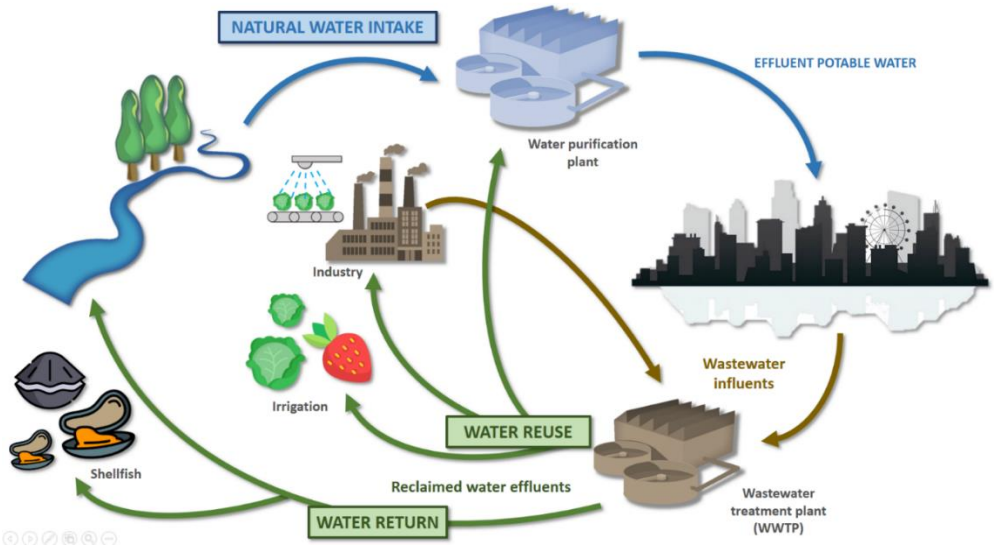


Figure 1. Urban water cycle in countries with efficient infrastructe

2.2. Control of viruses in water

A wastewater treatment plant's main goals are to (1) isolate the solid from the influent and concentrate the solids collected; (2) remove innocuous materials that will cause disturbance when the effluent and residuals are subjected to final disposal, and (3) maximize the reuse possibilities of treated wastewater and residuals (Englande et al. 2015). Pretreatment/primary treatment, secondary treatment, tertiary treatment, sludge treatment/stabilization, and final disposal or reuse treatments for residuals are the most characteristic steps in wastewater treatment facilities.

Pre-treatment techniques are employed to make wastewater suitable for later treatment procedures. Equalization, neutralization, and oil and grease separation are common procedures. For municipal WWTPs, the first treatment entails physical separation and often includes screening or comminution, grit removal, and sedimentation before secondary treatment (Dominguez and Gujer 2006).

Following pre-treatment, secondary treatment involves biological treatment steps because much of the organic material in wastewater is colloidal or

dissolved, and therefore the procedures outlined thus far will not be successful in treating it. It has previously been demonstrated that in the presence of microbes, oxygen, and nutrients, organic matter is oxidized to carbon dioxide and water (Limpiyakorn et al. 2005; Wang et al. 2012). Consequently, an efficient wastewater treatment requires an appropriate number of acclimated microorganisms able to metabolize the organic material. In turn, these microorganisms require nutrients, an oxygen supply, a means of facilitating interaction between the bacteria and organic matter, and a mechanism of containment.

Physically eliminating pathogens by conventional treatment and inactivating pathogens using UV light or chemical oxidants such as chlorine, chloramines, ozone, and chlorine dioxide are two common water treatment procedures used across the globe. Because viruses represent such minuscule particles, traditional treatment methods, such as filtration, are useless in physically eliminating them. The use of disinfectants is heavily dependent on the chemistry of the water and local restrictions. Free chlorine (the sum of hypochlorous acid and hypochlorite ions generated by the dissolution and hydrolysis of chlorine gas in water) is the most widely used disinfectant in the world, having been used to disinfect water since the early 1900s (Gall et al. 2015). These treatments show different efficiency among target human enteric viruses (Sirikanchana, Shisler, and Mariñas 2008).

Quality of water is being addressed in different ways depending on the country. Generally, wastewater treatment utilities test for total faecal coliform bacteria by plaque count in water supplies on a regular basis, but they do not test for infectious viruses since it is either difficult or impractical to identify or cultivate infectious virus particles in a cost-effective and timely way. Authorized treatment methods are based on bench-scale research in which a particular virus is subjected to a disinfectant under different environmental conditions until it is rendered inactive to 99.99% (U.S. EPA 2009). A new European regulation on minimum quality criteria (MQR) for water reuse (EC, 2020), which outlines the guidelines for the use of reclaimed water for agricultural irrigation, was released in May 2020. From June 26, 2023, this regulation will be immediately applicable in all Member States. However, since the report's publication in 2020 (Truchado et al. 2021), questions have arisen concerning potential non-compliance scenarios in European water reuse systems. Regulations for each disinfectant are determined by analysing a variety of enteric viruses and determining an acceptable dosage to properly inhibit the activity of the most robust enteric virus investigated. To comply with enteric virus rules, utilities must use a proper disinfectant dosage. According to EC, 2020/741 regulation, validation monitoring needs to assess whether the performance targets (log10 reduction) are met. The monitoring of validation shall involve the monitoring

of indicator micro-organisms associated with each group of pathogens: bacteria, viruses and protozoa. The indicator microorganisms selected in the regulation are *E. coli* for pathogenic bacteria, F-specific coliphages, somatic coliphages or coliphages for pathogenic viruses, and *Clostridium perfringens* spores or spore-forming sulfate-reducing bacteria for protozoa. Performance targets (log10 reduction) for the validation monitoring for the selected indicator microorganisms are reflected in Table 2 and shall be met at the point of compliance, taking into account the concentrations of the raw wastewater entering the urban WWTP. A minimum of 90 % of the validation samples has to reach or exceed the performance targets (EC 2020).

Reclaimed water quality	
Indicator microorganisms	Performance targets for the treatment chain (log10 reduction)
Escherichia coli	≥ 5.0
Total coliphages/F-specific coliphages/somatic coliphages/coliphages	≥ 6.0
Clostridium perfringens spores/spore-forming sulfate-reducing bacteria	≥ 4.0 (in case of *Clostridium perfringens* spores) ≥ 5.0 (in case of spore-forming sulfate-reducing bacteria)

Table 2. Validation monitoring of reclaimed water for agricultural irrigation from Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse (Text with EEA relevance).

2.3. Viral indicators for tracking domestic wastewater contamination in the aquatic environment

Aside from the limits of detection technologies, there is still no single optimal water treatment that will inactivate all type of viruses regardless of water quality. The scientific world is still baffled as to why viruses exhibit distinct profiles of disinfection resistance. For example, in comparison to other enteric viruses, human adenovirus is roughly five times more resistant to monochromatic (254 nm) UV inactivation (Eischeid, Meyer, and Linden, 2009). Also, even though bacteriophages are often employed as models to human enteric viruses, no bacteriophage has been shown to optimally reflect enteric viral behaviour for all disinfectants. Nonetheless, a key regulatory roadblock is that no single disinfection technology is efficient against all viruses and can be used in all water quality settings (Jalali Milani and Nabi Bidhendi, 2022).

Alternatively, an indicator might be used to measure the efficacy of wastewater and drinking water treatments, as well as to investigate pathogen abundance, persistence, adsorption, and movement in the aquatic environment. Quantitative monitoring of viral markers may also give information for microbial source tracking, transport modelling, and risk assessment. To evaluate levels of faecal pollution in water, faecal indicator bacteria (FIB; includes coliform bacteria, *Escherichia coli*, *Enterococcus*, and *Streptococcu*s spp.) have traditionally been utilized. Nonetheless, bacteria have been proven to be less resistant to wastewater treatments than human enteric viruses (Fong and Lipp 2005; Kim et al. 2009; Lin and Ganesh 2013; Sidhu et al. 2017). As a result, FIBs are poor predictors of viral contamination, which would imply that existing water quality monitoring programs based only on FIB are insufficient. As stated in Farkas et al. (2020), an optimal indicator for tracking wastewater viral contamination should also be (i) easy to detect and quantify, (ii) source-specific (to differentiate between pollution caused by animals and by humans), (iii) resistant to wastewater treatment processes, and (iv) persistent in the aquatic environment, with similar behaviour to viral pathogens (Farkas et al. 2020) (Figure 2). This would allow for continuous monitoring and reporting of contamination levels and the likelihood of pathogen presence.

Although several enteric viruses found in wastewater have the potential to be utilized as markers, not all of them meet these criteria. Furthermore, since several enteric viruses are zoonotic (e.g., HAstV, RVs, and HEV; Table 1), their presence in the environment might be due to animal farming and agricultural operations rather than human waste. HAV and HEV viruses are common in less industrialized areas, but they have recently been described as emergent pathogens in highly developed areas (Bosch et al. 2016). Furthermore, in

temperate regions, enteroviruses, noroviruses, and sapoviruses exhibit strong seasonality, peaking in the summer (enteroviruses) or the winter (noroviruses and sapoviruses). As a result, these viruses are not detected in wastewater or polluted environments throughout the year (Farkas, Cooper, et al. 2018; Pons-Salort et al. 2018; Prevost et al. 2015). HAdVs, Polyomaviruses (PyVs), and AiVs are regularly detected in wastewater and contaminated areas without any discernible periodicity, suggesting that they might be useful as faecal indicators (Rachmadi, Torrey, and Kitajima 2016; Rames et al. 2016). Bacteriophages that infect bacteria in the human gut are also found in wastewater. To test for wastewater pollution, somatic coliphages (phages that infect *E. coli*) and F-specific RNA bacteriophages (FRNAP; phages that infect bacteria *via* the F-pili) are routinely utilized (EC 2020). However, they should be handled with care since not all strains are solely associated with human contamination (Jofre et al. 2016). Bacteriophages that infect *Bacteroides* spp. may also be used to detect wastewater pollution. A new group of viruses known as crAss-like phages has been found among these phages. CrAssphage, a bacteriophage discovered in human faecal metagenomes (type genome NC_024711.1), is a member of the typical gut virome which has co-evolved with humans (Dutilh et al. 2014). More crAss-like sequences have been discovered since the publication of the first crAssphage genome, and one phage has been isolated. However, they have a lot of genetic variability, and the crAssphage and the isolated crass-like phage are not the same genus (Shkoporov et al. 2018). CrAssphage is made up of a collection of viruses having nucleotide similarities to the crAssphage *sensu stricto* (NC_024711.1) identified by Dutilh et al. (2014) and measured by Stachler et al. (2017) since the taxonomy of crAss-like phages has yet to be established (Dutilh et al. 2014; Stachler, Kelty, Sivaganesan, Li, Bibby, and Orin C. Shanks 2017). A plant virus, the pepper mild mottle virus (PMMoV; family *Virgaviridae*), is frequently found in human wastewater and has been detected in contaminated surface and groundwater as well as drinking water (Symonds et al. 2018). Consumption of infected peppers (*Capsicum* spp.) and food items containing contaminated peppers is the principal source of PMMoV in human excreta (Zhang et al. 2005). PMMoV has been proposed as a useful indicator for wastewater contamination (Kitajima, Sassi, and Torrey 2018; Symonds et al. 2018), but its size and shape (17 x 300 nm rod-shaped capsid) differ from other pathogenic viruses with icosahedral capsids, and thus its fate and behaviour in the environment may differ (Kitajima et al. 2018; Shirasaki et al. 2017; Wetter, 1984).

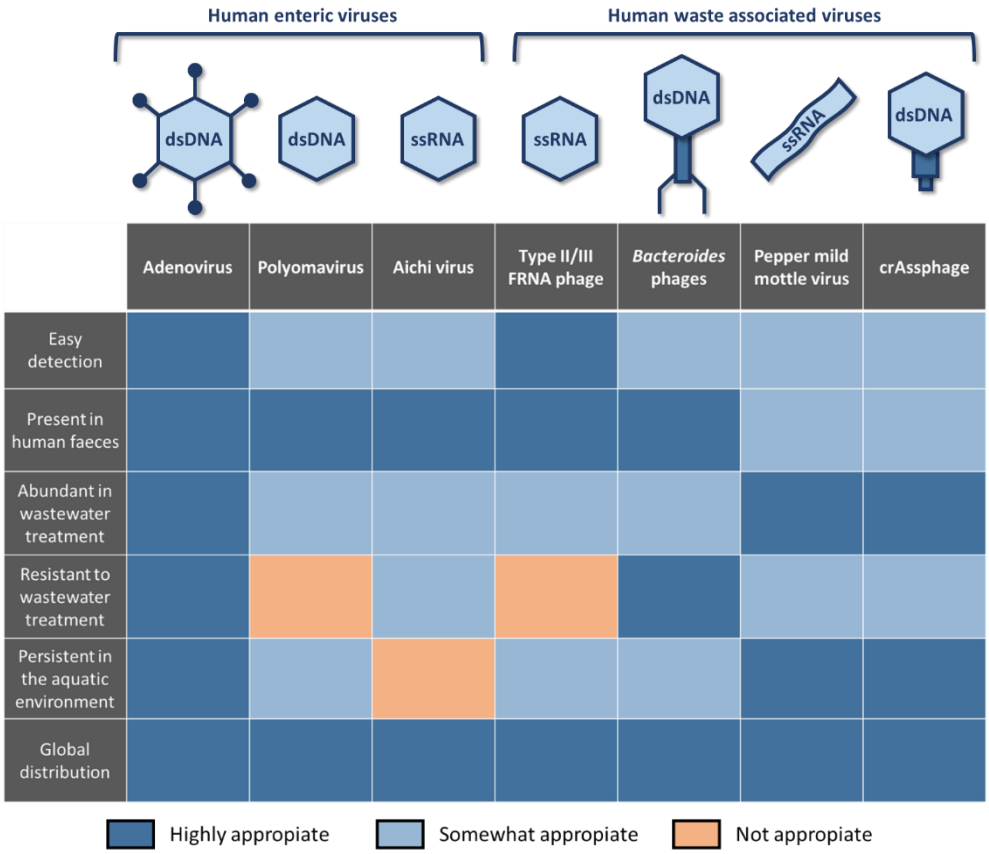


Figure 2. Viral indicators for tracking domestic wastewater contamination in the aquatic environment. Adapted from (Farkas et al. 2020)**.**

Limitations on the detection and quantification of viruses in water

Despite the fact that governments and the scientific community have agreed to control the viral population in various types of waters, the analytical methods for quantification and, consequently, the reduction of levels required to ensure minimal risk to the exposed population, are in a state of uncertainty (Gerba et al. 2018). In order to finally build effective water reclamation systems, it is obvious that approaches to concentrate and quantify human enteric viruses in environmental waters need to be improved. For a very long time, technologies based on cell culture have been employed to detect some infectious enteric viruses in water samples. However, water concentration processes are time-consuming, and the majority of them call for specialised

equipment (Nordgren et al. 2009). As an alternative, molecular detection techniques have been shown to be effective for the quick, accurate, and reliable detection and quantification of enteric viruses in water samples (Farkas, McDonald, et al. 2018; Katayama et al. 2008; Simmons and Xagoraraki 2011). However, it is impossible to distinguish between inactivated and potentially infectious viruses using molecular-based techniques. In terms of methodology, infectious virus presence is generally quantified through infection of a confluent monolayer of a specific host cell line which is then covered in a semi-solid medium to impede excessive viral spread and by finally counting visible infected areas named as "plaques" which are surrounded by non-infected cells. This procedure takes more time, labour, skill, and costly equipment than detecting faecal coliform bacteria (Fong and Lipp 2005). Besides, some of these viruses are difficult (i.e. HEV, human norovirus, and HAV) or impossible to replicate in cell culture. Furthermore, cell culture-based methods, for example, need a 10-day incubation time to detect replicating HAdVs. As a result, typical viral growth tests by cell-culture are either unavailable or take too long to be applied in samples collected from WWTPs. While ELISA and polymerase chain reaction (PCR)-based methods may be used to identify viral proteins or genomes quickly, they do not differentiate between infectious and non-infectious viral particles. Alternatively, integrated cell culture-PCR (ICC-PCR) decreases the time required for cell-culture based tests while still allowing infectious viruses to proliferate in host cells (Fong and Lipp 2005). However, the use of cell culture is still required to demonstrate viral infectiousness, which is impracticable for microbiological analysis laboratories. Although breakthroughs have been made in concentrating viruses from huge quantities of water, a simple, quick method of detecting infectious enteric viruses has yet to be developed (Ikner, Gerba, and Bright 2012).

2.4. Viral concentration procedures in waters

Detection of human enteric viruses in many kinds of aquatic habitats, such as raw and treated wastewater, surface water, groundwater, ocean, and even treated drinking water, has been the topic of many investigations to date (Fong and Lipp 2005; Gerba, Kitajima, and Iker 2013). Given that human enteric viruses are often present in low amounts in environmental water samples, it is critical to begin these analyses by concentrating the viruses into reduced sample volumes to improve the assay sensitivity (Haramoto et al. 2018a). The design and implementation of approaches for concentrating viruses have aided in the identification of a variety of viruses utilizing culture- or molecular-based tests (Cashdollar and Wymer 2013; Ikner et al. 2012).

3. Molecular methods

3.1. RT-qPCR and Digital RT-qPCR

Nowadays, it is possible to collect quantitative information on the viral genomes present in the water thanks to the fast improvement of molecular biology methods like quantitative PCR (qPCR)(Corpuz et al. 2020). Nonetheless, even though qPCR provides highly accurate quantitative data, concentrations obtained using this method should be interpreted with caution due to potential efficiency losses during the complex detection process which includes the following steps: water sample concentration and processing; RNA extraction; (RT-)qPCR of targeted viruses; PCR inhibition assessment by process controls throughout sample collection and processing; PCR standard curve preparation; and recovery efficiency testing (Ahmed et al. 2020; Corpuz et al. 2020; Haramoto et al. 2018b).

PCR has become the instrument of choice for precise amplification of DNA *in vitro*. The main principle of PCR has not changed since 1985, and it still comprises primers, DNA polymerase, nucleotides, particular ions, and DNA template, as well as cycles that include DNA denaturation, primer annealing, and extension. The advent of PCR has stimulated study in many areas of biology, and this technique has contributed considerably to the present knowledge in many fields.

The introduction of the notion of measuring DNA amplification in real time by fluorescence monitoring was the most significant milestone in PCR use (Higuchi et al. 1992; Holland et al. 1991). Fluorescence is monitored after each cycle in qPCR, and the intensity of the fluorescent signal represents the instantaneous quantity of DNA amplicons in the sample at that exact time. The use of standard reference material of the targeted nucleic acid sequence allows the quantification of the samples.

PCR has been utilized in pathogen detection because of its ability to amplify a particular piece of DNA (specificity). With the increased availability of sequencing data, it is now practically viable to develop qPCR tests for any microbe of interest (groups and subgroups of microorganisms, for example).

Pros and cons of using qPCR in detection and quantification of pathogens

The key benefits of qPCR are that it can identify and quantify target nucleotide sequences in a variety of matrices quickly and accurately. Furthermore, since no additional sample processing is necessary after the amplification, qPCR is safer in terms of preventing cross-contamination. A large dynamic range for

quantification and multiplexing of amplification of many targets into a single reaction are two further benefits of qPCR (Klein 2002). In diagnostic qPCR analyses that depend on the presence of internal amplification controls, the multiplexing option is critical for detection and quantification (Bustin et al. 2009; Kubista et al. 2006; Yang and Rothman 2004).

When it comes to identifying and quantifying viral pathogens in water sampless, there are many factors to consider: the target of interest (DNA or RNA); the availability of a reference standard material; the presence of inhibitors; and result interpretation of qPCR.

Reverse transcription qPCR (RT-qPCR) adds an additional step when converting RNA to cDNA by using a reverse transcriptase enzyme. The resulting cDNA acts as template for the posterior PCR step, allowing the amplification of target in RNA sequences. As a result, (RT)-qPCR along with qPCR has become a must-have technological method for the quantification, identification, and genotyping of RNA viral pathogens (Yang and Rothman 2004).

Digital PCR

In the recent years there has been a notable increase in the use of a modern PCR technique called digital PCR (dPCR) for environmental samples analysis (Heijnen et al. 2021; Kuypers and Jerome 2017). Different from conventional PCR, the original sample is separated into a large number of partitions where dPCR individually takes place. Even though it has greater complexity and slower throughput, there are many advantages over qPCR. These include absolute quantification without a standard curve, improved precision, improved accuracy in the presence of inhibitors, and more accurate quantitation when amplification efficiency is low (Kuypers and Jerome 2017).

3.2. Capsid integrity quantitative PCR to assess virus potential infectivity

Molecular methods based on qPCR are rapid and have been effectively used to over the last two decades to evaluate viral loads in the aquatic environment and to meet food safety regulations (Bosch et al. 2018; Gerba and Betancourt 2019). While qPCR is robust, cost-effective, and particularly sensitive and specific, it has the serious flaw of being unable to distinguish between infectious and non-infectious viral particles, resulting in an overestimation of the number of virions present in a sample (Chhipi-Shrestha, Hewage, and Sadiq 2017). Novel approaches, such as changing the length of the PCR product, or amplifying less stable mRNA after reverse transcription to DNA (Ho

et al. 2016; Ko et al. 2003; Polston et al. 2014; Wu et al. 2019) have shown limited robustness and sensitivity. Capsid integrity qPCR, in which samples are pre-treated with the intercalating azo dyes propidium monoazide (PMA), ethidium monoazide (EMA), or their derivates PMAxx and PEMAX, is one of the most well-known qPCR modifications for inferring viral infectivity (Table 3). Platinum compounds, such as platinum chloride (IV) ($PtCl_4$) or cisplatin (CDDP), have some advantages over other photoactivable viability dyes, such as PMA and EMA (Elizaquível, Aznar, and Sánchez 2014; Puente et al. 2020a; Randazzo, Piqueras, et al. 2018; Randazzo, Vasquez-García, et al. 2018), in that they do not require a photoactivation instrument and are less expensive (Karami et al. 2014; Soejima and Iwatsuki 2016). Furthermore, owing to turbidity, suspended solids, and density of concentrated samples, photoactivation of azo dyes may be hindered in complex matrices such as wastewater and molluscs (Leifels et al. 2020; Polo et al. 2021). Capsid-integrity RT-qPCR was first reported nearly two decades ago by (Nogva et al. 2003) to allow the identification of viable but non-cultivable bacteria. This method was successfully optimized to remove putatively false-positive qPCR signals deriving from virions with broken capsids in complex matrices such as sewage and surface water (Coudray-Meunier et al. 2013; Leifels et al. 2016; Parshionikar et al. 2010; Randazzo et al. 2018a; Sánchez et al. 2012a). Based on the concept that an azo dye can only enter viral particles with a disrupted capsid to covalently and permanently bind with viral DNA or RNA, this preparation prevents the amplification of nucleic acids due to the detachment of the polymerase when it comes into contact with the dye-genome complex. Following that, only genomic targets originating from intact virions are amplified, whereas nucleic acids that are free (outside the virus particle) or belong to non-infectious viruses are not amplified by qPCR/RT-qPCR. This approach of indirect viable assessment has been particularly effective for viruses for which cell cultivation-based detection has proven problematic, although it has yet to be thoroughly verified (Estes et al. 2019). One notable drawback of intercalating dyes is their inability to discriminate viruses that have lost their infectivity due to damaged nucleic acids but whose capsid is still intact, which is a common occurrence after UV-C treatment (Leifels et al. 2015). Furthermore, a technique's effectiveness is influenced by a variety of elements, including virus type, inactivation method, dye type, contaminated matrix, and concentration. As seen by the wide variety of capsid integrity pretreatment conditions reported in the literature, incubation conditions and light source are also important in the application of capsid integrity qPCR (Randazzo et al. 2016)(Table 3). More recently, this approach has been used to measure coronavirus virion integrity, utilizing either platinum compounds or photoactivable dyes (Blondin-Brosseau et al. 2021; Puente et al. 2020a; Wurtzer et al. 2021).

Reagents	Concentrations	Virus	Incubation conditions		Light exposure condition		References
			Temp.	Time (min)	Time (min)	Source	
Monoazide dyes							
EMA	5-100 µM	HAV, RV	4 °C	120	15	LED	(Coudray-Meunier et al. 2013)
	20--250 µM	HuNoV	5 °C	30	15	LED	(Fraisse et al. 2018)
	20 µM	HAV, HAdV 41, Coxsackievirus B2 (CV-B2), HuNoV	On ice, RT	10, 30	15	LED	(Moreno, Aznar, and Sánchez 2015; Prevost et al. 2016; Randazzo et al. 2016)
	25 µM	MNV	RT	10	15	LED	(Lee et al. 2015)
	40 µM	HAdV	On ice	30	15	LED	(Leifels et al. 2016, 2019)
	50 µg/mL (or 119 µM)	Aichi virus 1 (AiV), HAdV5, PV1	4 °C	30	3	Halogen	(Canh et al. 2018; Sangsanont et al. 2014)
	1-10,000 ng/mL (or 0.0024–24 µM)	PV1	4 °C	30	0–5	Halogen	(Kim et al. 2011)
	0.1-100 µM	Murine hepatitis virus (MHV), SARS-CoV-2	4 °C	30	3	Halogen	(Canh, Torii, Yasui, Kyuwa, and Katayama 2021a)
	100 µM	HAdV5, PV1, RV, MNV, Aichi virus 1 (AiV)	n/a	5	5	Halogen	(Canh et al. 2019; Leifels et al. 2015)
	100 µg/mL (238 µM)	Avian influenza virus (AIV)	RT	5	10	Halogen	(Graiver et al. 2010)
PMA	5-100 µM	HAV, RV	4 °C	120	15	LED	(Coudray-Meunier et al. 2013)
	10 µM	MS2 coliphage	RT	5	10	Halogen	(McLellan, Lee, and Habash 2016)
	20 µM	HAdV 41, CV-B2	On ice	30	15	LED	(Prevost et al. 2016)
	20-250 µM	HuNoV	5 °C	30	15	LED	(Fraisse et al. 2018)
	25-200 µM	HAV	n/a	5	15	LED	(Fuster et al. 2016)
	40 µM	HAdV, RV, EV	On ice	30	15	LED	(Leifels et al., 2019, 2016)
	50 µM	HuNoVs, HAdV-2, mengovirus (MgV), Tulane virus (TV), HAV	RT	10, 15	10, 15	LED	(Blanco et al. 2017; Kralik et al. 2016; Moreno et al. 2015; Quijada et al. 2016; Randazzo et al. 2016; Zhou et al. 2017)
	50-1000 µM	HAdV, CV, PMMoV	RT	10	15	LED	(Shirasaki et al. 2020)
	50 and 100 µM	HAV	RT	5	15	LED	(Sánchez, Elizaquível, and Aznar 2012b)
	100 µM	Bacteriophage T4	RT	5	15	LED	(Fittipaldi et al. 2010)
	100 µM	AiV, Snow Mountain virus (SMV), HuNoV, HAdV5, PV1, RV, MNV	4 °C, RT	5, 30, 60	3, 5	Halogen	(Canh et al. 2019; Escudero-Abarca et al. 2014; Leifels et al. 2015)
	100, 200 µM	PV1, Echovirus 7, CV-B5, HuNoV	n/a	5	3	Halogen	(Parshionikar et al. 2010)
	200 µM	Bacteriophage phiX174, HuNoV	RT	5, 10	10, 20	LED	(Ho et al. 2016; Jeon et al. 2020)

	10-300 µM	HuNoV, PMMoV	RT	5	15	LED	(Lee et al. 2018)
	250 µM	MNV	RT	10	15	LED	(Lee et al. 2015)
	348 µM	PV, HuNoV, MNV	RT	5	3	Halogen	(Karim et al. 2015)
	0.1-100 µM	MHV	RT	30	3	Halogen	(Canh, Torii, Yasui, Kyuwa, and Katayama 2021a)
PMAxx	20-250 µM	HuNoV	5 °C	30	15	LED	(Fraisse et al. 2018)
	50-1000 µM	HAdV, CV, PMMoV	-	10	15	LED	(Shirasaki et al. 2020)
	50 µM	HAV, HuNoV	4 °C, RT	10	15	LED	(J. Chen et al. 2020; Randazzo et al. 2016)
	50-250 µM	HuNoV	RT	10	15	LED	(W. Randazzo et al., 2018a)
	100 µM	HEV, HAV, CV-B5, SARS-CoV-2	On ice, RT	30	15	LED	(Walter Randazzo et al., 2018; Wurtzer et al., 2021)
	100, 250 µM	Porcine epidemic diarrhea virus (PEDV)	RT	10	15	LED	(Puente et al. 2020a)
	200-400 µM	MNV, MgV	RT	15	15	LED	(Razafimahefa et al. 2021)
PEMAX	50-200 µM	HuNoV	RT	30	15	LED	(Gyawali and Hewitt 2018)
	50 µM	HuNoVs	RT	10	15	LED	(Randazzo et al. 2016)
Platinum compounds							
CDDP	0.1-1000 µM	MHV, SARS-CoV-2	RT	30	–	–	(Canh, Torii, Yasui, Kyuwa, and Katayama 2021b)
	50-500 µM	PEDV	RT	30	–	–	(Puente et al. 2020a)
	1000 µM	PMMoV, AiV	37 °C	30	–	–	(Canh et al. 2019; Canh, Torii, Furumai, et al. 2021)
	50-2500 µM	HuNoV	5 °C	30	–	–	(Fraisse et al. 2018)
$PtCl_4$	50-2500 µM	HuNoVs, HAV	5, 37 °C	10–120	–	–	(J. Chen et al., 2020)
	50-1000 µM	HEV, HAV, PEDV, HuNoVs, HAV	4–5 °C, RT	30	–	–	(J. Chen et al., 2020; Puente et al., 2020; Walter Randazzo et al., 2018)
H_2PtCl_6, Cis-DEP, $Pt(PPh_3)_4$	50-2500 µM	HuNoV	5, 37 °C	10–120	–	–	(Fraisse et al. 2018)
Palladium compounds							
$Pd(Oac)_2$, $PdCl_2(cod)$, BBPC	50-2500 µM	HuNoV	5, 37 °C	10–120	–	–	(Fraisse et al. 2018)

Table 3 Treatment conditions of capsid-integrity reagents for viruses in literature. n/a, not available; -, not applicable; RT, room temperature. Hepatitis E (HEV), Hepatitis A (HAV), Human norovirus (HuNoV), Human adenovirus (HAdV), Murine hepatitis virus (MHV), Murine norovirus (MNV), Aichivirus (AiV), Poliovirus (PV), Avian Influenza virus (AIV), Tulane virus (TV), Rotavirus (RV), Coxsackievirus (CV), Enterovirus (EV), Snow Mountain virus (SMV), Pepper mild mottle virus (PMMoV), Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Porcine epidemic diarrhea virus (PEDV). Adapted from (Canh et al. 2022).

3.3. Porcine Gastric Mucin (PGM) capsid-integrity assay

There are other capsid integrity assays that may be utilized to circumvent inhibition in viability RT-qPCR procedures caused by suspended organic compounds. These assays are normally based on viral capsid attachment to antibodies or specific organic molecules. The porcine gastric mucin (PGM) test has been used as a quick and easy way to check the integrity of norovirus capsids. Previous studies have shown how PGM can bind to norovirus and Tulane virus capsid proteins with a high affinity (Dancho, Chen, and Kingsley 2012; Li and Chen 2015; Suresh, Harlow, and Nasheri 2019). As a result, when PGM-coated magnetic beads (PGM-MBs) or multi-well plates coated with PGM are combined with a sample, the viruses' capsids attach to PGM, which can then be sorted from free viral nucleic acids and non-infectious norovirus particles using magnetic forces or by washing steps, respectively. The PGM test developed by Farkas et al. (2018) was used to evaluate norovirus inactivation in wastewater, river and estuary water, and sediment samples with great success (Farkas, Cooper, et al. 2018). Furthermore, PGM-MBs are extensively utilized to predict the hazards associated with shellfish and fresh vegetables (Bartsch et al. 2016; Tian et al. 2012; Ye et al. 2014).

3.4. Metagenomics and high-throughput sequencing for environmental samples

Metagenomics is a term used for experiments in which all nucleic acids in a certain sample are sequenced (Wooley, Godzik, and Friedberg 2010). Since viruses lack a universally conserved motif such as the 16S rRNA gene for bacteria, viral metagenomics appear to be the best tool to recover full and partial genomes of all viruses present in a given sample (Bai et al. 2022). Isolation of viral particles, extraction of viral nucleic acid, reverse transcription

(RNA to cDNA), nucleic acid segmentation (not needed for third-generation or targeted sequencing), and high-throughput sequencing of cDNA or DNA fragments are the general steps in the viral metagenome assembly process. Finally, raw or assembled sequences are subsequently annotated and aligned with reference genomes or specific genes. Different metagenomic procedures for viral analyses are schematized in Figure 3. Untargeted viral metagenomics has several advantages over other methods for identifying viral pathogens (Bergner et al. 2019): firstly, there is no need to target a specific pathogen or obtain sequence information for that pathogen; secondly, multiple pathogens can be detected in a single sample; and finally expensive and often unsuccessful culturing or immunologic assays can be avoided (Hasiów-Jaroszewska, Boezen, and Zwart 2021). Due to its sensitivity, extensive detection range, and precise information on the discovered virus, viral metagenomics offers a lot of possibilities in the monitoring of viruses in the food chain, environment, and in disease outbreaks (Aarestrup et al. 2012; Adriaenssens et al. 2021; Deurenberg et al. 2017; McCall et al. 2021; Nieuwenhuijse and Koopmans 2017).

Metagenomic sequencing has previously been used to describe global viral diversity (virome) in ocean samples (Hingamp et al. 2013), and untargeted metagenomic sequencing has been used to identify a variety of viruses in sewage samples in multiple studies (Cantalupo et al. 2011; Guajardo-Leiva et al. 2020; Martínez-Puchol et al. 2020; Nieuwenhuijse et al. 2020; Yang et al. 2021). Nonetheless, metagenomics approaches present some limitations that must be taken into account. For example, there is a higher presence of bacterial genomes rather than viral genomes in environmental and sewage water samples. Also, both interpretation and analysis of metagenomic data are very complex and difficult to handle because of all the "data-cleaning" steps which are difficult to automate and the fact that there is still a very poor general knowledge on the virome of these types of samples. These limitations make it necessary to add enrichment techniques to the protocols to increase the amount of viral sequences in the samples (Ajami et al. 2018; Gebremedhn et al. 2020; Hall et al. 2014; Lewandowska et al. 2017). Finally, there has also been a notably strong bias towards viruses isolated from western countries when looking for sequence similarities among samples from all over the globe. As an example, sewage viromes from Nigeria, Nepal, Bangkok, and California, showed differences in the subsets of detected human viruses with California presenting far more sequence similarities with the NCBI GenBank reference sequences than the others (Ng et al. 2012).

Sequencing reads with no sequence similarity to existing reference databases have also been discovered in sewage and environmental metagenomics research. Unmapped sequences account for 37 to 66 % of all sequences (Paul G. Cantalupo et al. 2011; Ng et al. 2012). However, it is still unclear if such

sequences reflect new viruses that can be spread by food or water. Nonetheless, these findings demonstrate that untargeted metagenomic sequencing has the potential to detect both new and well-known human viruses.

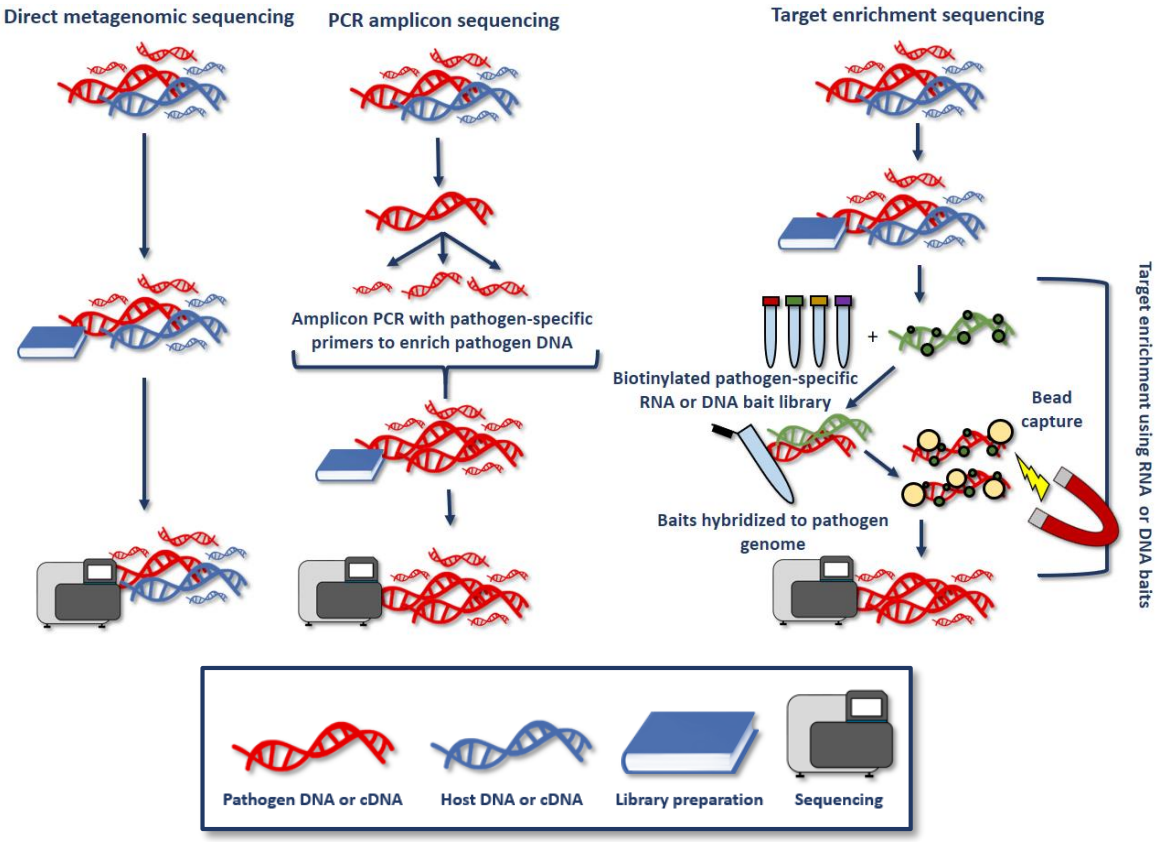


Figure 3. Procedures for sequencing viral genomes. Adapted from (Houldcroft et al., 2017)**.**

4. Wastewater based epidemiology

In the aquatic environment, most faecal-oral-transmitted viruses are very resistant, and they may remain at high levels despite reclamation treatments applied for drinking water and sewage treatment (Kim et al. 2012; Kotwal and Cannon 2014). Since each person sheds roughly 100 g faecal matter each day, and 10^5 to 10^{11} enteric virus particles per gram of stool are discharged daily from an infected individual, these viruses may be detected in sewage (Bosch 1998; Fechner, Fenske, and Jahreis 2013; Timm et al. 2013). Several approaches for detecting viruses in sewage, known as wastewater-based epidemiology (WBE) have been developed (Calgua et al. 2013; Koo et al. 2012; Rodríguez et al. 2013). As part of the World Health Organisation's (WHO) polio eradication mission, several protocols have been developed for poliovirus monitoring (Levitt et al. 2014). Noroviruses and other viruses have also been detected in water using these approaches (Fernandez-Cassi et al. 2018a; Nenonen et al. 2012; Rusiñol et al. 2014). Concentration and identification of human viruses using PCR and sequencing techniques may be utilized as an early warning system for epidemics caused by faecally-shredded viruses. The analysis of community wastewater samples in recent years has made it possible to study the seasonality of certain viruses throughout the year, as in the case of noroviruses, where several studies have highlighted their greater presence in winter than in summer resembling the illness incidence within the population (Muscillo et al. 2013). Furthermore, qPCR or metagenomics data obtained from WBE can be compared with clinical samples from the same population finally serving as a perfect complement for disease transmission modelling. For example, variants of a pathogenic viruses identified in clinical samples can be looked for in wastewater to assess whether these variants represent a public risk to community spread or not (Ai et al. 2021). In addition, WBE provides a lot of information at theon a community level at a very low cost and free from ethical complications as there is no need for invasive tests on affected individuals. As an additional advantage with respect to clinical samples data, the results from wastewater analysis also take into account that share of the population that may be asymptomatic (Ahmed, Angel, et al. 2020; Y. Chen et al. 2020). Finally, it’s notable that not only the faecally transmited viruses can be looked for in wastewater, but all those that are present in faeces and urine, such as, for example, the avian influenza virus (AIV).

5. COVID-19 pandemic

Coronavirus Disease 2019 (COVID-19) is an infectious illness caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). In November of 2019, the first known case was discovered in Wuhan, China (Liu, Kuo, and Shih, 2020). Since then, the virus has spread globally, resulting in a pandemic that has caused over 600 million confirmed cases and cost more than 6 million human lives (WHO 2022b). COVID-19 symptoms vary, but often most include fever, cough, headache, exhaustion, breathing difficulties, and loss of smell and taste (da Rosa Mesquita et al. 2021). Symptoms might appear anywhere from one to fourteen days after being exposed to the virus. At least one-third of those who are afflicted do not show any signs or symptoms (Esakandari et al. 2020). In addition, for the majority (81%) of those whose symptoms are manifest, these are mild to moderate (up to mild pneumonia). Severe symptoms are more likely to emerge in the elderly. Some persons continue to have a variety of symptoms (long COVID) months after recovery, and organ damage has been reported (Alimohamadi et al. 2020). SARS-CoV-2 is spread via the air when droplets and airborne particles harbouring the virus are inhaled (Falahi and Kenarkoohi 2020). Transmission may also occur if infected fluids are splashed or sprayed in the eyes, nose, or mouth, as well as through contaminated surfaces (Falahi and Kenarkoohi 2020; Lotfi, Hamblin, and Rezaei 2020). As there is evidence of SARS-CoV-2 faecal excretion, the possibility of transmission via the faecal-oral route has been suggested (Y. Wu et al. 2020). Since the beginning of the pandemic, many diagnostic procedures have been established. Lately, several COVID-19 vaccines have been authorized and supplied in a large number of countries, which have led to promising widespread immunization results (Feikin et al. 2022; Harder et al. 2021). The standard diagnostic approach is nucleic acid amplification by RT-qPCR from a nasopharyngeal swab sample, but viral presence has also been detected by transcription-mediated amplification (TMA), or reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Eftekhari et al. 2021).

OBJECTIVES

II. OBJECTIVES

Background and current status

Foodborne viruses, or enteric viruses, are among the major health risks associated with food consumption, and thus have an impact on food safety. WHO reported that 120 million cases of gastroenteritis caused by human noroviruses occur annually (WHO 2017). Enteric viruses are responsible for a variety of pathologies: from usually mild gastroenteritis to more serious pathologies such as acute hepatitis, myocarditis, or even meningitis or aseptic encephalitis. In recent years, there has been an increase in the number of foodborne outbreaks caused by these pathogens. Moreover, an increased incidence of hepatitis E cases associated with meat consumption has recently been demonstrated (Kupferschmidt 2016).This is a major public health concern, causing serious health complications in some population groups, such as immunocompromised people or the elderly. This situation also represents important economic implications globally. Enteric viruses are mainly transmitted via the faecal-oral route and can therefore be potentially present in food directly contaminated with faecal matter or through contaminated water. The importance of enteric viruses in the field of food safety is highlighted by the interest shown by various international organisms. In this regard, the European Food Safety Agency (EFSA) has also published several guidelines on the relevance and control of viruses in food; in line with the guidelines on general hygiene principles for the control of viruses in food (CX/FH 10/42/5) developed by the Codex Commission (Ricci et al. 2017). These documents underline that the control of risks in food should start during agricultural or animal production and continue throughout the food chain ("from farm to table"). Water is ingested as part of the diet, used for irrigation of crops, for washing and cleaning of food during food preparation, and is also an essential part of many food products. The presence of human enteric viruses (e.g. human norovirus, HAV, etc.) in water is also well documented and can pose a significant threat to consumers' health (Katayama et al. 2008).

Currently, the control of human enteric viruses in water is based on the use of bacterial indicators which, very often, do not correlate with the presence of these (Lin and Ganesh 2013; Staley et al. 2012). In this sense, PCR and RT-qPCR analysis for the amplification of specific genes of pathogenic viruses of public interest is presented as the most cost-effective and simplest method to determine the presence of these pathogens in water. However, these techniques have the limitation of not being able to distinguish between infectious viruses and non-infectious viral particles. Recently, the advent of capsid-integrity PCRs and RT-qPCRs assays has allowed more accurate

estimates of the presence of infectious viruses in aquatic matrices (Leifels et al. 2016, 2020).

Lately, high-throughput sequencing (HTS) techniques have arisen as a great tool to gain an in-depth understanding of the diversity of microbial populations, offering the possibility to simultaneously analyse all genomic sequences present in a single sample (Bergner et al. 2019; Haramoto et al. 2018a; Nieuwenhuijse et al. 2020).

General and specific objectives

The main objective of this thesis aimed to **detect and track viruses of public health interest in waters through the combination of molecular and metagenomic procedures.** Initially, the interest was mainly focused on enteric viruses and at a later stage also SARS-CoV-2.

For this purpose, several specific objectives have been considered:

1. To develop procedures for enteric viruses' concentration in water samples.

2. To monitor the prevalence of enteric viruses and viral indicators in influent and effluent water samples from wastewater treatment plants.

3. To characterise the virome of influent and effluent samples from water treatment plants.

4. To implement a SARS-CoV-2 monitoring system in wastewater.

5. To develop and optimize rapid molecular methods to infer SARS-CoV-2 viral infectivity.

RESULTS

Chapter 1. Development of procedures to concentrate and detect Hepatitis E virus in water samples.

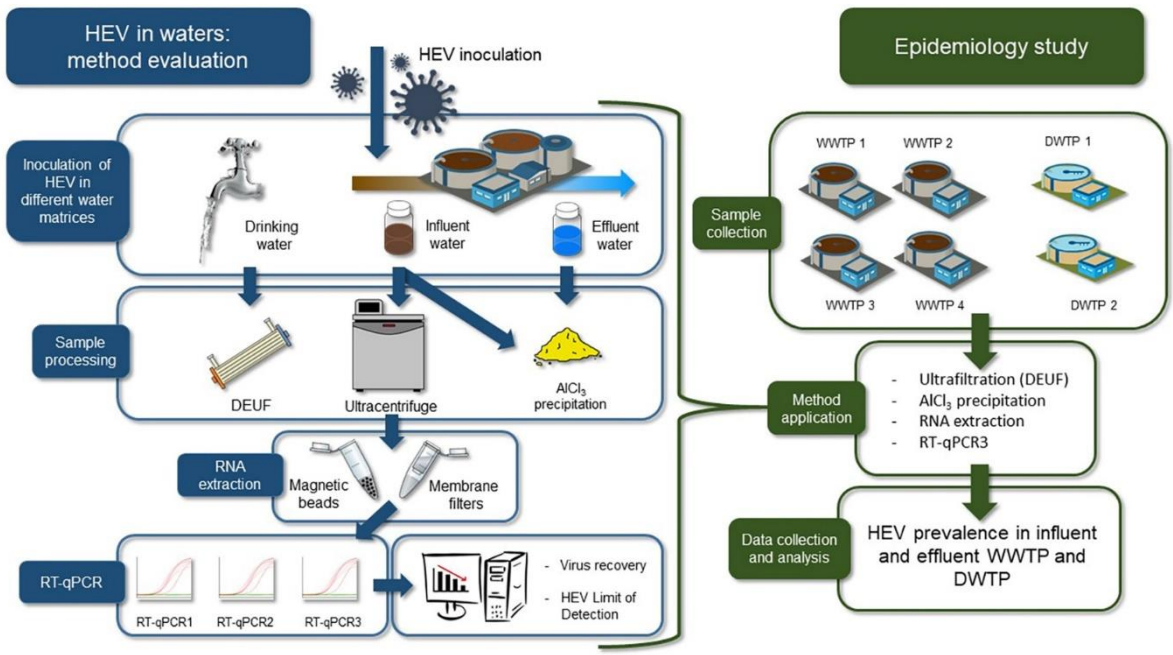
III. RESULTS

Chapter 1. Development of procedures to concentrate and detect Hepatitis E virus in water samples

1.1. HEV Occurrence in Waste and Drinking Water Treatment Plants

This section is an adapted version of the following published research article:

Cuevas-Ferrando E, Randazzo W, Pérez-Cataluña A and Sánchez G (2020). **HEV Occurrence in Waste and Drinking Water Treatment Plants.** Front. Microbiol. 10:2937.
DOI: https://doi.org/10.3389/fmicb.2019.02937



1. Abstract

Hepatitis E virus (HEV), particularly zoonotic genotype 3, is present in environmental waters worldwide, especially in industrialized countries. Thus, monitoring the presence of HEV in wastewater treatment plants (WWTPs) is an emerging topic due to the importance of reusing water on a global level. Given the limited data, this study aimed to monitor the occurrence of HEV in influent and effluent water in waste- and drinking-water treatment plants (WWTPs and DWTPs). To this end, different procedures to concentrate HEV in influent and effluent water from WWTPs and DWTPs were initially evaluated. The evaluated procedures resulted in average HEV recoveries of 15.2, 19.9, and 16.9% in influent, effluent, and drinking water samples, respectively, with detection limits ranging from 10^3 to 10^4 international units (IU)/L. Then, a one-year pilot study was performed to evaluate the performance of the selected concentration method coupled with three RT-qPCR assays in influent and effluent water samples from four different WWTPs. HEV prevalence in influent water varied based on both the RT-qPCR assay and WWTP, while HEV was not detected in effluent water samples. In addition, HEV prevalence using only RT-qPCR3 was evaluated in influent (n = 62) and effluent samples (n = 52) from four WWTPs as well as influent (n = 28) and effluent (n = 28) waters from two DWTPs. The present study demonstrated that HEV circulated in the Valencian region at around 30.65% with average concentrations of 6.3×10^3 IU/L. HEV was only detected in influent wastewater samples, effluent samples from WWTPs and influent and effluent samples from DWTPs were negative. However, given that the infective dose in waterborne epidemics settings is not yet known and the low sensibility of the assay, unfortunately, no direct conclusion could be achieved on the risk assessment of environmental contamination.

1. Introduction

Hepatitis E virus (HEV) is a human enteric virus that mainly causes self-limiting acute viral hepatitis. According to the World Health Organization, 20 million cases of hepatitis E and 44,000 deaths occur worldwide every year1. HEV is an emerging foodborne pathogen (Harrison and DiCaprio, 2018), and the incidence of confirmed cases in the European Union has steadily increased over the last decade (Kupferschmidt, 2016; Ricci et al., 2017).

Hepatitis E infections are caused by a small (27–34 nm), positive-sense, single-stranded RNA virus (approx. 7.2 kb size) that belongs to the Hepeviridae family (Sooryanarain and Meng, 2019; Van der Poel and Rzezutka, 2019). HEV is excreted in feces as non-enveloped virions but circulates in the blood in a membrane-associated, quasi-enveloped form (Yin et al., 2016). HEV is classified into eight genotypes, of which genotype 1 (G1) and G2 are specific to humans. HEV G3, G4, and G7 are zoonotic genotypes that infect humans and animals and have been isolated in different animal species, especially in pigs (Van der Poel, 2014; Sooryanarain and Meng, 2019). The different HEV genotypes have different geographical distributions2. For example, HEV G1 and G2 are predominantly transmitted via the fecal-oral route in Asia, Africa, and Central America, usually through the consumption of contaminated drinking water (Khuroo et al., 2016; Van der Poel and Rzezutka, 2019). In contrast, HEV G3 and G4 are endemic in industrialized countries and transmitted primarily via the consumption of animal meats or direct contact with infected animals (Sooryanarain and Meng, 2019).

Hepatitis E virus transmission to humans through water has been largely demonstrated for HEV G1 and G2, primarily in developing countries, but transmission is also suspected for the zoonotic genotypes since HEV G3 and G4 have been detected in different types of environmental waters (Miura et al., 2016; Haramoto et al., 2018; Fenaux et al., 2019; Van der Poel and Rzezutka, 2019). Given the authorities' concerns, several surveillance studies conducted in different geographic regions have assessed the presence of HEV in urban wastewater with highly variable occurrence (Fenaux et al., 2019). However, few studies have focused on effluent wastewater or drinking water (Fenaux et al., 2019; Purpari et al., 2019; Van der Poel and Rzezutka, 2019). In addition, available data must be interpreted with caution due to the lack of standardized HEV detection procedures and the substantial differences among studies in terms of volume of samples, concentration methods, and RT-qPCR (Fenaux et al., 2019).

To overcome these challenges, this study initially evaluated the performances of different concentration methods, RNA extraction kits, and RT-qPCR protocols in detecting and quantifying HEV in influent and effluent wastewater samples as well as in drinking water samples (Graphical Abstract). After method evaluation, the presence of HEV was monitored in influent and effluent waters from four municipal wastewater treatment plants (WWTPs) and two drinking water treatment plants (DWTPs) in the metropolitan region of Valencia (Spain).

1. Materials and methods

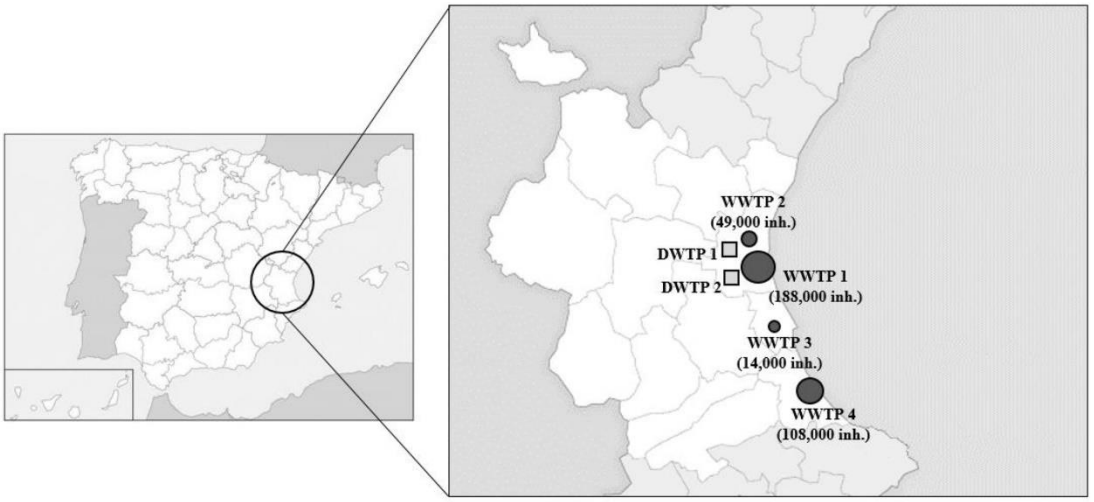
Virus Strains

Fecal sample containing HEV genotype 3f was used in the study. Fecal sample (10% wt/vol) was suspended in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Thermo Fisher Scientific) (pH 7.2). The mix was then vigorously vortexed and centrifuged at 1,000 × g for 5 min to obtain a final 10% (wt/vol) fecal suspension. The supernatant was stored at −80°C in aliquots. The first WHO international standard for HEV nucleic acid amplification technique (NAT)-based assays (code 6329/10) was purchased from Paul-Ehrlich-Institut (Germany). This standard corresponds to HEV genotype 3a positive plasma measured in international units (IU) and containing 250,000 IU/mL and it was used for RT-qPCR quantification, as detailed below (Baylis et al., 2013). Mengovirus (MgV) vMC0 (CECT 100000) was used as a process control.

Sampling Sites

Influent and effluent water samples were collected from four WWTPs and two DWTPs located in the Valencian region, Spain (Figure 1). The collected samples were transferred to the laboratory immediately, and subsequently concentrated as described below.

Figure 1. Map of the sampling locations. WWTP, wastewater treatment plant (squares); DWTP, drinking water treatment plants (circles). Symbols are sized according to the number of inhabitants.



Concentration Procedure Comparison in Influent Wastewater

Influent water samples collected from WWTP1 were artificially inoculated with 5 log IU/L of HEV and 7 log PCRU/L of MgV, spiked as process control.

Initially, the performance of two concentration methods was evaluated: an ultracentrifugation-based method (referred as UC) and an aluminum hydroxide adsorption-precipitation method (referred as Al). For UC method, 35 mL of influent water were centrifuged at 141,000 × g for 2 h 30 min at 4°C. The pellet was then incubated on ice for 30 min with 5 mL of 0.25 N glycine buffer (pH 9.5) and then the solution neutralized with 19 mL of PBS. Suspended solids were removed by centrifugation at 12,000 × g for 15 min. Viruses were finally recovered by ultracentrifugation at 505,000 × g for 1 h at 4°C and subsequently eluted in 1 mL of PBS (Rodríguez-Díaz et al., 2009).

For Al method, 35 mL of influent water were adjusted to pH 6.0 and Al(OH)3 precipitate formed by adding 1 part 0.9N AlCl3 solution to 100 parts of sample. The pH was readjusted to 6.0 and sample mixed using an orbital shaker at 150 rpm for 15 min at room temperature. Then, viruses were collected by centrifugation at 1,700 × g for 20 min. The pellet was resuspended in 1.75 mL of 3% beef extract pH 7.4, and samples were shaken for 10 min at 150 rpm. Concentrate was recovered by centrifugation at 1,900 × g for 30 min and pellet

resuspended in 1 mL of PBS (AAVV, 2018; Randazzo et al., 2019) and stored at −80°C. Experiments were performed in duplicate.

Detection Limit in Influent and Effluent Wastewater

The limit of detection (LoD95%) was obtained by artificially inoculating HEV at 5, 4, 3, and 2 log IU/L in 35 mL for influent water or in 200 mL for effluent water.

Samples were spiked with MgV (7 log PCRU/L) as a process control. Then, virus particles were concentrated by the previously described Al method and RNA extracted using two kits and analyzed by RT-qPCR1 and RT-qPCR2 (detailed below). For each method and contamination level, a PBS sample without influent or effluent water were included to assess potential matrix effects. Experiments were performed in duplicate by concentrating two independent samples for each condition tested.

Concentration Procedure Comparison and Detection Limit in Drinking Water

Drinking water samples (20 L) were artificially inoculated with HEV at 7, 6, 5, and 4 log IU/L. In addition, MgV was spiked and used as process control. HEV primary concentration was performed by a Dead End Hollow Fiber Ultrafiltration (DEUF) using single-use Rexeed-25A dialysis filters (Asahi Kasei Medical Co., Ltd.) with a molecular mass cutoff of 30 kDa, a surface area of 2.5 m2, a fiber inner diameter of 185 µm and a priming volume of 137 mL (Borgmästars et al., 2017). A peristaltic pump (model FH100, Thermo Fisher Scientific) was used for all experiments.

In brief, the Rexeed-25A filters were blocked with 6.25% fetal bovine serum by circulating the blocking solution for 5 min followed by 2 h incubation at room temperature. Afterward, filter was properly assembled and flushed with 1 L of sterile water at 2,900 mL/min and then with the 20 L of inoculated drinking water samples. Subsequently, filter was assembled for a back-flush elution with 500 mL of sterile water supplemented with 0.001% Antifoam, 0.01% NaPP, and 0.01% Tween 80.

Two different approaches were evaluated for secondary concentration: a precipitation with polyethylene glycol (PEG) and a centrifuge filtration procedure by Amicon® Ultra-15 tubes (Merck Millipore Ltd.). For PEG precipitation, 300 mL of concentrate were transferred to two 250 mL

centrifugation tubes, 150 mL of eluate for each tube. Then, 2 g of beef extract (Laboratorio Conda) were added into each tube and shaken until completely dissolved. Then, 50 mL of PEG/NaCl 5× were added and incubated overnight at 4°C in an orbital shaker at 150 rpm. Finally, the samples were centrifuged at 10,000 × g for 30 min and resulting pellets resuspended in 1 mL PBS.

For secondary concentration by centrifuge filtration, 15 mL volume was added to Amicon® Ultra-15 tube and concentrated via centrifugation at 4,000 × g for 15 min. This step was repeated three times using the same ultrafilter for a total of 45 mL sample processed. Then the concentrated viruses were recovered in 1 mL PBS. The viral concentrates were stored at −80°C until further processed. Experiments were performed in duplicate by concentrating two independent samples for each condition tested.

RNA Extraction and RT-qPCR Assays

Two different commercial extraction kits were used for RNA extraction. The extraction using the NucleoSpin®RNA virus kit (Macherey-Nagel GmbH & Co.) (referred as MN) was performed according to the manufacturer's instructions with some modifications. Briefly, 150 µL of each concentrated sample was mixed with 25 µL Plant RNA Isolation Aid (Ambion) and 600 µL of lysis buffer from the NucleoSpin® RNA virus kit and subjected to pulse-vortexing for 1 min. Afterward, the homogenate was centrifuged for 5 min at 10,000 × g to remove the debris. The supernatant was subsequently processed according to the manufacturer's instructions. An additional extraction was carried out using the NucliSENS® miniMAG® system (BioMérieux SA) (referred as NS) and according to manufacturer instructions. In particular, the sample volume was 500 µL and the elution volume was 100 µL. Resultant RNA was analyzed using the RNA UltraSense One-Step kit (Invitrogen SA) and RT-qPCR performed as described in Schlosser et al. (2014) for HEV (referred as RT-qPCR1) and as in ISO 15216-1:2017 for MgV (Supplementary Table 5). For both RT-qPCR assays, undiluted and 1/10 diluted RNA was tested to check for RT-qPCR inhibitors.

Moreover, RNAs were also quantified using the ceeramTOOLS® Hepatitis E Virus Detection KHEV commercial kit (BioMérieux SA) (referred as RT-qPCR2) provided with an internal amplification control.

In all experiments, all samples were run in duplicate and different controls were used, including negative process, extraction and RT-qPCR controls, and controls for extraction efficiency.

Hepatitis E virus was quantified by plotting the quantification cycles (Cqs) to an external standard curve built with the International Standard WHO HEV RNA (code 6329/10). Moreover, extraction efficiencies were calculated and used as quality assurance parameters according to ISO 15216-1:2017 (2017).

Analysis of Naturally Contaminated Influent and Effluent Wastewater

A total of 62 influent and 52 effluent wastewater samples were investigated for the occurrence of HEV as hereafter detailed.

Initially, influent (n = 32) and effluent (n = 32) water samples were collected from four municipal WWTPs located in the Valencian region (eastern Spain), from May 2018 to March 2019 (Figure 1). Two-hundred milliliters of influent and effluent water samples were processed using the Al procedure. Mengovirus was used as process control. RNA extraction was performed using the NucleoSpin® RNA Virus kit (MN kit) and HEV RNA quantified by both RT-qPCR1 and RT-qPCR2. In addition, RNA samples were analyzed by a third RT-qPCR assay (referred as RT-qPCR3, Supplementary Table 5; Girón-Callejas et al., 2015). Additional influent (n = 30) and effluent (n = 20) samples were further collected in June, August, and October 2018 and from April 2019 to August 2019 and analyzed by RT-qPCR3 only.

Analysis of Drinking Water Samples

A total of 28 influent and 28 effluent water samples were collected from two municipal DWTPs (Figure 1) in October and November 2018. The samples were maintained under refrigeration (4°C) for transportation and processed within 24 h. Water samples (20 L) were dechlorinated with sodium thiosulphate (10% wt/vol) after collection, added with mengovirus and concentrated using the Rexeed-25A filters and PEG precipitation, as detailed above. Resultant RNA was extracted by the NucleoSpin® RNA Virus kit (MN kit) and detected by RT-qPCR3.

Statistical Analysis

Results were statistically analyzed and significance of differences was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all cases, a value of $p < 0.05$ was deemed significant. Spearman's rank-order correlation coefficient (ρS) was determined

between inhabitants and HEV positive samples by using Statistica software (StatSoft Inc., Tulsa, OK, United States). The estimated probability of detection with 95% confidence (LoD95%) was calculated by using the PODLOD calculation program (version9) (Wilrich and Wilrich, 2009) for all water samples.

Ethics Statement

Faecal samples were collected at Hospital Clínico Universitario de Valencia (Valencia, Spain). The study was approved by the Comisión de Ética en Investigación Experimental of the University of Valencia (Spain), in accordance with the World Medical Association's Declaration of Helsinki and the relevant European and Spanish guidelines and regulations.

2. Results and discussion

Detection Limit and Efficiency of the Procedure to Concentrate HEV in Influent Water

One major limitation in understanding HEV transmission in contaminated waters is the lack of standardized and validated methods (Ricci et al., 2017). Thus, to provide data on the performance of the HEV detection methods in environmental waters, an ultracentrifugation-based protocol (UC) was compared to an aluminum precipitation procedure (Al) using artificially inoculated influent water samples. The mean HEV recoveries obtained with the UC concentration procedure ranged from 7.98 to 16.83% using MN kit and from 10.24 to 55.08% using NS kit. The Al procedure resulted in mean HEV recovery values ranging from 7.00 to 20.54% and from 10.18 to 90.19% using MN and NS kits, respectively (Table 1).

Table 1. Performance of concentration methods (ultracentrifugation and aluminum precipitation), RNA extraction kits and RT-qPCR assays for HEV detection in artificially inoculated influent water samples.

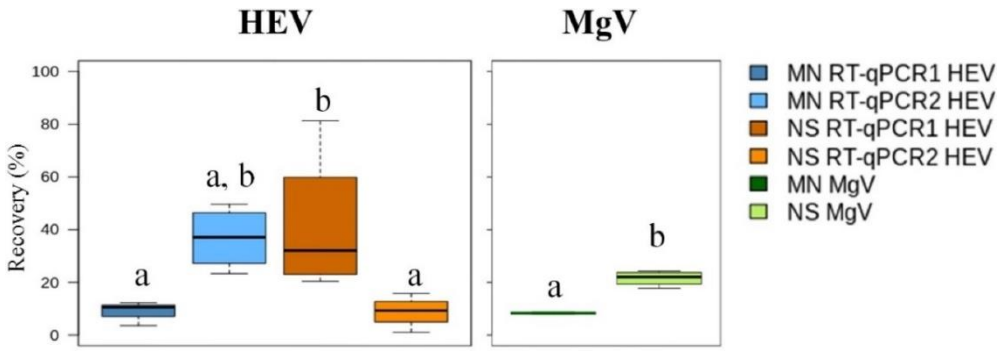
Extraction kit	RT-qPCR	Ultracentrifugation		Aluminum precipitation	
		Mean HEV recovery (min–max) (%)	Mean mengovirus recovery (%)	Mean HEV recovery (min–max) (%)	Mean mengovirus recovery (%)
MN	RT-qPCR1	16.83*A* (13.33 – 21.68)	13.76 ± 4.59A	20.54*A* (17.06 – 24.10)	13.67 ± 2.4A
	RT-qPCR2	7.98*A* (7.75 – 8.30)		7.00*A* (5.45 – 8.58)	
NS	RT-qPCR1	55.08*A* (49.24 – 60.84)	23.31 ± 2.46A	90.19*AB* (84.16 – 96.22)	54.45 ± 17.06B
	RT-qPCR2	10.24*A* (8.95 – 12.64)		10.18*A* (8.54 – 11.82)	

MN: NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.). NS: NucliSENS© miniMag® system (BioMérieux SA). RT-qPCR1: Schlosser et al., 2014. RT-qPCR2: ceeramTOOLS® Hepatitis E Virus Detection KHEV kit (BioMérieux SA). Within each column, different letters denote significant differences among methods (P < 0.05).

The Al procedure was selected for the determination of LoD95% since an ultracentrifuge is not required. To determine LoD95%, influent water was artificially inoculated with MgV together with four levels of HEV and samples concentrated according to the Al procedure. RNA extraction from concentrates was performed using MN and NS kits and subsequently analyzed by RT-qPCR1 and RT-qPCR2.

The mean HEV recovery values obtained using the MN and NS kits ranged from 8.81 to 36.8% and from 8.90 to 41.45%, respectively (Figure 2 and Supplementary Table 1), and no statistically significant differences were observed ($P > 0.05$). On average, LoD95% was 2.9×10^5 IU/L for MN kit and 2.2×10^6 IU/L for NS kit, calculated according to Wilrich and Wilrich (2009). Accordingly, LoD95% increased approximately 10-fold when NS was compared to MN extraction procedure. Overall, the MN kit combined with RT-qPCR1 provided the best LoD95%, which was similar to or slightly higher than those previously reported for other enteric viruses in influent waters (approx. 104–105 genome copies/L) (Nordgren et al., 2009; Randazzo et al., 2019).

Figure 2. Median HEV recovery (%) in influent water samples using the aluminum protocol and comparing two extraction kits and two RT-qPCRs assays. MN: NucleoSpin®RNA virus kit (Macherey-Nagel GmbH & Co.); NS: NucliSENS® miniMag® system (BioMérieux SA); RT-qPCR1: Schlosser et al., 2014; RT-qPCR2: ceeramTOOLS® Hepatitis E Virus Detection KHEV kit (BioMérieux SA). Within each virus, different letters denote significant differences among methods ($P < 0.05$).

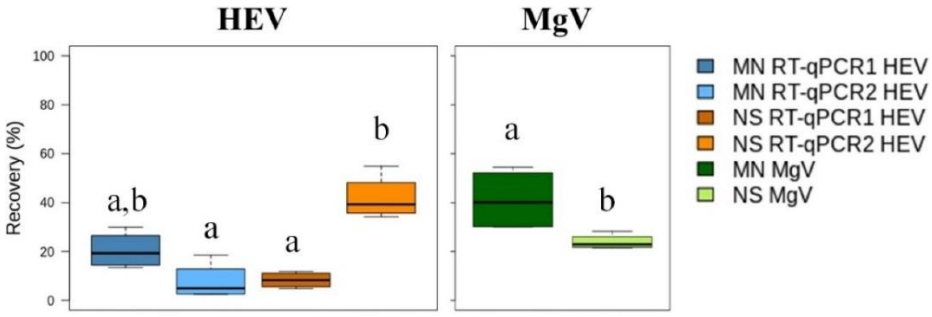


The MgV recovered using the MN and NS kits ranged from 7.92 to 8.72% (8.34% mean) and from 17.76 to 24.29% (21.56% mean), respectively (Figure 2 and Supplementary Table 1). These results support previously reported MgV recoveries in influent waters (Miura et al., 2016). Because only 35 mL of sample are needed for the analysis and ultracentrifugation is not required, the procedure is a potential alternative method for routine influent water screening.

Detection Limit and Efficiency of the Al Procedure to Concentrate HEV in Effluent Water

Few studies over the last decade have assessed the presence of HEV in effluent water samples due in part to the lack of validated procedures (Fenaux et al., 2019). Therefore, the performance of the Al concentration method was analyzed using effluent water samples that were collected downstream from WWTP1 and artificially spiked with four levels of HEV and with MgV, as process control. The MN and NS extraction kits and RT-qPCR1 and RT-qPCR2 were used for sample processing. Viral recovery and HEV LoD95% were determined and the results are shown in Figure 3 and Supplementary Table 2.

Figure 3. Median HEV recovery (%) in effluent water samples using the aluminum protocol and comparing two extraction kits and two RT-qPCRs assays. MN: NucleoSpin®RNA virus kit (Macherey-Nagel GmbH & Co.); NS: NucliSENS® miniMag® system (BioMérieux SA); RT-qPCR1: Schlosser et al., 2014; RT-qPCR2: ceeramTOOLS® Hepatitis E Virus Detection KHEV kit (BioMérieux SA). Within each virus, different letters denote significant differences among methods ($P < 0.05$).



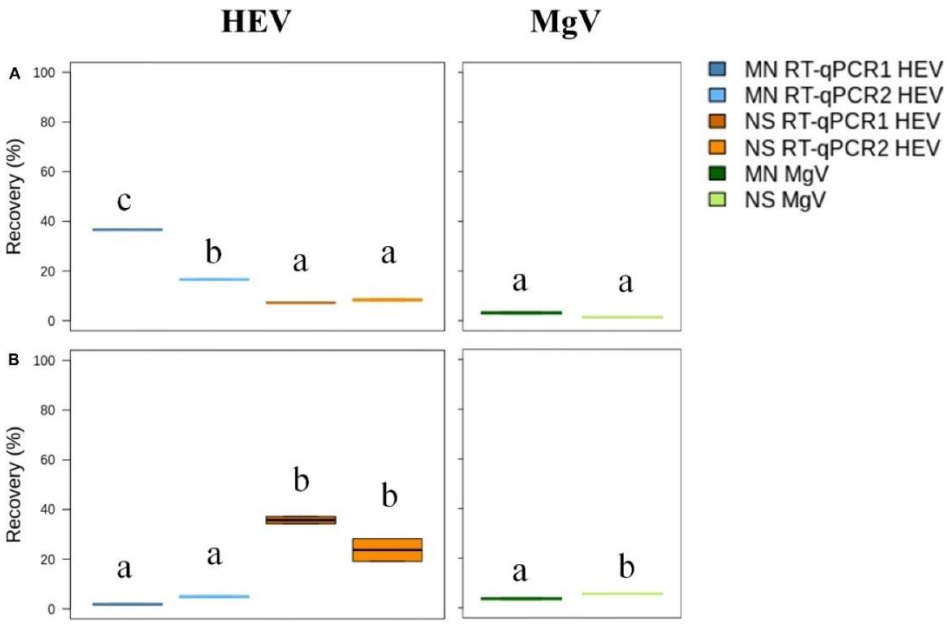
MgV recoveries using the MN and NS kit ranged from 30.08 to 54.50% (41.17% mean) and from 21.52 to 28.32% (23.90% mean), respectively, which are slightly higher than the 8–13% recovery rates of cross-flow ultrafiltration reported previously (Miura et al., 2016). The mean recovery of HEV ranged from 8.33 to 30.01% using the MN kit and from 7.72 to 41.90% with the NS kit. LoD95% was 1.25 × 104 IU/L regardless of the RNA extraction and RT-qPCR used (Supplementary Table 2).

Detection Limit and Efficiency of the Procedure to Concentrate HEV in Drinking Water

Prior to concentration, 20 L tap water samples were added with four different concentrations of HEV, and MgV, as a whole process control. Primary virus concentration was performed using DEUF with Rexeed-25A filters, resulting in an average eluate volume of 605 ± 38.22 mL. Then, the secondary concentration was evaluated comparing in parallel a PEG precipitation and a centrifuge filtration. DEUF ultrafiltration combined with PEG precipitation and MN kit resulted in HEV mean recovery of 16.6 to 36.6%, while recoveries ranged from 7.2 to 8.3% for NS kit (Figure 4A and Supplementary Table 3). The centrifuge filtration procedure using MN and NS resulted in mean HEV recovery values ranging from 1.8 to 4.9% and 23.7 to 35.7%, respectively (Figure 4B and Supplementary Table 4). A minimum recovery rate of 1% MgV

was achieved from all procedures, validating the results. For all the tested procedures, the HEV LoD95% in drinking water was of 6.2 × 103 IU/L (Supplementary Tables 3, 4).

Figure 4. Median HEV recovery (%) in drinking water by Rexeed 25AX ultrafiltration followed by precipitation with polyethylene glycol (A) or centrifuge filtration with Amicon filters (B). MN: NucleoSpin®RNA virus kit (Macherey-Nagel GmbH & Co.); NS: NucliSENS® miniMag® system (BioMérieux SA); RT-qPCR1: Schlosser et al., 2014; RT-qPCR2: ceeramTOOLS® Hepatitis E Virus Detection KHEV kit (BioMérieux SA). Within each virus, different letters denote significant differences among methods (P < 0.05).

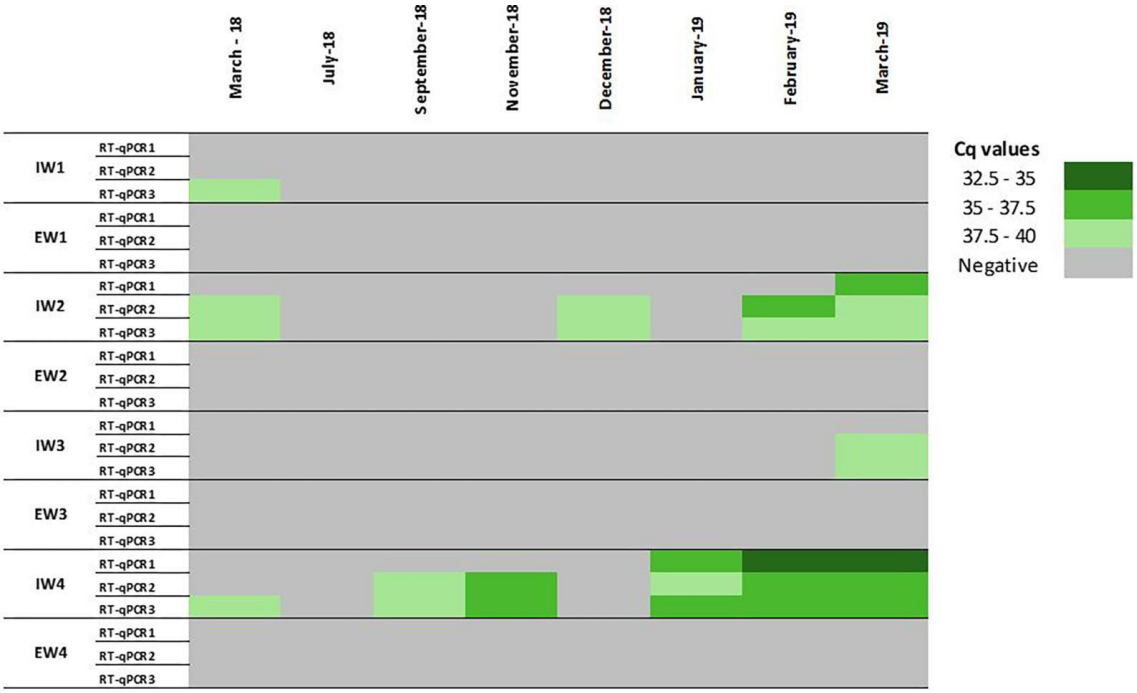


Performance of RT-qPCR Assays of HEV in Naturally Contaminated Wastewater Samples

A lack of information on HEV viral loads before and after treatments applied in WWTPs has been identified (Fenaux et al., 2019). In the present study, a total of 64 samples were collected upstream (n = 32) and downstream (n = 32) of four WWTPs, and these samples were concentrated according to the Al procedure combined with the MN kit and analyzed by different RT-qPCRs assays. To improve the sensibility of the RT-qPCR assays, the initial 35 mL influent water sample volume was increased to 200 mL. Initially, two different

RT-qPCRs were applied to assess HEV occurrence. Surprisingly, RT-qPCR1 showed a limited number of positives compared to RT-qPCR2 (Figure 5) despite a previous study reported similar performance of these assays in influent water samples (Randazzo et al., 2018). Suspecting that a different HEV genotype was circulating, a third RT-qPCR assay was included in the study. In particular, a method widely used in clinical and environmental virology firstly described by Jothikumar et al. (2006) and modified by Girón-Callejas et al. (2015) (RT-qPCR3) was applied to retest samples. All the samples had a minimum recovery rate of 1% MgV, validating the results. Overall, out of 32 influent water samples, 12 were positive for at least one of the three HEV RT-qPCR assays, and an overall HEV prevalence of 37.5% was found. Different numbers of positive samples and different prevalence rates were recorded during the comparison of the three RT-qPCR assays (Figure 5). In particular, prevalence rates of 12.5, 28.5, and 37.5% in influent waters were recorded for the RT-qPCR1, RT-qPCR2, and RT-qPCR3 assays, respectively. Although RT-qPCR1 fail to detect HEV in several samples, lower Cq values were observed in samples collected from January 2019 on (Figure 5). The observed differences may be due to HEV genotype variability. Unfortunately, conducted genotyping analyses did not solve the question because of the negative results, likely due to the low genome titers in the samples. Therefore, due to the high variability of the HEV genotypes (Smith et al., 2013, 2016), the RT-qPCR assays used for environmental analyses must be carefully checked to avoid false negative results.

Figure 5. Occurrence of HEV in influent (IW) and effluent (EW) waters by comparing three RT-qPCR assays. RT-qPCR1: Schlosser et al. (2014); RT-qPCR2: ceeramTOOLS Hepatitis E Virus Detection KHEV kit (BioMérieux SA); RT-qPCR3: Girón-Callejas et al. (2015) (cf., Supplementary Table 5 for details of assays).



Surveillance of HEV in Influent and Effluent Water Samples From WWTPs and DWTPs

Data about the occurrence of HEV in Spanish wastewaters are scarce, therefore the number of influent and effluent samples were expanded and 62 influent and 52 effluent water samples were analyzed by RT-qPCR3 (Table 2). In the current study HEV is widely disseminated (30.65%) in Valencian influent waters even though the prevalence rates among WWTPs varies widely (Supplementary Figure 1). For example, WWTP4 had a prevalence rate of 73.33% (11/15) using the RT-qPCR3 assay. As a public health concern, this WWTP receives domestic sewage from several municipalities, accounting for 108,000 inhabitants, even though we cannot exclude streams from pig farms or other agricultural run-offs. In contrast, WWTP1 (14,000 inhabitants) and WWTP2 (188,000 inhabitants) showed only 12.5 and 13.3% prevalence, respectively. These data show no correlation between HEV prevalence and the number of inhabitants served by WWTPs ($\rho S = 0.26$).

Table 4. Prevalence and HEV loads (IU/L) from four WWTPs (n = 114) and two drinking water treatment plants (n = 56) using RT-qPCR3.

Type of water sample	Treatment plant	No of samples analyzed	No of positive samples	HEV prevalence (%)	Viral load(log IU/L) (range: min–max)
Influent	WWTP1	16	2	12.50	3.11–3.57
	WWTP2	16	4	25.00	3.11–3.82
	WWTP3	15	2	13.33	3.11–3.79
	WWTP4	15	11	73.33	3.11–4.31
Effluent	WWTP1	13		0	ND
	WWTP2	13		0	ND
	WWTP3	13		0	ND
	WWTP4	13		0	ND
Influent	DWTP1	14		0	ND
	DWTP2	14		0	ND
Effluent	DWTP1	14		0	ND
	DWTP2	14		0	ND

ND, not detected (below of limit of detection). WWTP, wastewater treatment plant; DWTP, drinking water treatment plant.

Studies conducted in Barcelona (Spain) have shown similar prevalence (from 13.5 to 43.5% in influent waters, with absence or low detection of HEV in effluent waters (Clemente-Casares et al., 2003; Rodriguez-Manzano et al., 2010; Rusiñol et al., 2015).

The present study showed HEV contamination in influent waters ranging from approximately 1.3×10^3–3.5×10^4 IU/L using the RT-qPCR3 assay (Table 2), which is consistent with previously reported levels (Fenaux et al., 2019). HEV genomes were not detected in effluent waters (Table 2). These results are consistent with most of the studies published in Europe (Fenaux et al., 2019), even those done after a confirmed outbreak (Miura et al., 2016). This suggests that treatments applied at WWTPs (Supplementary Figure 1) are efficient in removing HEV despite the fact that a reduction of 1–2 log would result in concentrations below the LoD95%. Thus, further improvements are needed to

increase the sensitivity of the methods applied for virus concentration in effluent waters.

Additionally, a total of 56 samples were collected upstream (n = 28) and downstream (n = 28) of two DWTPs, and 20 L water samples were concentrated by DEUF using Rexeed-25A filters combined with PEG precipitation, the MN kit and analyzed by RT-qPCR3. None of the influent and effluent samples were positive for HEV despite all the samples had a minimum recovery rate of 1% MgV (Table 2).

3. Conclusion

Hepatitis E virus is considered an emerging pathogen in industrialized countries, especially in Europe, and analytical procedures for estimating HEV concentrations in water samples are required. Among the different methodologies evaluated in this study, HEV concentration with aluminum hydroxide was able to detect HEV in influent and effluent water samples. However, the limited sensitivity of the method could be improved, for example by increasing the sample volume. The procedure for drinking water includes a DEUF step using a 30 kDa membrane to reduce the sample volume from 20 to 200 L to approximately 500 mL. Overall, the results showed that HEV is efficiently recovered from spiked drinking water samples processed using a PEG secondary concentration and the MN extraction kit.

This study also confirms that the selection of the RT-qPCR assays is critical since the overall performance of the methods varied considerably, most likely based on the circulating strains. In particular, this aspect remarkably affects genotyping results and thus epidemiology and traceability investigations.

Wastewater is an important environmental source for studying the epidemiology of viral pathogens transmitted via the fecal-oral route, and the current study demonstrated that HEV circulated in the Valencian region at around 30.6% during 2018–2019. No HEV was detected in effluent samples from WWTP and influent and effluent samples from DWTP. However, given that the infective dose in waterborne epidemics settings is not yet known and the low sensibility of the assay, unfortunately, no direct conclusion could be achieved on the risk assessment of environmental contamination.

4. Supplementary material

Supplementary Table 1. Limit of detection of HEV in influent wastewater samples.

Extraction method	RT-qPCR	Levels of inoculated HEV (IU/ 0.035L)				$LoD_{95\%}$ (IU/L)	Mean mengovirus recovery (min-max) (%)
		≈1 x 10^5	≈1 x 10^4	≈1 x 10^3	≈1 x 10^2		
MN	RT-qPCR1	4/4* 8.81**A (3.57 – 12.27)	4/4	4/4	0/4	3.57 x 10^4	8.34A (7.92 – 8.72)
	RT-qPCR2	4/4 36.81A, B (23.34 – 49.64)	3/4	1/4	0/4	5.43 x 10^5	
NS	RT-qPCR1	4/4 41.45B (20.39– 81.22)	0/4	0/4	0/4	4.29 x 10^6	21.56B (17.76 – 24.29)
	RT-qPCR2	4/4 8.90A (1.12 – 15.81)	4/4	3/4	0/4	7.57 x 10^4	

LoD95: limit of detection calculated according to Wilrich and Wilrich (2009); Within each column, different letters denote significant differences among methods (P < 0.05); *HEV positive/total numbers of samples; **Mean HEV recovery (min-max) (%).

Supplementary Table 2. Limit of detection of HEV in effluent water samples using the aluminium protocol.

Extraction method	RT-qPCR	Levels of inoculated HEV (IU/ 0.2 l) ≈2 x 10^5	≈2 x 10^4	≈2 x 10^3	≈2 x 10^2	$LoD_{95\%}$ (IU/L)	Mean mengovirus recovery (min-max) (%)
MN	RT-qPCR1	4/4* 30.01**A, B (15.48 – 46.85)	4/4	4/4	0/4	1.25 x 10^4	41.17A (30.08 – 54.50)
	RT-qPCR2	4/4 8.33A (2.55 – 18.53)	4/4	4/4	0/4	1.25 x 10^4	
NS	RT-qPCR1	4/4 7.72A (4.84 –11.85)	4/4	4/4	0/4	1.25 x 10^4	23.90B (21.52 – 28.32)
	RT-qPCR2	4/4 41.90B (34.19 – 54.85)	4/4	4/4	0/4	1.25 x 10^4	

LoD95: limit of detection calculated according to Wilrich and Wilrich (2009); Within each column, different letters denote significant differences among methods (P < 0.05); *HEV positive/total numbers of samples; **Mean HEV recovery (min-max) (%).

Supplementary Table 3. Limit of detection of HEV in tap water by Rexeed 25AX ultrafiltration followed by precipitation with polyethylene glycol.

Extraction method	RT-qPCR	Levels of inoculated HEV (IU/ 20 l) ≈1 x 10^7	≈1 x 10^6	≈1 x 10^5	≈1 x 10^4	$LoD_{95\%}$ (IU/L)	Mean mengovirus recovery (min-max) (%)
MN	RT-qPCR1	4/4* 36.65**C (36.39-36.90)	4/4	4/4	0/4	6.2 x 10^3	3.14A (2.65 – 3.63)
	RT-qPCR2	4/4 16.59B (16.24-16.94)	4/4	4/4	0/4	6.2 x 10^3	
NS	RT-qPCR1	4/4 7.24A (7.18-7.29)	4/4	4/4	0/4	6.2 x 10^3	1.39A (1.34 – 1.45)
	RT-qPCR2	4/4 8.33A (7.86-8.80)	4/4	4/4	0/4	6.2 x 10^3	

LoD95: limit of detection calculated according to Wilrich and Wilrich (2009); Within each column, different letters denote significant differences among methods (P < 0.05); *HEV positive/total numbers of samples; **Mean HEV recovery (min-max) (%).

Supplementary Table 4. Limit of detection of HEV in tap water by Rexeed 25AX ultrafiltration follow by centrifuge filtration with Amicon filters.

Extraction method	RT-qPCR	Levels of inoculated HEV (IU/ 20 l) ≈1 x 10^7	≈1 x 10^6	≈1 x 10^5	≈1 x 10^4	$LoD_{95\%}$ (IU/L)	Mean mengovirus recovery (min-max) (%)
MN	RT-qPCR1	4/4* 1.82**A (1.45-2.19)	4/4	4/4	0/4	6.2 x 10^3	3.72A (3.27 – 4.17)
	RT-qPCR2	4/4 4.90A (4.38-5.41)	4/4	4/4	0/4	6.2 x 10^3	
NS	RT-qPCR1	4/4 35.70B (34.19-37.21)	4/4	4/4	0/4	6.2 x 10^3	5.72B (5.64 – 5.79)
	RT-qPCR2	4/4 23.69B (19.15-28.23)	4/4	4/4	0/4	6.2 x 10^3	

LoD95: limit of detection calculated according to Wilrich and Wilrich (2009); Within each column, different letters denote significant differences among methods (P < 0.05) *HEV positive/total numbers of samples; **Mean HEV recovery (min-max) (%).

Supplementary Table 5. List of primers and probes used in this study for HEV analysis.

Assay	Amplification region	Primers and probe	Sequence 5'-3'	RT-qPCR conditions	Location*	Reference
RT-qPCR1	ORF3	HEV.Fa HEV.Fb HEV.R HEV.P	GTGCCGGCGGTGGTTTC GTGCCGGCGGTGGTTTCTG GCGAAGGGGTTGGTTGGATG FAM-TGACMGGGT/ZEN/TGATTCTCAGCC/3IABkFQ	RT 50 °C for 30' 95 °C for 15' PCR (45x) 95 °C for 10" 55 °C for 20" 72 °C for 15"	5296–5377 (81 nt)	Schlosser et al. (2014) with modified probe
RT-qPCR2	ORF3	N/A	N/A	RT 45 °C for 10' 95 °C for 10' PCR (40x) 95 °C for 15" 60 °C for 45"	N/A	Ceeram (hepatitis@ceeramTools)
RT-qPCR3	ORF3	JVHEVF JVHEVRmod JVHEVPmod	GGTGGTTTCTGGGGTGAC AGGGGTTGGTTGGRTGRA TGATTCTCAGCCCTTCGC	RT 50 °C for 30' 95 °C for 2' PCR (45x) 95 °C for 15" 60 °C for 40"	5304–5373 (69 nt)	Jothikumar et al. (2006) modified; Girón-Callejas, Clark, Irving, & McClure (2015)

∗ Location in reference to WHO International Standard for HEV RNA, HRC-HE104 strain, accession no. AB630970 (Baylis et al., 2013).

Supplementary Figure 1. Reclamation processes applied in the four wastewater treatment plants selected in this study.

		WWTP 1	WWTP 2	WWTP 3	WWTP 4
Pretreatment	Coarse screening	X	X		X
	Fine screening	X		X	
	Sifting	X	X	X	X
	Flow homogenization tank	X			X
	Grit removal	X	X	X	X
	Grease removal	X	X	X	X
Primary treatment	Physicochemical treatment	X			X
	Decantation	X	X		X
Secondary treatment	Activated sludge	X			X
	Extended aeration		X	X	
	Nitrogen removal	X	X	X	X
	Phosphor removal	X		X	
	Coagulation – Flocculation	X			
	Filtration	X			
Tertiary treatment	UV	X		X	
	Chlorination		X		X

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RESULTS

Chapter 2. Impact of library preparation when characterising the virome of influent and effluent samples from wastewater treatment plants.

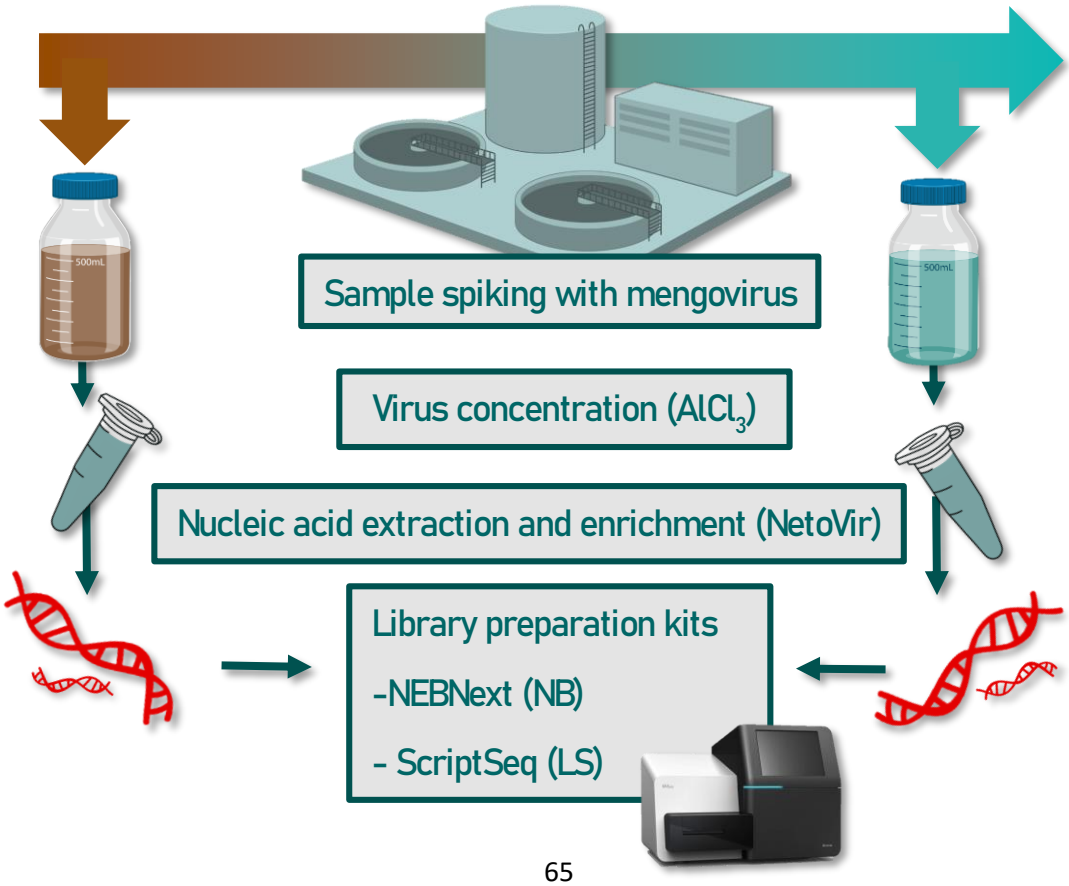
Chapter 2. Impact of library preparation when characterising the virome of influent and effluent samples from wastewater treatment plants.

2.1. Bias of library preparation for virome characterization in untreated and treated wastewaters

This section is an adapted version of the following published research article:

AlbaPérez-Cataluña, Enric Cuevas-Ferrando, Walter Randazzo, Gloria Sánchez (2021) **Bias of library preparation for virome characterization in untreated and treated wastewaters**. Science of The Total Environment, 144589

DOI: https://doi.org/10.1016/j.scitotenv.2020.144589



Highlights

- Work-flow procedure for the characterization of virome in wastewaters
- Sequencing libraries lead to different virome profiles.
- Not all proposed viral indicators correlate with the presence of enteric viruses.

1. Abstract

The use of metagenomics for virome characterization and its implementation for wastewater analyses, including wastewater-based epidemiology, has increased in the last years. However, the lack of standardized methods can lead to highly different results. The aim of this work was to analyze virome profiles in upstream and downstream wastewater samples collected from four wastewater treatment plants (WWTPs) using two different library preparation kits. Viral particles were enriched from wastewater concentrates using a filtration and nuclease digestion procedure prior to total nucleic acid (NA) extraction. Sequencing was performed using the ScriptSeq v2 RNA-Seq (LS) and the NEBNext Ultra II RNA (NB) library preparation kits. Cleaned reads and contigs were annotated using a curated in-house database composed by reads assigned to viruses at NCBI. Significant differences in viral families and in the ratio of detection were shown between the two library kits used. The use of LS library showed Virgaviridae, Microviridae and Siphoviridae as the most abundant families; while Ackermannviridae and Helleviridae were highly represented within the NB library. Additionally, the two sequencing libraries produced outcomes that differed in the detection of viral indicators. These results highlighted the importance of library selection for studying viruses in untreated and treated wastewater. Our results underline the need for further studies to elucidate the influence of sequencing procedures in virome profiles in wastewater matrices in order to improve the knowledge of the virome in the water environment.

Keywords

Wastewater; Metagenomics; Enteric viruses; Viability RT-qPCR.

1. Introduction

The reuse of water, including for irrigation, cooling, and other non-potable applications is an emerging topic due to climate change and water scarcity. Treatment and regeneration of household sewage water in urban regions are usually performed by wastewater treatment plants (WWTPs); however, they are not always able to completely eliminate the microbiological risks present in treated wastewaters (Chalmers et al., 2010; Randazzo et al., 2019; Sano et al., 2016). Fecal bacteria have traditionally been used as indicators for the presence of pathogenic microorganisms even though they fail to detect the presence of human pathogenic enteric viruses (Eslamian, 2016; Gerba et al., 2013; Kitajima et al., 2014). Thus, several viruses (i.e. crAssphage, Pepper mild mottle virus, adenovirus, polyomavirus, …) have been proposed as indicators because of their similarity to pathogenic viruses in terms of environmental stability and resistance to wastewater sanitation treatments (Farkas et al., 2020). The presence of human enteric viruses in treated wastewaters has been well documented (Gerba et al., 2018; Randazzo et al., 2019; Sano et al., 2016), posing public health risk-related concerns also because of their stability into the environment. Thus far, nearly one hundred different types of human enteric viruses are known, which cause a variety of illnesses and diseases in humans (Fong and Lipp, 2005), primarily gastroenteritis and hepatitis, and new pathogenic strains and species continue to be discovered. Among others, the viruses most commonly detected in untreated and treated wastewaters include human norovirus, adenovirus (AdV), enterovirus (EV), sapovirus (SaV), astrovirus (HAstV), rotavirus A (RV), and hepatitis A and E viruses (HAV and HEV) (Haramoto et al., 2018). Surveillance of human enteric viruses in untreated and treated wastewaters is performed by molecular procedures (e.g., real time PCR (qPCR) or digital PCR (dPCR)) (Haramoto et al., 2018). Currently, a wastewater-based epidemiology surveillance has been globally implemented to monitor COVID-19 disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with notable implications for public health response in local settings (Bivins et al., 2020; Polo et al., 2020). These approaches require reference sequences for primer and probe design which limit the number and variety of viruses to be analyzed. Alternatively, recent shotgun or untargeted metagenomic approaches enable the simultaneous identification of viral sequences from a sample, referred to as 'virome', which is a diverse community of mainly eukaryotic RNA and DNA eukaryotic viruses and bacteriophages. Virome characterization in wastewater provides a potential solution to the challenges associated with the traditional surveillance of viruses in sewage (Nieuwenhuijse and Koopmans, 2017).

In this pilot study, we have used metagenomics analyses using two different library preparation kits for metagenomic sequencing to characterize the virome composition in influent and effluent samples from four different WWTPs. Thus, the objectives of this study were to: 1) evaluate different sequencing libraries for virome characterization; and 2) investigate virome distribution and diversity in influent and effluent samples.

1. Material and methods

1.1. Sample processing

Five-hundred mL of influent (IW) and effluent (EW) grab samples from four different WWTPs were collected in November 2018 in Valencia (Spain). Treatment plants differed in the number of equivalent population, the volume of treated wastewater and the disinfection treatments (Table S1). Escherichia coli counts, expressed as Most Probably Number (MPN), were performed using the Colilert® kit (IDDEX Laboratories, Spain) following the ISO 9308-2:2012 standard on the same sampling day. Samples were kept for further analyses at −20 °C and thawed for 12 h at approximately 20 °C before processing. After thawing, 200 mL of each sample were inoculated with 7 log PCRU/L of mengovirus (MgV) vMC0 (CECT 100000), used as a process control. Samples were processed using the aluminum-based precipitation protocol described elsewhere (AAVV, 2018; Randazzo et al., 2019). Briefly, 200 mL of sample was adjusted to pH 6.0. The Al(OH)3 precipitate was performed mixing 1 part of AlCl3 0.9 N per 100 parts of sample and the solution was mixed at 150 rpm for 15 min. Then, samples were centrifuged at 1700 ×g for 20 min and the pellet was resuspended in 10 mL of 3% beef extract (pH 7.4) and shaken at room temperature (RT) for 10 min at 150 rpm. Finally, samples were centrifuged for 30 min at 1900 ×g and the resulting pellet was resuspended in 1 mL phosphate saline buffer (PBS, pH 7.4) and stored at −80 °C.

1.2. Sample processing for metagenomics

Viral particles were enriched from sample concentrates (n=8) following the NetoVIR protocol, which includes both filtration and nuclease digestion steps (Conceição-Neto et al., 2015). In brief, 500 µL of concentrates were homogenized using the MP FastPrep24 5G (MP Biomedicals, Spain) for 40 s at a speed of 6.0. The homogenate was centrifuged at 16,000 ×g for 3 min and 200 µL of the supernatant was filtered through a 0.8 µm PES filter (Sartorius,

UK) to remove large particles. The filtrate was incubated with benzonase (Millipore, Spain) and micrococcal nuclease (New England Biolabs, USA) enzymes at 37 °C for 2 h to degrade free nucleic acids. Capsid protected viral nucleic acids were extracted with the NucleoSpin®RNA virus kit (Macherey-Nagel GmbH & Co., Germany), according to the manufacturer's instructions, without adding carrier RNA. Thus, both DNA and RNA viral nucleic acids were concomitantly extracted. Nucleic acids were eluted in 50 µL RNase-free water. Libraries were generated from 1 to 50 ng of a DNA-RNA sample using two different library preparation kits. The first library preparation kit was the ScriptSeq v2 RNA-Seq Library Preparation Kit (Illumina, USA), referenced as LS, with slight modifications. An initial denaturation step (95 °C for 5 min) was added to the protocol, and PCR cycles were increased to 20 to obtain enough library concentration to sequence. Additionally, the RT enzyme from the original library preparation kit was substituted by the AMV Reverse Transcriptase (Promega, Spain). The second library preparation kit was the NEBNext Ultra II RNA Library Prep Kit (New England BioLabs Inc., Ipswich, UK) (referenced as NB) following manufacturer's instructions. The two libraries compared in this study differ in terms of fragmentation times, enzymes, cDNA synthesis conditions, primers used in the PCR, as well as the conditions for aforesaid amplification (Table S2). Libraries were normalized, pooled, and sequenced using the NextSeq™ 500 platform (Illumina), following the manufacturer's protocol, with a configuration of 150 cycles paired-end reads. Sequencing was performed by Lifesequencing S.L. (Valencia, Spain).

1.3. Data analyses

Obtained reads were cleaned for adaptor removal using cutadapt software (Martin, 2011) with a minimum overlap of 5 nucleotides between read and adaptor and a maximum error rate of 0.1. Reads were cleaned with the reformat.sh script from BBMap software (sourceforge.net/projects/bbmap/) in order to remove nucleotides from both ends with Phred scores lower than 20 and reads shorter than 50 bp. Cleaned reads were merged in to single reads with FLASH v1.2.11 (Magoč and Salzberg, 2011) allowing outies. Additionally, cleaned reads were assembled with Ray 2.3.1. (Boisvert et al., 2012) using 31-mers.

Merged reads and contigs were taxonomically annotated using BLASTn algorithm (Boratyn et al., 2013) with a manually curated in-house database constructed with all the viral sequences (NCBI:txid10239; release May 5, 2020) available at GenBank.

(https://www.ncbi.nlm.nih.gov/nuccore/?term=viruses%5Borganism%5D). For the BLASTn analysis of viral reads against this curated in-house database, a cut off of 70% of query sequence coverage and 80% of identity was used, respectively. Rarefaction curves and diversity indexes Shannon and Simpson were calculated with R package vegan v2.5-6 (https://github.com/vegandevs/vegan).

1.4. Virus quantification

For virus quantification an optimized viability RT-qPCR was applied as previously described (Randazzo et al., 2019). In brief, 150 µL sample concentrates were added to 50 µM PMAxx (Biotium, USA) and 0.5% Triton 100-X (Thermo Fisher Scientific, Spain) and incubated in the dark at RT for 10 min at 150 rpm. Then, samples were exposed to photo-activation using a photo-activation system (Led-Active Blue, GenIUL, Spain) for 15 min. RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions including the Plant RNA Isolation Aid (Ambion, Spain) pretreatment. Primers, probes and RT-qPCR conditions for norovirus GI, norovirus GII, RV, HAV, HEV, mengovirus and HAstV quantification have been previously reported (Randazzo et al., 2019; Cuevas-Ferrando et al., 2020).

For crAssphage quantification by qPCR, the primer set CPQ_064 described by Stachler et al. (2017) was used. PCR conditions were an initial denaturation step of 30 s at 95 °C followed by 45 cycles of 5 s at 95 °C and 30s at 60 °C. The Premix Ex Taq master mix for probe-based real-time PCR kit (Takara, France) was used for the reaction. For the crAssphage quantification, the standard curve was performed with a customized gBlock® fragment (Integrated DNA Technologies, Spain) of 228 bp that contained the crAssphage sequences used for amplification.

Limit of quantification, qPCR efficiency and standard curve R2 values for all the tested genes are displayed in Table S3. For all RT-qPCR assays, undiluted and ten-fold diluted RNA was tested to check for RT-qPCR inhibitors.

1.5. Correlation and similarity analyses

Correlation analyses were carried out between data sets obtained by both libraries at family level, and between metagenomics and RT-qPCR results using the R package Hmisc v4.2-0 (https://CRAN.R-project.org/package=Hmisc) and applying the Spearman method (ρ). Significance was set at 0.05. Representation of correlation matrix values was performed with the R library corrplot v0.84 (https://CRAN.R-project.org/package=corrplot).

For each individual sample, the Jaccard index was used to analyze the similarity among results obtained with both libraries. Calculations were performed using R package betapart v1.5.2 (Baselga, 2010) taking into account the beta.JAC values representing the overall beta diversity for each sample pair.

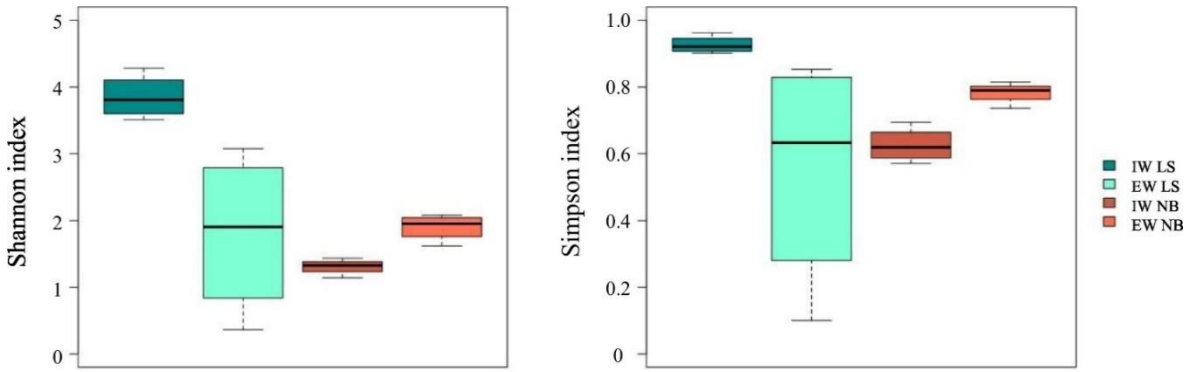
2. Results

2.1. Overview of bias due to library preparation

Each concentrated sample was sequenced using two sequencing libraries: the ScriptSeq v2 RNA-Seq Library Preparation Kit (LS) and the NEBNext Ultra II RNA Library Prep Kit (NB). The average number of reads was 3.2 and 11.5 million for LS and NB libraries, respectively. Rarefaction analyses showed that 5 out of 8 samples sequenced by the LS library reached the plateau, while 2 out of 8 samples sequenced by NB library reached it. Despite that, remaining samples were close to stabilization with both libraries (Fig. S1). Merged viral reads were annotated through a BLASTn comparison with the curated in-house database that comprised all the viral sequences (CDS and complete genomes) available at GenBank. For the LS library, the percentage of viral reads ranged from 0.6% to 2.4% in influent and from 0.4% to 4.4% in effluent samples. For NB library, the BLASTn analysis showed a high number of sequences ascribed to the same taxon, suggesting an overrepresentation due to sequencing bias, representing between 33 and 60% of the total viral reads. For that reason, the relative calculations of subsequent analyses were made also taking into account this overrepresentation. These corrected calculations will be called NB-corrected. For the NB library, viral reads ranged from 38% to 58% in influent and from 14% to 24% in effluent samples. Taking into account the results of NB-corrected, these percentages ranged from 9% to 12% and from 7% to 12% in influent and effluent samples, respectively. Shannon and Simpson diversity

indexes were calculated for each type of sample (influent or effluent) and for each library (LS or NB) (Fig. 1). Shannon indexes were higher in influent samples sequenced with LS library (mean values of 3.85±0.33 for LS and 1.30±0.12 for NB); however, for effluent samples both indexes showed similar means (1.81±1.21 for LS and 1.90±0.2 for NB), being effluent samples sequenced with LS library more variable (0.36–3.07). Similar results were obtained for Simpson index, even though the mean values for influent samples were highly different (0.93±0.02 for LS and 0.62±0.05 for NB). Raw data was deposited at SRA under the Bioproject PRJNA67378 with the following accession numbers: SAMN16633937-SAMN16633944 for ScriptSeq v2 RNA-Seq Library Preparation Kit samples and SAMN16634071-SAMN16634078 for NEBNext Ultra II RNA Library Prep Kit samples.

Fig. 1. Shannon and Simpson diversity indexes for viral species in influent (IW) and effluent (EW) samples processed by using ScriptSeq v2 RNA-Seq Library Preparation kit (LS) and NEBNext Ultra II RNA Library Prep Kit libraries (NB) for metagenomics characterization.



2.2. Mengovirus recovery

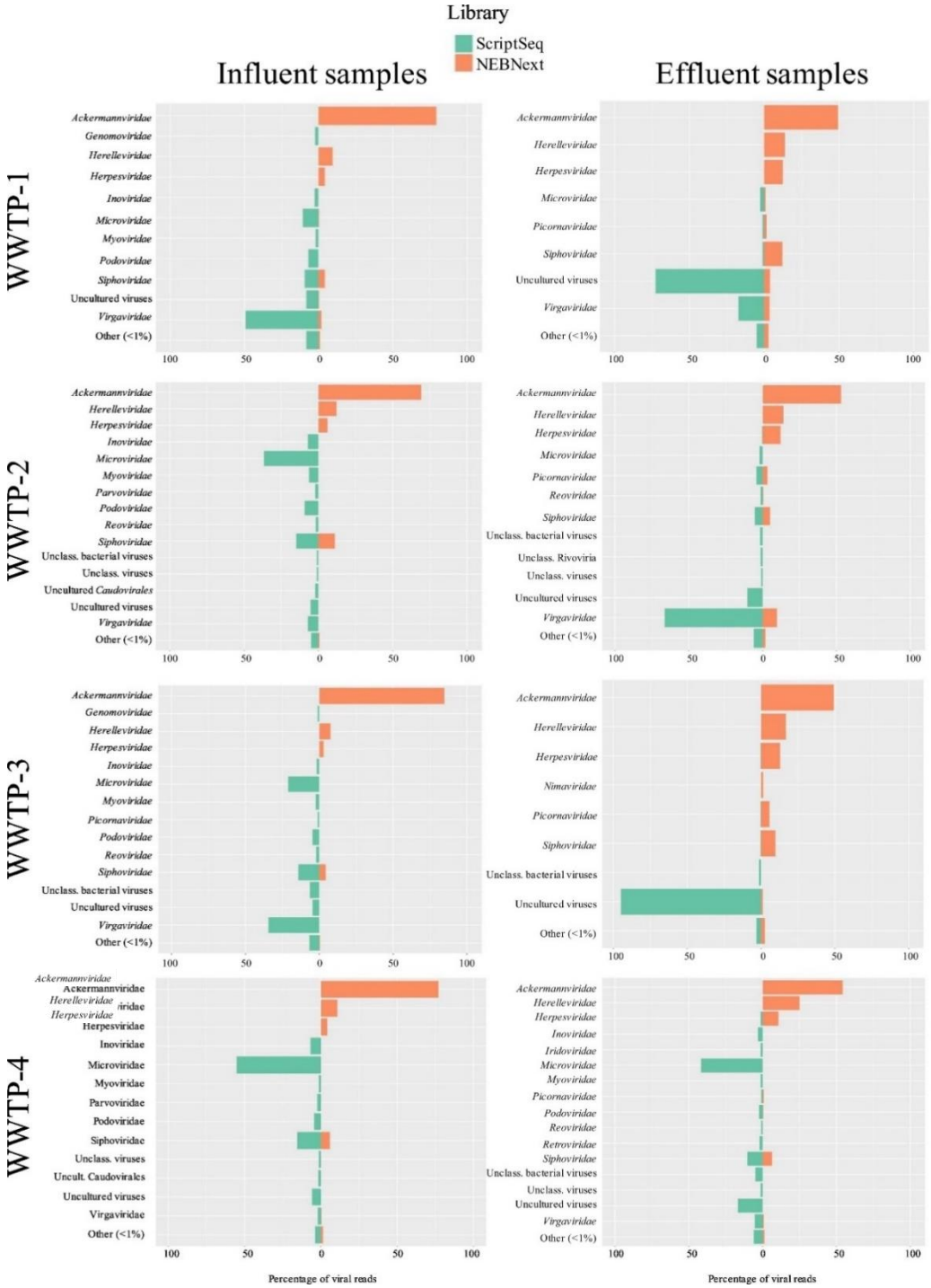
Mengovirus (MgV) was used as a process control to analyze the performance of each library to recover reads and the entire genome of MgV. Its recovery, represented as the percentage of viral reads and the percentage of MgV isolate M genome (L22089.1) obtained for each sample with each library, was different depending on the library used. For LS library, the percentage of viral reads of MgV ranged from 0.05% to 0.79% in influent and from 0.35% to 3.68% in effluent samples. For NB libraries, these values ranged from 0.01% to 0.16%

in influent samples, and from 0.63% to 5.77% in effluent samples. However, the percentage of MgV reads with the NB-corrected values were higher in effluent samples, ranging from 1.38% to 11.38%. For the analysis of the recovery of MgV genome, assembled contigs belonging to this species were compared with the genome of Mengovirus isolate M (L22089.1). LS library genome recovery ranges from 6.0% to 95.1%. The highest recovery was obtained in the sample IW3. On the other hand, the coverage of this genome by the NB library ranged from 98.4% to 100% (EW3).

2.3. Virome comparison

Regarding the virome composition for each library at family levels, results showed high differences between the two approaches (Fig. 2). While the most represented families with the LS library were Virgaviridae, Microviridae and Siphoviridae; the most abundant families with the NB library were Ackermannviridae and Helleviridae. These differences allowed the detection of some viral families depending on the library used. For example, families as Rhabdoviridae, Pospiviroidae or Mitoviridae were only detected when the NB library was used for sequencing. Also the taxa uncultured human fecal virus (NCBI:txid239364) and uncultured marine virus (NCBI:txid186617) were only detected with the NB approach. Regarding the families detected with both libraries, only Genomoviridae showed total correlation (ρ=1) between values obtained with both libraries in influent and effluent samples. For influent wastewater samples, families Nairoviridae and Virgaviridae showed total correlations; however, this correlation was only observed for Parvoviridae in effluent samples. Other families showed high correlations (ρ=0.8) in influent wastewaters, as Peribunyaviridae and Picornaviridae; while Podoviridae, Poxviridae, Reoviridae and Virgaviridae families showed high correlations (ρ=0.8) in effluent samples. Jaccard indexes showed similarities between the same sample sequenced with each library that ranged from 0.76 (IW1) to 0.91 (IW2), with mean values of 0.83±0.09 for IWs and 0.81±0.04 for EWs.

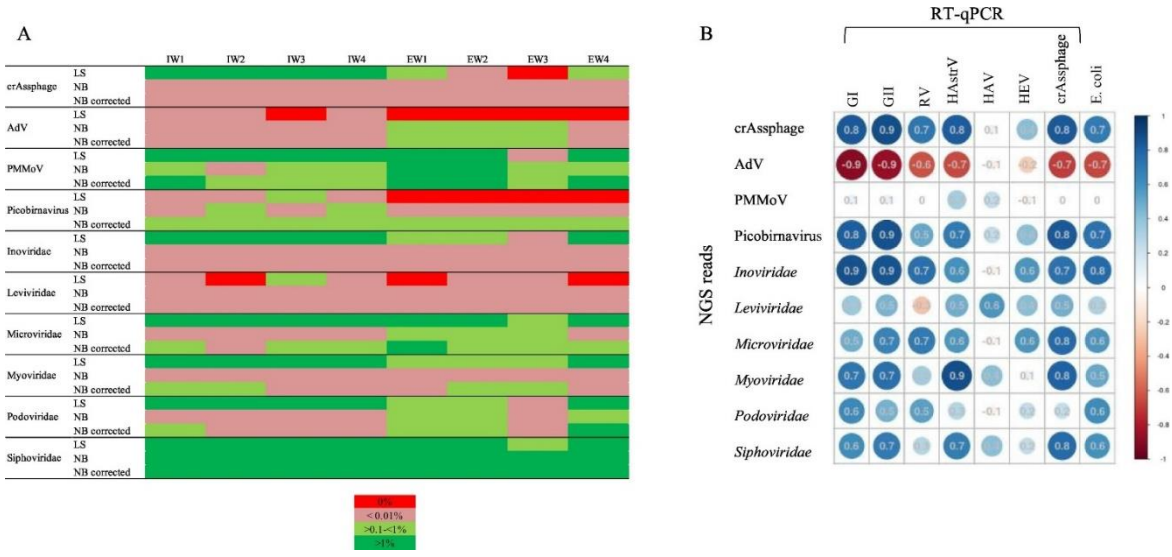
Fig. 2. Relative abundance at family level of the viral population detected in influent and effluent samples from four different WWTPs by metagenomics with ScriptSeq v2 RNA-Seq Library Preparation kit (LS, green) and NEBNext Ultra II RNA Library Prep Kit (NB, orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



2.4. Analyses of viral fecal indicators by NGS and correlation with enteric viruses detected by RT-qPCR

Each of the libraries used in this study showed different power for detecting fecal indicators. Similarly, influent and effluent samples showed different detections rates (Fig. 3A). For example, LS library detected CrAssphage with read percentages higher than 1% but these percentages decreased to less than 0.01% with NB library. Most importantly, LS library was unable to detect the fecal indicator adenovirus in effluent wastewaters, while the NB library detected adenoviruses in percentages between 0.1 and 1%. The same scenario was observed for the Picobirnavirus indicator. However, indicators families as Inoviridae, Microviridae, Myoviridae and Podoviridae showed a better detection with the LS library. Siphoviridae family detection did not show differences in its detection capacity between the two different tested libraries, with the exception of sample EW3 (Fig. 3A).

Fig. 3. Viral indicators analysis in influent (IW) and effluent (EW) samples. Panel A, Detection of viral indicators with ScriptSeq v2 RNA-Seq Library Preparation kit (LS) and NEBNext Ultra II RNA Library Prep Kit (NB and NB-corrected). Panel B, Correlation matrix between the reads of viral indicators obtained by NGS and the load of enteric viruses (RT-qPCR) and E. coli counts.

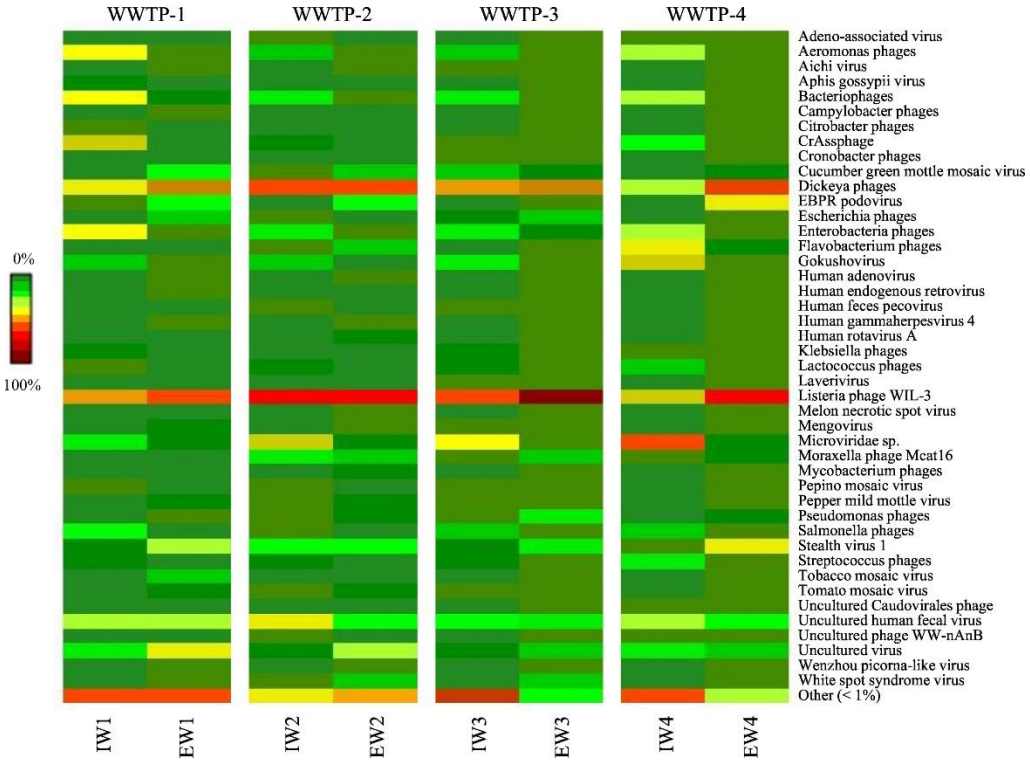


Correlation between the number of reads of proposed viral indicators obtained with both libraries and the quantifications obtained by RT-qPCR for enteric viruses along with the E. coli counts were calculated. Fig. 3B shows the Spearman values of correlation (ρ) calculated with 95% confidence. Norovirus GI and GII showed high correlation values (ρ>0.8) with the indicators crAssphage, Picobirnavirus and Inoviridae. The highest correlation values (ρ=0.7) between RV and indicators reads were crAssphage, Inoviridae and Microviridae. For HAstrV, high correlations (ρ>0.8) only occurred with crAssphage and Myoviridae. HAV and HEV did not correlate with any of the indicators. Interestingly, the proposed indicator AdV showed negative correlations with all the enteric viruses analyzed (ρ>−0.7), with the exception of HAV and HEV. Results obtained by NGS for the pepper mild mottle virus (PMMoV), also proposed as viral indicator, showed no correlation with any of the enteric viruses.

2.5. Virome comparison between influent and effluent wastewaters

Clean reads obtained from each library and each sample were assembled and contigs longer than 200 bp were used for taxonomical classification by using BLASTn algorithm and the in-house database. Due to the different results observed at reads level for each library, contigs classification obtained with both libraries for each sample were merged for results representation and virome analysis. The relative abundances of different taxa are shown in Fig. 4. As observed in the heatmap graphic, the most abundant viruses were bacteriophages, as Dickeya phage or Listeria phage WIL-3, even with higher percentages in effluent samples. The higher detection of these phages in treated samples, as occurred with other species (i.e. cucumber green mottle mosaic virus, EBPR podovirus 2, PMMoV, Stealth virus 1, or Tobacco and tomato mosaic viruses), can be due to the decrease of other viruses after wastewater treatment that allows their detection. Similarly, this effect could be the responsible of the detection of some viruses in effluent samples that were not detected in influent samples, as the case of human adenovirus and human gammaherpesvirus. Some viruses were only found in high percentages in influent samples, as the indicator crAssphage, some Aeromonas phages, Escherichia phages or viruses belonging to the Microviridae family. Wastewater treatments could be the factor that produce this decrease; however, more studies along time from the same WWTPs must be performed in order to ensure the effect of performed treatments.

Fig. 4. Heatmap showing the virome composition at species level obtained by merging the results of ScriptSeq v2 RNA-Seq Library Preparation kit (LS) and NEBNext Ultra II RNA Library Prep Kit (NB). Only species with percentages higher than 1% are shown.



3. Discussion

The virome of wastewaters has been previously characterized from samples collected around the world (Adriaenssens et al., 2018; Aw et al., 2014; Cantalupo et al., 2011; Fernández-Cassi et al., 2018; Furtak et al., 2016; Nieuwenhuijse et al., 2020; Rusiñol et al., 2020; Strubbia et al., 2019a, Strubbia et al., 2019b; Wang et al., 2018); however, much less is known about the virome of effluent samples as only one study has analyzed two effluent samples collected in the UK (Adriaenssens et al., 2018). As far as we know, this is the first study that concomitantly analyzes the RNA and DNA viruses present in influent and effluent samples besides providing a comparison of viruses profiles detected using different sequencing library kits.

Results obtained in our study showed high differences regarding not only viruses, but also the power of detection of viral fecal indicators. Both aspects

are important for the use of random metagenomics as tool for specific detection. Our results evidenced the influence of the library used for virome studies together with their variability. Additionally, by using MgV as process control for both metagenomic and RT-qPCR analyses, we further assessed the sensitivity of each library, being higher when using NB library. Recoveries of MgV complete genome were between 6.0 and 95.1% for LS library and between 98.4 and 100% for NB library. In contrast, in a recent study, MgV reads were not recovered from spiked water and sediment samples (Adriaenssens et al., 2018). According to the authors, this was likely due to an inclusion of an inactivation step of the DNase at 75 °C, which potentially exacerbated the effect of the RNase step (Adriaenssens et al., 2018). The use of models of a virus of interest when comparing sequencing libraries can be an excellent tool for the library selection.

For the analysis of the virome of influent and effluent wastewaters, results obtained by both libraries were merged. Phages as crAssphage, Aeromonas phages, Escherichia phages or viruses belonging to the Microviridae family were found in high percentages in influent wastewaters. The absence of these viruses in effluent samples can be due to the sanitation treatments applied in WWTPs, even though further analysis that includes a wider sampling design needs to be performed. These results are in line with previous studies showing a high abundance of bacteriophages families (Aw et al., 2014; Cantalupo et al., 2011; Fernández-Cassi et al., 2018; Rusiñol et al., 2020; Wang et al., 2018) in influent sewage samples. Nevertheless, other studies showed Virgaviridae as the most represented viruses (Furtak et al., 2016). Differences in virome profiling with other studies might be due to the influence of library sequencing and the intrinsic characteristics of the virome related to the sample itself and the area of study. On the other hand, the higher presence of some viruses or even its detection only in effluent samples could be produced by the decrease of other viruses that allowed its detection.

The presence of pathogenic viruses is an important aspect for defining the final use of treated waters as it may be the case of irrigation. Due to their high environmental resistance, the presence of human enteric viruses has been reported in treated wastewaters (Adriaenssens et al., 2018). However, some of these pathogenic viruses are not always detected by metagenomics analyses. For instance, in the study by Fernández-Cassi et al. (2018), human adenoviruses (HAdV) reads were not detected in samples concentrated from 10 L of wastewater. Adenoviridae was also not detected in the study of Adriaenssens et al. (2018), in which the sample was concentrated from 1 L of wastewater. In our study, concentrating 200 mL of effluent samples, we were

able to detect HAdV in percentages between 0.16% and 0.35%. In contrast, percentages of HAdV in influent wastewaters were lower than 0.01%. Overall, the majority of the annotated virome belonged to bacteriophages. This indicates that metagenomics is poor in sensitivity when used to detect a low abundance of viral pathogens against a large background of bacteriophages, as occurred for the enteric viruses detected by viability RT-qPCR. For example, in the present study, norovirus genomes could not be retrieved from the reads as reported elsewhere (Adriaenssens et al., 2018; Fernández-Cassi et al., 2018; Strubbia et al., 2019b). In the current study, the number of generated paired reads per sample was 3.2 and 11.5 million for LS and NB, respectively; while Adriaenssens et al. (2018) reported between 10 and 110 million, increasing significantly the probability to retrieve full or partial viral genomes. Alternatively, methods to detect and characterize specific viruses have been described and rely on the selection of target RNA prior to library preparation through a capture using VirCapSeq-VERT target enrichment, as reported for norovirus (Strubbia et al., 2019b).

4. Conclusion

The use of metagenomics for virome characterization and its implementation for wastewater analyses has increased in the last years (Nieuwenhuijse and Koopmans, 2017). However, the major problem of this approach is the lack of standardized procedures and the substantial differences among studies; thus, available data must be interpreted with caution. The present study showed a procedure that allows the detection and the characterization of viral populations in untreated and treated wastewater samples. Overall, this study sheds light on the diversity of the viral communities in untreated and treated wastewaters providing valuable information also in terms of viral fecal indicators. The study also evidences the bias on virome profiles obtained by tested sequencing libraries. Our results underline the need for further studies to elucidate the influence of sequencing procedures in virome profiles in wastewater matrices in order to improve the knowledge of the virome in the environment.

5. Supplementary data

Figure S1. Rarefaction curves obtained for ScriptSeq v2 RNA-Seq Library Preparation kit (LS, Panel A) and NEBNext® Ultra™ II RNA Library Prep Kit (NB, Panel B).

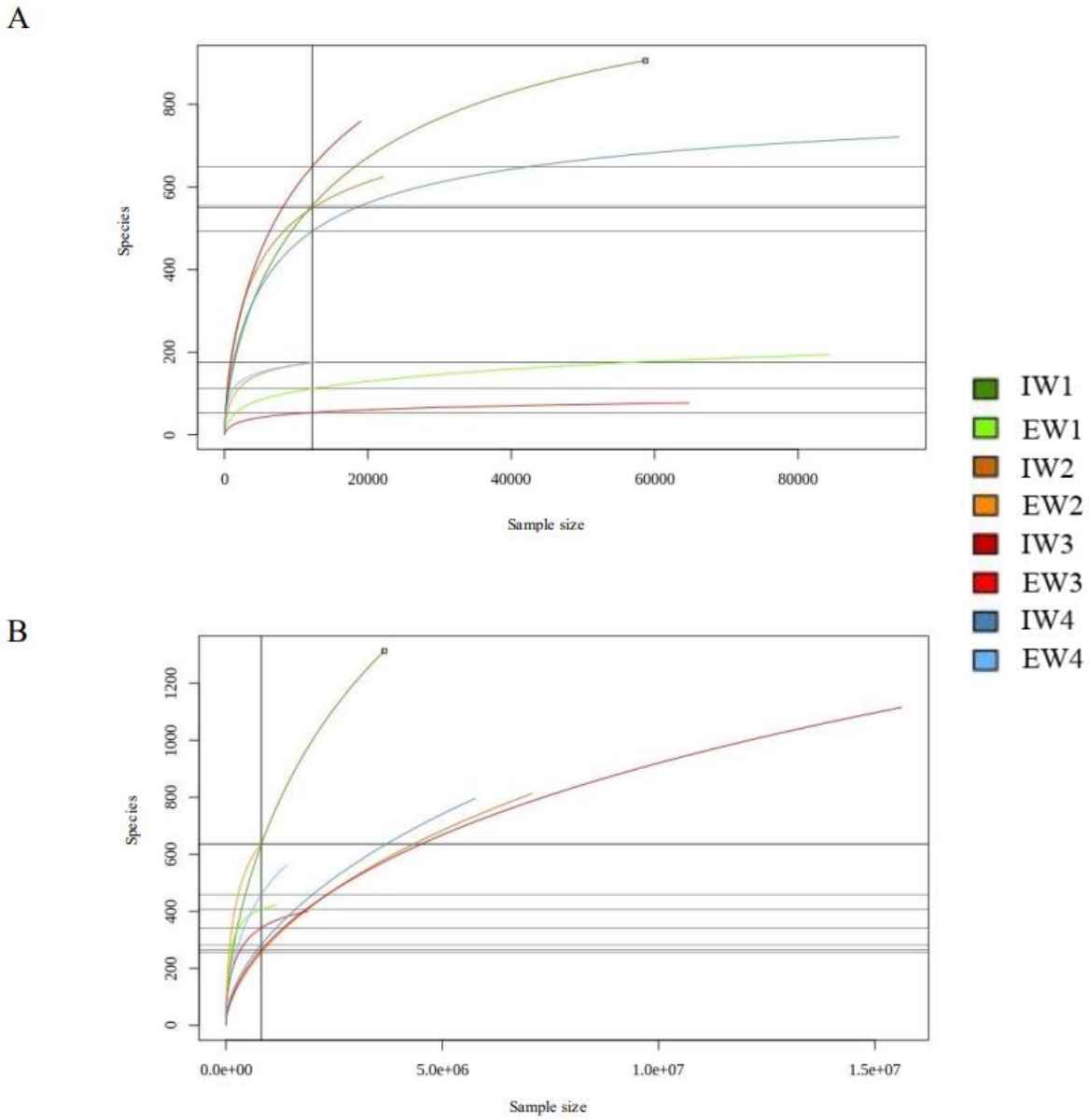


Table S1. Characteristic and wastewater treatments for each analyzed WWTP.

		WWTP1	WWTP2	WWTP3	WWTP4
Population equivalents		152,063	37,974	25,059	100,062
Flow (m³/day)		31,129	8,834	4,152	35,813
Pretreatment	Coarse screening	X	X		X
	Fine screening	X		X	
	Sifting	X	X	X	X
	Flow homogenization tank	X			X
	Grit removal	X	X	X	X
	Grease removal	X	X	X	X
Primary treatment	Physicochemical treatment	X			X
	Decantation	X	X		X
	Activated sludge	X			X
Secondary treatment	Extended aeration		X	X	
	Nitrogen removal	X	X	X	X
	Phosphor removal	X		X	
	Coagulation - Flocculation	X			
	Filtration	X			
Tertiary treatment	UC	X		X	
	Chlorination		X		X

Table S2. Characteristics of the library preparation kits used in the study. LS, ScriptSeq v2 RNA-Seq Library Preparation kit; NB, NEBNext® Ultra™ II RNA Library Prep Kit.

Step	LS	NB
Fragmentation	85°C/5 min	94°C/15 min
cDNA synthesis	StarScript AMV RT 25°C/5 min + 42°C/20 min	NEBNext enzymes 25°C/10min + 42°C/15 min + 70°C/15min
End repair/tagging	Random hexamer 25°C/15 min + 95°C/3 min	NEBNext Ultra II End Prep 20°C/30 min + 65°C/30 min
Barcodes	From Epicentre	From NEBNext 20°C 15 min
PCR primers	Kit primers	P5 and P7 Primers
PCR conditions	Initial denat 95°C 5 min Denat 95°C 30 secs Ann 55°C 30 secs Extension 68°C 3 min X20 Final Ext 68°C 7 min	Denat 98°C 30 secs Denat98°C 10 secs Ann/Ext 65°C 75 secs X20 Final Exten 65°C 5 min

Table S3. Analytical parameters of the RT-qPCRs used for the quantification of enteric viruses and of the qPCR for the quantification of crAssphage.

Virus	Standard curve	PCR Efficiency (%)	R^2	LoQ (gc/PCR)
Norovirus GI	y = -3.3211x + 39.016	100	1	3.6
Norovirus GII	y = -3.3212x + 39.586	100	1	61.3
HAV	y = -3.3212x + 39.634	100	1	94.1
HAstV	y = -3.338x + 37.78	99	0.989	22.8
RV	y = -3.3158x + 37.431	100	0.995	18.0
HEV	y = -3.5008x + 38.564	93	0.997	2.7
MgV	y = -3.603x + 38.019	90	0.993	13.3
CrAssphage	y= -3.4903 + 43.630	93	0.992	312.3

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RESULTS

Chapter 3. Prevalence of enteric viruses and viral indicators in influent and effluent water samples from wastewater treatment plants.

Chapter 3. Prevalence of enteric viruses and viral indicators in influent and effluent water samples from wastewater treatment plants

3.1. Monitoring human viral pathogens reveals potential hazard for treated wastewater discharge or reuse

This section is an adapted version of the following published research article:

Cuevas-Ferrando E, Pérez-Cataluña A, Falcó I, Randazzo W and Sánchez G (2022) **Monitoring Human Viral Pathogens Reveals Potential Hazard for Treated Wastewater Discharge or Reuse**. Front. Microbiol. 13:836193. DOI: 10.3389/fmicb.2022.836193

1. Abstract

Wastewater discharge to the environment or its reuse after sanitization poses a concern for public health given the risk of transmission of human viral diseases. However, estimating the viral infectivity along the wastewater cycle presents technical challenges and still remains underexplored. Recently, human-associated crAssphage has been investigated to serve as viral pathogen indicator to monitor fecal impacted water bodies, even though its assessment as biomarker for infectious enteric viruses has not been explored yet. To this end, the occurrence of potentially infectious norovirus genogroup I (GI), norovirus GII, hepatitis A virus (HAV), rotavirus A (RV), and human astrovirus (HAstV) along with crAssphage was investigated in influent and effluent water sampled in four wastewater treatment plants (WWTPs) over 1 year by a PMAxx-based capsid integrity RT-qPCR assay. Moreover, influent and effluent samples of a selected WWTP were additionally assayed by an in situ capture RT-qPCR assay (ISC-RT-qPCR) as estimate for viral infectivity in alternative to PMAxx-RT-qPCR. Overall, our results showed lower viral occurrence and concentration assessed by ISC-RT-qPCR than PMAxx-RT-qPCR. Occurrence of potentially infectious enteric virus was estimated by PMAxx-RT-qPCR as 88–94% in influent and 46–67% in effluent wastewaters with mean titers ranging from 4.77 to 5.89, and from 3.86 to 4.97 log10 GC/L, with the exception of HAV that was sporadically detected. All samples tested positive for crAssphage at concentration ranging from 7.41 to 9.99 log10 GC/L in influent and from 4.56 to 6.96 log10 GC/L in effluent wastewater, showing higher mean concentration than targeted enteric viruses. Data obtained by PMAxx-RT-qPCR showed that crAssphage strongly correlated with norovirus GII ($\rho = 0.67$, $p < 0.05$) and weakly with HAstV and RV ($\rho = 0.25–0.30$, $p < 0.05$) in influent samples. In effluent wastewater, weak ($\rho = 0.27–0.38$, $p < 0.05$) to moderate ($\rho = 0.47–0.48$, $p < 0.05$) correlations between crAssphage and targeted viruses were observed. Overall, these results corroborate crAssphage as an indicator for fecal contamination in wastewater but a poor marker for either viral occurrence and viral integrity/infectivity. Despite the viral load reductions detected in effluent compared to influent wastewaters, the estimates of viral infectivity based on viability molecular methods might pose a concern for (re)-using of treated water.

2. Introduction

The microbiological analysis of raw and treated wastewater has become a hot topic in recent years due to the emerging concerns on the disease/pathogen epidemiological tracking (known as wastewater-based epidemiology, WBE) and on the safety of wastewater discarding and reusing. Monitoring of wastewater has already been implemented with success for a long time on the tracking of chemical pollutants, drug spread within communities, and antibiotic resistance genes (ARGs) (Mercan et al., 2019; de Oliveira et al., 2020). Over the last years, molecular analysis detection of viruses in wastewater samples has allowed disease surveillance as for poliovirus during the global eradication program (Asghar et al., 2014), re-emerging zoonotic pathogens such as hepatitis E virus (Miura et al., 2016; Cuevas-Ferrando et al., 2020), human enteric viruses (Hellmér et al., 2014; Prevost et al., 2015; Santiso-Bellón et al., 2020), and very recently severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Bivins et al., 2020b; Randazzo et al., 2020).

Human enteric viruses are the causative agents of viral gastroenteritis, hepatitis, and other diseases which predominantly transmit through the fecal–oral route (Oude Munnink and van der Hoek, 2016). Viral spread is mainly associated to person-to-person contact and ingestion of contaminated food and waters since enteric viruses are shed at huge concentrations of up to 1013 particles per gram by both symptomatic and asymptomatic individuals (Carter, 2005; Bosch et al., 2008; Okoh et al., 2010). Group A rotavirus (RV), norovirus genogroups I (GI) and II (GII), hepatitis A virus (HAV), and human astrovirus (HAstV) are the main causative agents of water−associated viral gastroenteritis and hepatitis outbreaks worldwide (Bosch et al., 2008).

Human enteric viruses show higher resistance to decontamination treatments generally applied by wastewater treatment plants (WWTPs) such as chlorination and UV radiation (Gerba et al., 2018). Consequently, reclamation in WWTP does not usually achieve total removal of viral particles from sewage (Ramírez-Castillo et al., 2015) and they are commonly found in effluent water samples analyses (Sano et al., 2016; Farkas et al., 2018).

In the context of the exacerbated water shortage, the use of reclaimed water for irrigation, recreational, or industrial applications has become a strategy to tackle this critical problem (Barcelo and Petrovic, 2011). From a public health perspective, monitoring not only the occurrence but also the infectivity of viral human pathogens may permit to estimate the adequacy of current water reclamation systems. To approach this issue, targeting specific human viral

pathogens or a properly selected indicator constitutes alternative strategies to pursue.

Traditionally, methods based on cell culture have been used in clinical virology to test for viral infectivity, which show considerable limitations when applied in environmental samples because of the co-contamination of multiple virus species, the absence of permissive cell lines for certain viruses, and the cytotoxic effect of wastewater in cell culture (Gerba et al., 2018; Randazzo et al., 2018a). In recent years, there has been an enormous progress in detecting enteric viruses in water samples by real-time polymerase chain reaction (qPCR) methods (Katayama et al., 2008; Simmons and Xagoraraki, 2011; Farkas et al., 2018). Even so, molecular assays do not discriminate between viruses with infective capacity, inactivated viruses, and free genome. To overcome this limitation, viability markers and binding assays have been coupled to qPCR detection to evaluate the integrity of the viral capsid as estimate for viral infectivity also in water samples (Parshionikar et al., 2010; Kim et al., 2011; Coudray-Meunier et al., 2013; Prevost et al., 2016; Randazzo et al., 2016, 2018b,c; López-Gálvez et al., 2018; Tian et al., 2018; Leifels et al., 2019, 2020; Shirasaki et al., 2020; Canh et al., 2021a). Of note, such approach has been very recently implemented for SARS-CoV-2 detection in wastewater (Canh et al., 2021b; Cuevas-Ferrando et al., 2021).

Capsid integrity is a strong indicator of virus infectivity, as virions with an accessible genome yield reduced qPCR signals, improving the molecular estimation of virions (Leifels et al., 2020). As an alternative, fecal indicator bacteria (FIB) have traditionally been used to estimate fecal contamination of environmental waters, even though surveillance data demonstrated FIB may not always correlate with human enteric viruses (Sano et al., 2016; Amarasiri et al., 2017; Sidhu et al., 2017; Gerba et al., 2018). Recently, viruses such as crAssphage (cross-assembly phage), tobacco mosaic virus (TMV), and pepper mild mottle virus (PMMoV) have been suggested as indicators for either human fecal contamination and viral human pathogen removal throughout wastewater reclamations in the WWTPs (Kitajima et al., 2014; García-Aljaro et al., 2017; Farkas et al., 2019, 2020; Symonds et al., 2019; Bivins et al., 2020a; Tandukar et al., 2020; Wu et al., 2020).

Thus, the primary goal of this study was defining the occurrence of infectious human enteric viruses in influent and effluent wastewater by rapid molecular methods and, secondly, testing the hypothesis of whether crAssphage would serve as indicator for viral infectivity.

To this end, we monitored, by PMAxx-RT-qPCR over a 1-year period, the occurrence of intact capsid potentially infectious RNA enteric viruses (i.e., norovirus GI and GII, HAV, RV, and HAstV) and crAssphage in influent and effluent water samples collected from four WWTPs in the Valencian Region (Spain). Moreover, we compared in situ capture RT-qPCR (ISC-RT-qPCR) and PMAxx-RT-qPCR assays as alternative estimates for viral infectivity using influent and effluent samples of a selected WWTP longitudinally over a year. Finally, this work contributes on the expansion of the actual data pool on spatiotemporal viral monitoring studies in raw and treated wastewater and increases the significance of qPCR results for public health, economic, and QMRA purposes.

3. Materials and Methods

Sampling Site and Sample Collection

Influent (n = 48) and effluent (n = 48) wastewater samples were collected regularly each month (from November 2018 to October 2019) from four WWTPs located in the Valencian region in Spain (Figure 1). Reclamation processes applied in each sampled wastewater treatment plant are described in Table 1. Samples were grabbed early in the morning (7–12 a.m.) by collecting 500–1,000 ml of water in sterile HDPE plastic containers (Labbox Labware, Spain). Collected samples were transferred on ice to the laboratory, kept refrigerated at 4°C and concentrated within 24 h as reported below.

Figure 1. Geographical localization of wastewater treatment plants in Valencian region (Spain) and their population coverages included in the study. Diamonds on the figure are scaled according to population (inhabitants, inh).

Table 1. Reclamation processes of wastewater treatment plants.

Step	Treatment	WWTP 1	WWTP 2	WWTP 3	WWTP 4
	Coarse screening	X	X		X
	Fine screening	X		X	
Pretreatment	Sifting	X	X	X	X
	Flow homogenization tank	X			X
	Grit removal	X	X	X	X
	Grease removal	X	X	X	X
Primary treatment	Physicochemical treatment	X			X
	Decantation	X	X		X
Secondary treatment	Activated sludge	X			X
	Extended aeration		X	X	
	Nitrogen removal	X	X	X	X
	Phosphorus removal	X		X	
	Coagulation—Flocculation	X			
	Filtration	X			
Tertiary treatment	UV	X		X	
	Chlorination		X		X

Escherichia coli Counts and Physicochemical Characterization of Influent and Effluent Wastewater Samples

Influent and effluent wastewater samples were characterized by determining significant physicochemical parameters. Escherichia coli was determined as the Most Probable Number (MPN)/100 ml according to EN ISO 9308-2 (2014). The total alkalinity (referred as TA) was determined by titration by measuring the UV-Vis absorbance following the methyl orange method and expressed in mg/L CaCO3. The chemical oxygen demand (COD, mg/L O2) was determined by measuring the UV-Vis absorbance on an AP3900 laboratory robot coupled with a DR3900 spectrophotometer (Hach) following the potassium dichromate method. Total suspended solids (TSS) were determined by filtration by using glass fiber filters and results expressed in mg/L. Turbidity (Nephelometric Turbidity Unit, NTU) were determined by TU5200 Laser Turbidimeter and the oxidation-reduction potential (ORP, expressed in mV) by HQ 40D digital multi meter (Hach, United Kingdom). Physicochemical analyses and E. coli counts were performed at GAMASER laboratories (Valencia, Spain).

Virus Suspensions

Feces positive for norovirus GI, norovirus GII, and HAstV (courtesy of Dr. Buesa from Hospital Clínico Universitario, University of Valencia, Spain) were resuspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M NaNO3 (Panreac, Spain), 1% beef extract (Conda, Spain), and 0.1% Triton X-100 (Thermo Fisher Scientific, Spain) (pH 7.2), vortexed and centrifuged at 1,000 × g for 5 min. The supernatants were extracted, the RNA stored at -80°C in aliquots to be used as positive amplification controls. The human RV strain Wa (ATCC VR-2018), the cytopathogenic HM-175 strain of HAV (ATCC VR-1402), and mengovirus vMC0 (CECT 100000) were propagated in MA-104, FRhK, and HeLa cell monolayers, respectively. Semipurified stocks were thereafter produced in the same cells by low-speed centrifugations of infected cell lysates (3,000 × g for 20 min). RNA extracted from infected cell lysates was used as positive amplification control and mengovirus (MgV) was used as process control as suggested in ISO 15216-2:2019 (microbiology of the food chain) for sample concentration validation (Randazzo et al., 2019).

Wastewater Concentration

Influent and effluent water samples were artificially inoculated with approximately 7 log10 PCR units (PCRU)/L of MgV, as process control. Samples were concentrated through an aluminum hydroxide adsorption-precipitation method (AAVV, 2018; Randazzo et al., 2019). Briefly, 200 ml of sample was adjusted to pH 6.0 and Al(OH)3 precipitate formed by adding 1 part 0.9 N AlCl3 solution to 100 parts of sample. Then, pH was readjusted to 6.0 and sample mixed using an orbital shaker at 150 rpm for 15 min at room temperature. Next, viruses were collected by centrifugation at 1,700 × g for 20 min. The pellet was resuspended in 10 ml of 3% beef extract pH 7.4, and samples were shaken for 10 min at 150 rpm. Finally, the concentrate was recovered by centrifugation at 1,900 × g for 30 min and the pellet was resuspended in 1 ml of PBS and stored at -80°C.

Viral Capsid Integrity Assays in Wastewater Samples

To assess the intact capsid condition of enteric viruses in influent and effluent wastewater, a main protocol based on capsid permeability to PMAxx viability dye (PMAxx-RT-qPCR) was used for all wastewater samples. Besides, an alternative method based on the specific binding ability to porcine gastric mucin (PGM) was run in parallel in samples from WWTP4 in order to evaluate its unreported capsid integrity discrimination efficiency on wastewater matrices.

For PMAxx-RT-qPCR, a previously optimized protocol was applied prior to nucleic acid extraction and RT-qPCR detection (Randazzo et al., 2016, 2018c; López-Gálvez et al., 2018). Briefly, the photoactivatable dye PMAxx™ (Biotium, United States) was added to 150 µl of each concentrated water sample at 50 µM along with 7.7 mmol/L Triton 100-X (Thermo Fisher Scientific, Spain) and incubated in the dark at room temperature for 10 min at 150 rpm. Later, samples placed in DNA LoBind 1.5 ml tubes (Eppendorf, Germany) were exposed to photoactivation using a Led-Active Blue system (GenIUL, Spain) for 15 min, and viral RNA was extracted and analyzed as described hereafter.

The *in situ* capture assay (ISC-RT-qPCR) was performed as previously reported (Wang and Tian, 2014; Falcó et al., 2019) in 24-well plates with some modifications. Briefly, each well was coated with 100 µl of PGM (100 µg/ml) in carbonate-bicarbonate buffer (pH 9.6) at 37°C for 1 h and then incubated overnight at 4°C. After being washed five times with 300 µl of PBS containing 0.05% Tween 20 and 0.3% BSA (PBSTB), wells were blocked with 300 µl of 3%

BSA in PBS at 37°C for 2 h. Next, wells were washed five times with PBSTB, and 300 µl of concentrated water samples and controls were added to the 24-well plate and incubated at 37°C overnight. Untreated viral suspensions and those treated at 99°C for 5 min were used as positive and negative controls, respectively. Finally, after washing five times with PBSTB, 100 µl of lysis buffer from the NucleoSpin RNA virus kit (Macherey-Nagel GmbH and Co., Germany) was added to each well. Then, viral RNA was extracted and analyzed as described hereafter.

RNA Extraction and Virus Quantification

Nucleic acids from each water sample were extracted following the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH and Co., Germany) manufacturer's instructions with some modifications. In short, 150 µl of each concentrated sample was added with 25 µl Plant RNA Isolation Aid (Ambion, United Kingdom) and 600 µl of lysis buffer from the NucleoSpin® RNA virus kit and subjected to pulse-vortexing. Then, the homogenate was centrifuged for 5 min at 10,000 × g for debris removal. The supernatant was subsequently processed according to the manufacturer's instructions. Presence of norovirus GI and GII, HAV, HAstV, RV, and MgV was detected in 96-well plates using the RNA UltraSense One-Step kit (Invitrogen SA, United States), while crAssphage occurrence was performed using the qPCR Premix Ex Taq™ kit (Takara Bio Inc.) on the LightCycler® 480 instrument (Roche Diagnostics, Switzerland). Moreover, undiluted and 10-fold diluted nucleic acid were tested in duplicate to check for inhibitors.

Different controls were used in all assays, including a concentration control to monitor the process efficiency of each sample (spiked MgV), a negative nucleic acid extraction control, and positive and negative RT-qPCR controls. Primers, probes, and RT-qPCR conditions used in this study are listed in Supplementary Table 1.

Standard curves were determined using the Public Health England (PHE) Reference Materials for Microbiology for norovirus GI (batch number 0122-17), norovirus GII (batch number 0247-17), and HAV (batch number 0261-2017) and reported as genomic copies (GC), while standard curves for RV, MgV, and HAstV were generated by amplifying 10-fold serial dilutions of viral suspensions in quintuplicates and calculating the number of PCR units (PCRU). Standard DNA material for crAssphage standard curve generation relied on a customized gBlock gene fragment (Integrated DNA Technologies, United

States) containing target sequence for CPQ_064 crAssphage primers set (Stachler et al., 2017). All (RT)-qPCR determinations followed quality control and quality assurance criteria included in EMMI Guidelines (Borchardt et al., 2021).

Statistical Analyses

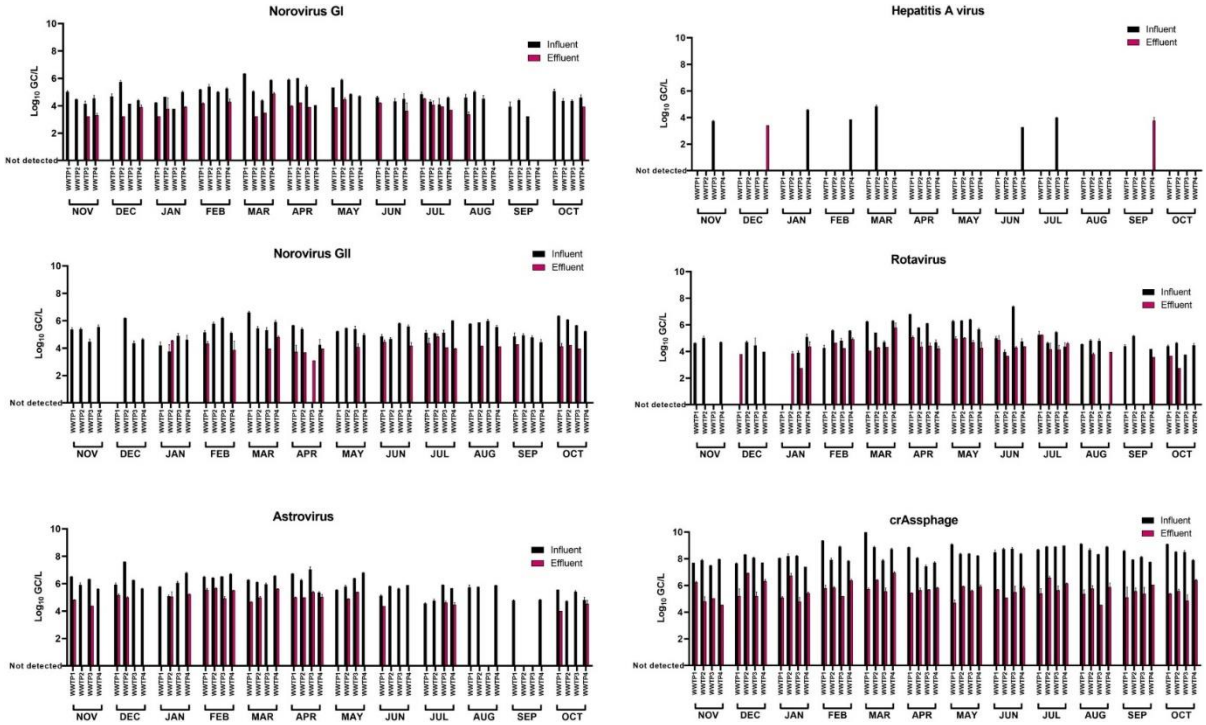
Statistical data processing was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, United States). The results were not normally distributed, so non-parametric Spearman's rank correlation analyses were performed to evaluate the strength of relationship between viral titers alone, and in combination with physicochemical parameters. In all cases, values of $p < 0.05$ were deemed significant. Effects of wastewater treatment plant's covered population, flow intake, and tertiary treatment (UV or chlorination) on crAssphage titers were analyzed by using the GraphPad Prism software. Correlation analyses among potentially infectious enteric viruses, crAssphage, E. coli, and physicochemical parameters were performed using viral loads, calculated as the product of viral titer per water flow for each WWTP. No log transformation was applied on data as Spearman's rank correlation is invariant under monotone transformations like the logarithm.

4. Results

Occurrence of Intact Capsid Enteric Viruses and CrAssphage in Influent and Effluent Wastewater Over a 1-Year Period

Influent and effluent wastewater samples from four WWTPs located in the Valencian region (Spain) were processed by PMAxx-RT-qPCR over a 12-month period (2018–19) to determine the occurrence of potentially infectious norovirus GI, norovirus GII, HAV, RV, and HAstV, along with crAssphage (Figure 2).

Figure 2. Occurrence of intact capsid enteric viruses and crAssphage in influent and effluent wastewater samples over a 1-year period. Capsid integrity was assessed by PMAxx-RT-qPCR. Colored bars represent mean Log10 GC/L values of two technical RT-qPCR replicates for each concentrated sample. Error bars indicate standard deviation.



It is worth to report that preliminary spiking experiments using murine norovirus (MNV, surrogate for human norovirus) and HAV assessed the effect of the wastewater concentration method on viral infectivity. According to the determination of the tissue culture infectious dose (TCID50/ml), no significant differences ($p > 0.05$) were observed among spiked and concentrated titers for both tested virus (data not shown).

The recoveries of MgV, spiked as viral process control, ranged between 1.18 and 37.80% (Supplementary Table 2); thus, results of targeted viruses were validated according to Haramoto et al. (2018) and the criteria included in the ISO 15216-1:2017 (recovery of control ≥1%). Viral titers of targeted viruses were not adjusted depending on the recovery of the concentration control (MgV) as back-calculation is not recommended (Haramoto et al., 2018). Norovirus GI, norovirus GII, and RV titers were 4.77 ± 0.65, 5.28 ± 0.63, and

5.08 ± 0.85 log10 GC/L in influent samples, and 3.86 ± 0.45, 4.13 ± 0.38, and 4.28 ± 0.64 log10 GC/L in effluent samples, respectively. HAstV showed the highest mean viral concentration among the five enteric RNA viruses in both influent (5.89 ± 0.68 log10 GC/L) and effluent (4.97 ± 0.43 log10 GC/L) samples. Moreover, HAstV was detected in 93.75% of influent water samples and in 50.0% of the effluent samples (Table 2). Overall, 93.8, 95.8, and 87.5% of influent samples (n = 48) and 52.1, 45.8, and 66.7% of effluent samples (n = 48) were positive for norovirus GI, norovirus GII, and RV, respectively. Finally, HAV was detected in 12 and 4.17% of the influent and effluent samples, respectively, and showed the lowest concentrations of 4.05 ± 0.56 log10 GC/L in influent samples and 3.60 ± 0.25 log10 GC/L in effluent samples. CrAssphage showed concentrations up to 3–4 log10 GC/L higher than targeted enteric RNA viruses ranging from 7.41 to 9.99 log10 GC/L in influent and from 4.56 to 6.96 log10 GC/L in effluent water samples, respectively. All samples tested positive for crAssphage and a mean decrease of 2.73 ± 0.68 log10 GC/L was observed in effluent compared to influent samples.

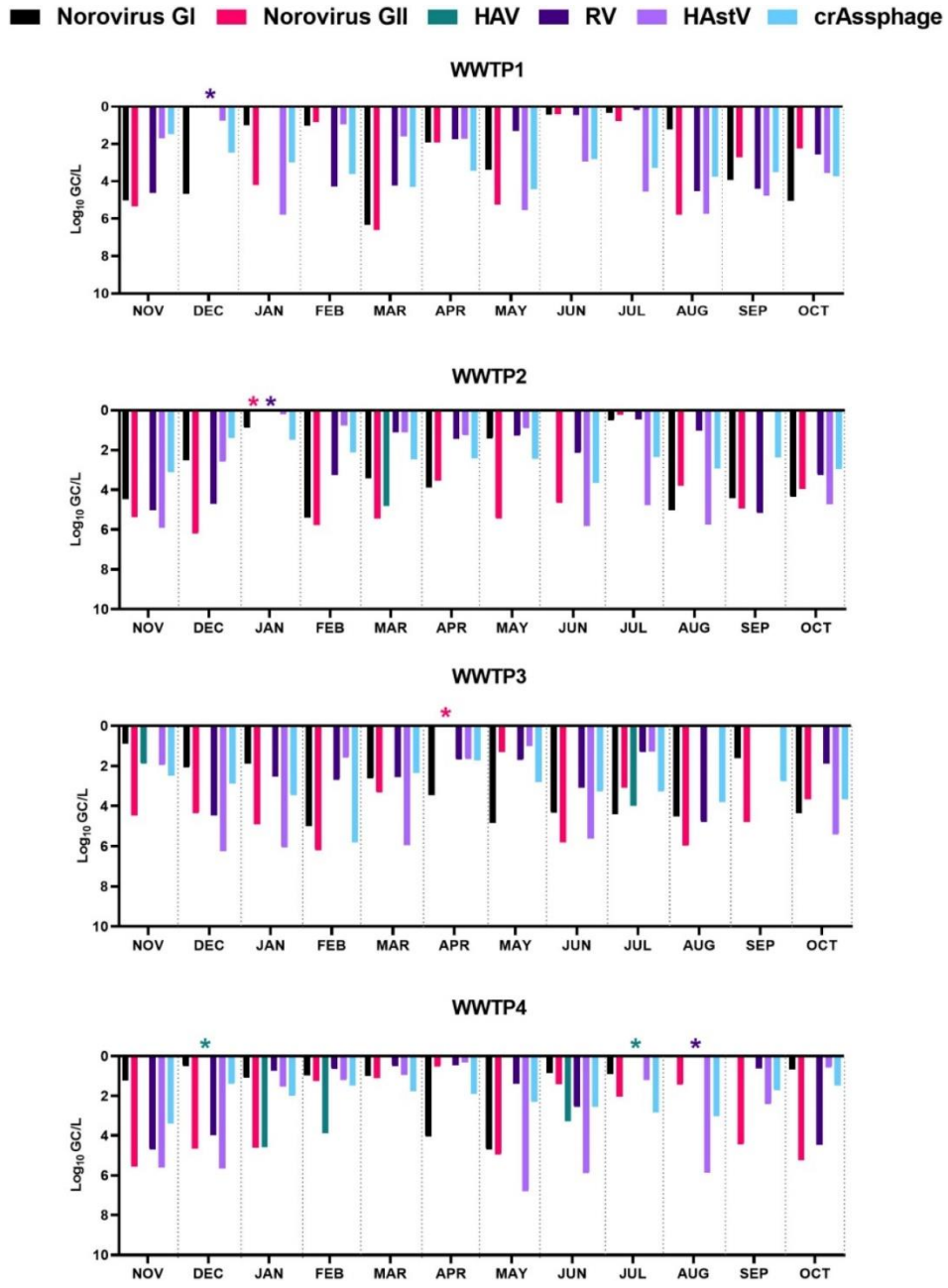
Table 2. Positive samples and percentiles of intact capsid enteric viruses assessed by PMAxx-RT-qPCR and in situ capture (ISC-)RT-qPCR assays in influent and effluent wastewater samples collected over a year from a selected wastewater treatment plant (WWTP4).

Virus	Wastewater sample	PMAxx-RT-qPCR	ISC-RT-qPCR
Norovirus GI	Influent (*n* = 12)	10 (83.3%)	7 (58.3%)
	Effluent (*n* = 12)	7 (58.3%)	3 (25%)
Norovirus GII	Influent (*n* = 12)	12 (100%)	9 (75%)
	Effluent (*n* = 12)	6 (50%)	1 (8.3%)
RV	Influent (*n* = 12)	11 (91.7%)	6 (50%)
	Effluent (*n* = 12)	9 (75%)	8 (66.7%)
HAstV	Influent (*n* = 12)	12 (100%)	12 (100%)
	Effluent (*n* = 12)	7 (58.3%)	6 (50%)

RV, rotavirus; HAstV, human astroviruses.

Considering enteric virus, our data showed mean log removals of 2.58, 3.70, 2.39, and 3.08 for norovirus GI, norovirus GII, RV, and HAstV, respectively (Figure 3).

Figure 3. Enteric virus and crAssphage mean log removal (Log10 GC/L) for each wastewater treatment plant (WWTP) over a 1-year period. HAV, hepatitis A virus; RV, rotavirus; HAstV, human astroviruses; asterisks (*) indicate complete viral removal as viral load was higher in effluent than in influent wastewaters; missing bars are used for no detected virus in influent wastewaters.



Viral removal separately calculated according to tertiary treatment, indicated log decreases of 3.65, 2.37, and 3.18 for norovirus GII, RV, and HAstV in UV-treated effluent wastewater (WWTP1 and WWTP3). Chlorination treatments in WWTP2 and WWTP4 determined log removals of 2.42, 2.97, and 3.75 for norovirus GII, RV, and HAstV. Viral removal differed between UV and chlorination showing 3.00 and 3.25 mean log reductions for UV, and 2.16 and 2.31 for chlorination for norovirus GI and crAssphage, respectively (Figure 3). None of the targeted viruses showed a sharp seasonal pattern (Figure 2). Extended data on viral quantification are presented in Supplementary Table 2.

Comparing PMAxx-RT-qPCR and ISC-RT-qPCR Assays

In order to assess the efficiency of two alternative capsid integrity assays, PMAxx-RT-qPCR, and ISC-RT-qPCR were compared for detecting potential infectious norovirus GI, norovirus GII, HAstV, and RV in wastewater samples collected from a selected WWTP (n = 24). HAV was not tested by ISC-RT-qPCR because of its sporadic detection.

Overall, ISC-RT-qPCR provided lower estimates of viral occurrence than PMAxx-RT-qPCR for all tested viruses, except for HAstV, that showed 100% of positive samples in influent samples regardless of the capsid integrity assay applied (Table 2). Specifically, norovirus GI, norovirus GII, RV, and HAstV were detected by ISC-RT-qPCR in 58, 75, 50, and 100% of influent and in 25, 8, 67, and 50% of the effluent water samples. On the other hand, PMAxx-RT-qPCR estimated the occurrence of norovirus GI, norovirus GII, RV, and HAstV in 94, 96, 88, and 94% of influent and in 52, 46, 67, and 50% of effluent samples.

Regarding viral concentration, viral titers based on PMAxx-RT-qPCR assay resulted higher than those obtained by ISC-RT-qPCR in 93.75% determinations (Supplementary Figure 1).

Escherichia coli Counts and Physicochemical Parameters

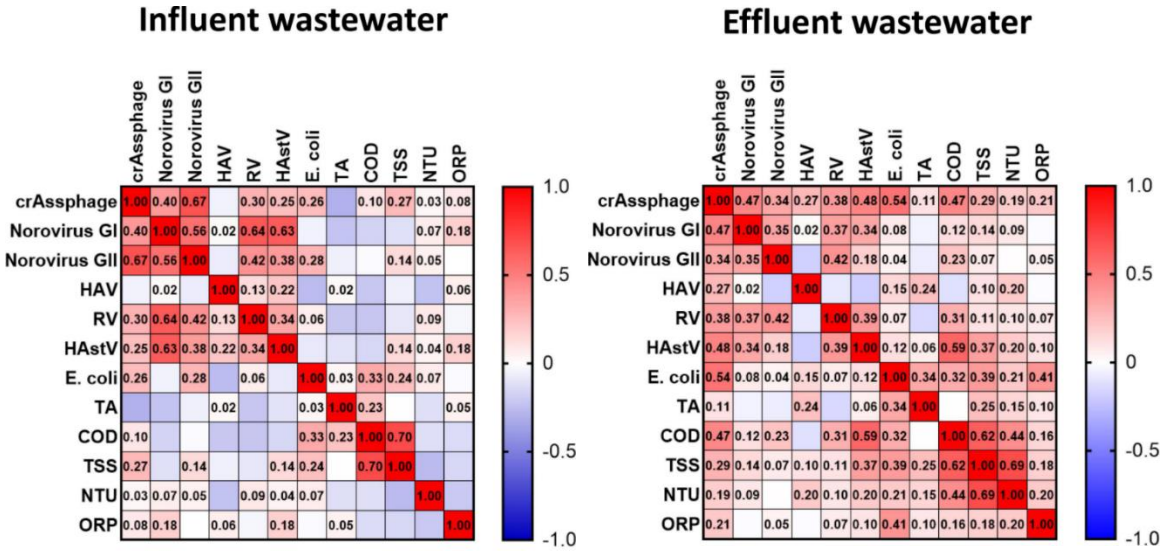
The E. coli counts and physicochemical parameters of influent and effluent wastewater samples are summarized in Supplementary Table 3. E. coli ranged from 3.96 to 8.19 log10 MPN/100 ml and from below the detection limit to 5.96 log10 MPN/100 ml in influent and effluent samples, respectively. Alkalimetric titration ranged from 58.30 to 744 mg/L CaCO3 and from 44.24 to 828 mg/L CaCO3 in influent and effluent samples, respectively. COD ranged from 28.7 to 5,768 and from 11.6 to 108 mg/l O2 in influent and effluent

samples, respectively. Suspended solids ranged from 69.2 to 582.3 mg/l, and from 0.9 to 63.6 mg/l in influent and effluent samples, respectively. Turbidity values ranged from 0 to 247 units in influent and from 0 to 30.02 units in effluent samples. The redox potential ranged from 1.9 to 270.4 and from 1.2 to 224 mV in influent and effluent samples, respectively.

CrAssphage as Fecal Viral Contamination Indicator of Potentially Infectious Enteric Viruses in Wastewater Samples

To further investigate the relationship among crAssphage, potentially infectious enteric virus, and physicochemical wastewater parameters, data sets were subjected to correlation analyses (Figure 4). Specifically, Spearman's rank correlation rho coefficients (ρ) were calculated for intact capsid viral loads (viral titer × water flow) detected by PMAxx-RT-qPCR, E. coli counts, and physicochemical parameters in both influent (n = 48) and effluent (n = 48) wastewater samples (Figure 4). Resulting ρ coefficients are described through this work as follows: weak correlation (0.2–0.39), moderate correlation (0.4–0.59), strong correlation (0.6–0.79), and very strong correlation (0.8–1). In influent waters, crAssphage showed strong correlation with intact capsid norovirus GII (ρ = 0.67), moderate correlation with intact capsid norovirus GI (ρ = 0.40), and weak correlation with HAstV, RV, and E. coli (ρ = 0.25–0.30). Among enteric viruses, a moderate correlation resulted between norovirus GI and norovirus GII (ρ = 0.56). None to poor correlations resulted among enteric viruses and physicochemical parameters. When analyzing effluent wastewater samples, crAssphage showed moderate correlation with E. coli (ρ = 0.54) and intact capsid HAstV (ρ = 0.48) and norovirus GI (ρ = 0.47). Weak correlations resulted between crAssphage and RV (ρ = 0.38) and norovirus GII (ρ = 0.34). In contrast, E. coli displayed no correlation with any of the tested enteric viruses in effluent wastewater samples (ρ = 0.01–0.15).

Figure 4. Spearman's rho coefficients (ρ) of correlation analyses of intact capsid enteric viruses loads, crAssphage, and physicochemical parameters in influent and effluent wastewaters. TA, total alkalinity; COD, chemical oxygen demand; TSS, total suspended solids; NTU, turbidity; ORP, oxidation-reduction potential.



Effect of Wastewater Treatment Plant Characteristics on CrAssphage Load

The effect of key characteristics (population coverage, flow intake, and tertiary treatment) (Figure 1 and Table 1) on crAssphage load was independently evaluated for each WWTP.

CrAssphage load levels in influent ($p = 0.039$) and effluent ($p = 0.007$) wastewater statistically differed among WWTPs. Univariate results showed that variability of crAssphage load was statistically dependent on population coverage and on flow intake ($p < 0.05$) for influent wastewater samples. Interestingly, crAssphage concentration in effluent samples was found to significantly differ depending on the tertiary treatment ($p = 0.016$), suggesting that UV might be more efficient than chlorination for crAssphage removal. This was also observed for norovirus GI, but not for the remaining enteric virus (Figure 3).

5. Discussion

The reuse of treated wastewater and its discharge into the environment poses a challenge for public health, as reclamation treatments needs to be adequate to provide water that is suitable for its intended purpose (e.g., irrigation, recreational, or drinking water). Governments and the scientific community agree on the need for monitoring the viral population in wastewater even though there is still much uncertainty on the analytical protocols to use as well as the load reduction needed for ensuring a minimal risk from exposure to reclaimed water (Gerba et al., 2018; Fenaux et al., 2019).

Capsid integrity is a strong indicator of virus infectivity and can be a worthy tool to adjust existent workflows and qPCR procedures to indicate the capability of viruses to infect humans, thus enhancing risk assessment inferred from monitoring programs (Leifels et al., 2020). However, the present investigation did not address the question on how to use data based on capsid integrity techniques as input for quantitative risk assessment; this query needs to be specifically explored in future work. A first step into this direction could be determining the relationships of viral infectious titers estimated by capsid integrity techniques and dose response resulting from clinical trials or known outbreaks.

Presence of Potentially Infectious Enteric Viruses and Indicators in Wastewaters

This study provides additional insights on the quantitative occurrence of intact capsid enteric viruses in influent and effluent samples, and their correlation with crAssphage as a proposed viral water quality indicator.

We repetitively detected potentially infectious enteric viruses, including norovirus GI, norovirus GII, HAstV, and RV, in both influent and effluent in four different WWTPs over a year. This was further confirmed analyzing longitudinally upstream and downstream wastewater of a selected WWTP by two alternative capsid integrity assays, PMAxx-RT-qPCR and ISC-RT-qPCR, even though with different percentages (Table 2). Such release of human enteric viruses in effluent wastewater is not surprising as viral infectivity has been advised using different viability dye pretreatments (Gyawali and Hewitt, 2018; Randazzo et al., 2019; Canh et al., 2021a) and definitively demonstrated by cell culture (Simmons and Xagoraraki, 2011). However, comparing the viral titers determined by capsid integrity assays among WWTPs may not be conclusive due to different ratios of infectivity characterizing each population

served by the sewerage system. This aspect could be additionally hindered for effluent wastewater samples exposed to different reclamation treatments (e.g., UV vs. chlorine) that distinctively affect viral morphology (e.g., nucleic acid vs. capsid), finally leading to diverse estimate of infectivity by capsid-integrity methods (Leifels et al., 2019).

Interestingly, a PMA-based capsid integrity assay was recently applied to assess the potential infectivity of novel HAV strains in treated wastewater in South Africa for which cell culture techniques may result to be not permissive (Rachida and Taylor, 2020).

Our results show that titers of viral particles with intact capsid in influent samples are comparable to those previously determined by RT-qPCR alone (Da Silva et al., 2007; Katayama et al., 2008; Kitajima et al., 2014; Montazeri et al., 2015; Haramoto et al., 2018), which suggests a high proportion of potentially infectious viruses, as expected.

However, capsid integrity RT-qPCR assays may not sharply discriminate infectious and inactivated viruses when subtle capsid alterations or genome damage occur because of the limited access to free RNA, the interaction with other compounds (e.g., organic acids), and the ineffective photoactivation (e.g., due to suspended solids, turbidity). These factors could differently affect capsid integrity RT-qPCR assays especially in complex matrices, such as wastewater, finally explaining the lower estimates for viral infectivity resulted from ISC-RT-qPCR compared to PMAxx-RT-qPCR. Thus, our findings further corroborate that PMAxx-RT-qPCR generally overestimate infectious viral particles (Leifels et al., 2015, 2020; López-Gálvez et al., 2018; Randazzo et al., 2018b, 2019). Nonetheless, capsid integrity RT-qPCRs better assess the potential risk of viral infection by providing more accurate information than conventional RT-qPCR alone that should be interpreted as a conservative approach.

Reduction of Potentially Infectious Enteric Viruses and Indicators During Wastewater Treatments

We observed reductions of 2–3 log10 on average between upstream and downstream wastewater, which do not comply with the most recent European legislation. Specifically, a ≥ 6 log10 decrease of rotavirus, total coliphages, or at least one of them (F-specific or somatic coliphages) is

indicated to validate monitoring programs of reclaimed water used for agricultural irrigation (Regulation (EU) 2020/741, 2020). However, specific guidelines should be defined globally as pointed out by the scientific community and water operators (Sano et al., 2016; Gerba et al., 2017).

In recent years, crAssphage has emerged as viral water quality indicator because of its specificity to human fecal pollution, its high concentrations in sewage, and its global presence (Farkas et al., 2019; Bivins et al., 2020a; Honap et al., 2020). Interestingly, KWR (Netherlands) included crAssphage to normalize SARS-CoV-2 titers in influent wastewater to monitor the COVID-19 pandemic (KWR, 2020), thus its potential as biomarker is not fully explored yet.

We detected crAssphage in all influent and effluent samples at mean concentrations of 8.37 ± 0.55 and 5.64 ± 0.59 log10 GC/L, respectively. These concentrations in influent wastewaters were roughly in line with the ones reported in the United Kingdom (Farkas et al., 2019), United States (Wu et al., 2020), and in a previous study conducted also in Spain (García-Aljaro et al., 2017). Slightly lower titers were reported in Thailand (Kongprajug et al., 2019) and in Italy (Crank et al., 2020). On the contrary, higher concentration of 10.98–12.03 log10 GC/L in influent and 7.45–8.62 log10 GC/L in effluent wastewaters were reported in Japan (Malla et al., 2019). These discrepancies might be due to the population served by WWTPs, the engineering characteristics of the sewer system (e.g., retention times, treatments, etc.), and the analytical method used for viral detection (wastewater concentration procedure, the genomic target, standards used to quantify viral concentrations), among other variables. Analyzing some of those variables, we observed statistically significant differences on crAssphage titers for served population, flow intake, and among WWTPs. This finding is in accordance to a previous report by Crank et al. (2020). Additionally, crAssphage concentrations in effluent wastewater were significantly lower when wastewater was exposed to UV than to chlorination. Thus, we further corroborate existing bibliography indicating the efficient viral disinfection applying UV light irradiation (Ali, 1997; Mezzanotte et al., 2007; Shah et al., 2011; Zyara et al., 2016). The increased mean removal in UV-treated wastewaters compared to chlorinated effluents can be extended to norovirus GI, but not for the other enteric viruses tested in this study (Figure 3).

CrAssphage as Indicator for the Potential Infectivity of Enteric Viruses in Wastewater

The correlation between crAssphage and human viral pathogens has been reported in recent studies investigating wastewaters (Farkas et al., 2019; Malla et al., 2019; Crank et al., 2020; Tandukar et al., 2020), sludge (Wu et al., 2020), and other fecal polluted waters (Jennings et al., 2020). However, no information was available to date on whether crAssphage would serve as indicator for the potential infectivity of enteric viruses in wastewater. In influent wastewater, we found crAssphage strongly correlated to intact capsid norovirus GII and moderately to norovirus GI. In effluent wastewater, crAssphage moderately correlated with potentially infectious HAstV and norovirus GI.

Overall, the consistent detection of crAssphage in all influent and effluent samples corroborates the phage as an indicator for fecal contamination in wastewater. However, correlation readouts do not solidly support the use of crAssphage as indicator for the presence of potentially infectious enteric virus in wastewater, which was the primary hypothesis tested in this study. Thus, a strategy that targets each viral contaminant should be preferred to the sole detection of phages and this applies for both investigation and monitoring purposes.

The results of the present study also demonstrated that E. coli, adopted in the current regulation as fecal biomarker, and physicochemical parameters are not well suited as indicators for the viral contamination of wastewater, according to previous reports (Stachler et al., 2018; Ahmed et al., 2020).

Limitation, Perspective, and Future Research

This study did not take into account environmental variables, such as rainfall and temperature, among others, that could have affected reported results.

Although analyzing the samples by RT-qPCR alone could have served as baseline to check the performance of PMAxx, previous studies already investigated the relationship of capsid integrity treatment on viral amplification signal reduction (Randazzo et al., 2016, 2019; Cuevas-Ferrando et al., 2020). Following a one-size capsid integrity treatment fits all approach and assuming it could lead to lower signal reduction (e.g., virus and matrix specificity: length and structure of genome targeted by the qPCR assays, the

influence of co-concentrated inhibitory substances, etc.), we tested the hypothesis to adapt existent workflows for improving risk assessment.

Also, the comparison of molecular results with cell culture would have soundly confirmed our findings. However, viral cell culture of environmental samples presents technical challenges that are difficult to overcome (e.g., contamination, toxicity, sensitivity), especially in a longitudinal monitoring study such this one. A similar consideration can be done for crAssphage (Shkoporov et al., 2018).

Our findings based on capsid integrity assays could boost the development of advanced quantitative microbial risk assessment (QMRA) models for determining the risk of infection in case of treated wastewater reuse. This warrants further investigation and constitutes the gap to fill in the future in order to better quantify the human health risk, provide robust information for decision-making, and support water quality regulation.

In conclusion, this work provides insights on the quantitative occurrence of crAssphage and intact capsid enteric viruses in influent and effluent wastewater, while correlation outcomes indicated that crAssphage is a poor indicator for enteric virus infectivity in reclaimed wastewater.

6. Supplementary material

Table S1. Primers, probes and (RT)-qPCR conditions used in the study.

Virus	Primers and probe	Sequence	RT-qPCR conditions	Reference
NoV GI	QNIF4	CGC TGG ATG CGN TTC CAT	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	(Anon n.d.)
	NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC		
	NVGG1p	TGG ACA GGA GAY CGC RAT CT		
NoV GII	QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	15216-1:2017. Microbiology of Food and Animal Feed — Horizontal Method for Determination of Hepatitis A Virus and Norovirus in Food Using Real-Time RT-PCR — Part 1: Method for Quantification,
	COG2R	TCG ACG CCA TCT TCA TTC ACA		
	QNIFs	AGC ACG TGG GAG GGC GAT CG		
HAV	HAV68	TCA CCG CCG TTT GCC TAG	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	15216-1:2017. Microbiology of Food and Animal Feed — Horizontal Method for Determination of Hepatitis A Virus and Norovirus in Food Using Real-Time RT-PCR — Part 1: Method for Quantification,
	HAV240	GGA GAG CCC TGG AAG AAA G		
	HAV150	CCT GAA CCT GCA GGA ATT AA		
RV	JVKF	CAG TGG TTG ATG CTC AAG ATG GA	RT: 50 °C for 30 min, Preheating: 95 °C for 15 min PCR (45 cycles) 94 °C for 10 s, 55 °C for 30 s, 72 °C for 20 s.	(Jothikumar, Kang, and Hill 2009)
	JVKR	TCA TTG TAA TCA TAT TGA ATA CCC A		
	JVKP	FAM-ACA ACT GCA GCT TCA AAA GAA GWG T		
HAstV	AstVorf1b+	AAG CAG CTT CGT GAC TCT GG	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 58 °C for 60 s, 65 °C for 60 s.	15216-1:2017. Microbiology of Food and Animal Feed — Horizontal Method for Determination of Hepatitis A Virus and Norovirus in Food Using Real-Time RT-PCR — Part 1: Method for Quantification,
	AstVorf1b-	AGC CAT CAC ACT TCT TTG GTC		
	AstVorf1bp			
MgV	Mengo 110	GCG GGT CCT GCC GAA AGT	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	15216-1:2017. Microbiology of Food and Animal Feed — Horizontal Method for Determination of Hepatitis A Virus and Norovirus in Food Using Real-Time RT-PCR — Part 1: Method for Quantification,
	Mengo 209	GAA GTA ACA TAT AGA CAG ACG CAC AC		
	Mengo 147	ATC ACA TTA CTG GCC GAA GC		
crAssphage	064F1	TGT ATA GAT GCT GCT GCA ACT GTA CTC	Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 5 s, 60 °C for 30 s	(Stachler, Kelty, Sivaganesan, Li, Bibby, and Orin C Shanks 2017)
	064R	CGT TGT TTT CAT CTT TAT CTT GTC CAT		
	064P1	CTG AAA TTG TTC ATA AGC AA		

Table S2. Intact capsid enteric viruses and crAssphage mean concentration values (log_{10} GC/L) and mengovirus recovery (%). *, indicate only one positive RT-qPCR replicate.

			Enteric viruses concentration (log_{10} GC/L)						
WWTP	**Month**	**Type**	**Norovirus GI**	**Norovirus GII**	**HAV**	**RV**	**HAstV**	**crAssphage**	**MgV Recovery (%)**
WWTP1	**NOV**	**Influent**	5.02±0.08	5.35±0.14	ND	4.62±0.03	6.51±0.01	7.7±0.0	4.84
		Effluent	ND	ND	ND	ND	4.82±0.01	6.24±0.07	3.05
WWTP2		**Influent**	4.46±0.03	5.38±0.1	ND	5.02±0.13	5.9±0.17	7.9±0.05	9.66
		Effluent	ND	ND	ND	ND	ND	4.81±0.35	1.66
WWTP3		**Influent**	4.12±0.22	4.47±0.19	3.77±0	ND	6.33±0.02	7.5±0.01	3.67
		Effluent	3.23*	ND	ND	ND	4.36±0.01	5.03*	3.65
WWTP4		**Influent**	4.54±0.22	5.55±0.14	ND	4.69±0.02	5.61±0.02	7.97±0.01	5.25
		Effluent	3.32±0.13	ND	ND	ND	ND	4.56*	1.94
WWTP1	**DEC**	**Influent**	4.67±0.2	ND	ND	ND	5.92±0.12	7.66±0.03	1.83
		Effluent	ND	ND	ND	3.79*	5.15±0.11	5.2±0.53	1.66
WWTP2		**Influent**	5.73±0.11	6.19±0.04	ND	4.71±0.07	7.6*	8.32*	15.15
		Effluent	3.23*	ND	ND	ND	5.01±0.04	6.92±0.01	1.57
WWTP3		**Influent**	4.14*	4.35±0.17	ND	4.46±0.55	6.25±0.02	8.08±0.04	1.91
		Effluent	ND	ND	ND	ND	ND	5.21±0.31	4.53
WWTP4		**Influent**	4.4±0.06	4.64±0.09	ND	3.98*	5.64±0.03	7.71*	1.21
		Effluent	3.91±0.16	ND	3.42±0	ND	ND	6.33±0.13	3.31
WWTP1	**JAN**	**Influent**	4.23±0.01	4.21±0.25	ND	ND	5.78±0.02	8.06*	1.68
		Effluent	3.23	ND	ND	ND	ND	5.09±0.07	10.43
WWTP2		**Influent**	4.65*	3.75±0.5	ND	ND	5.09±0.08	8.2±0.17	1.40

		Effluent	3.78±0.79	4.55±0.03	ND	3.84±0.16	5.05±0.35	6.73±0.17	1.49
WWTP3		Influent	3.78*	4.89±0.17	ND	3.91±0.13	6.06±0.11	8.22±0.03	1.32
		Effluent	ND	ND	ND	2.75*	ND	4.79±0.33	5.31
WWTP4		Influent	5±0.08	4.61±0.32	4.58±0.04	5.09±0.22	6.77±0.07	7.41*	13.20
		Effluent	3.91±0.02	ND	ND	4.36±0.37	5.23±0.01	5.42±0.07	5.85
WWTP1	FEB	Influent	5.19±0.02	5.15±0.14	ND	4.26±0.21	6.49±0.05	9.36*	2.10
		Effluent	4.17±0.04	4.32±0.16	ND	ND	5.54±0.11	5.79±0.23	12.44
WWTP2		Influent	5.39±0.16	5.78±0.11	ND	5.58±0.04	6.43±0.02	7.93±0.11	10.88
		Effluent	ND	ND	ND	4.65*	5.66±0.04	5.83±0.09	2.15
WWTP3		Influent	5.01±0.03	6.2±0.06	ND	4.81±0.18	6.51±0.01	8.91±0.05	12.00
		Effluent	ND	ND	ND	4.24*	4.92±0.13	5.2*	15.02
WWTP4		Influent	5.25±0.07	5.11±0.07	3.87±0	5.56±0.01	6.71±0.04	7.84±0.03	10.88
		Effluent	4.29±0.21	3.86±0.65	ND	4.91±0.1	5.51±0.02	6.37±0.11	9.54
WWTP1	MAR	Influent	6.34±0.01	6.61±0.07	ND	6.24±0.04	6.27±0.03	9.99±0.03	3.58
		Effluent	ND	ND	ND	4.04*	4.67±0.01	5.72±0.08	4.33
WWTP2		Influent	5.04±0.06	5.44±0.13	4.83±0.13	5.41*	6.11±0.02	8.86±0.07	1.53
		Effluent	3.23*	ND	ND	4.28±0.03	4.99±0.08	6.41±0.03	1.45
WWTP3		Influent	4.39±0.06	5.31±0.2	ND	4.71±0.09	5.94±0.11	7.88±0.07	5.05
		Effluent	3.5*	ND	ND	4.33*	ND	5.55±0.26	19.16
WWTP4		Influent	5.87±0.03	5.91±0.12	ND	6.3±0.03	6.57±0.04	8.72±0.07	10.39
		Effluent	4.89±0.09	4.8±0.09	ND	5.79±0.35	5.62±0.01	6.96±0.07	14.73
WWTP1	APR	Influent	5.9±0.05	5.65±0.03	ND	6.8±0.01	6.72±0.02	8.85±0.03	10.13
		Effluent	3.98±0.03	3.74±0.48	ND	5.06±0.09	5±0.03	5.43±0.01	10.12
WWTP2		Influent	6±0.01	5.39±0.1	ND	5.79±0.01	6.25±0.08	8.06±0.04	11.61
		Effluent	4.23*	3.69*	ND	4.34±0.34	4.98±0.01	5.65±0.15	7.70
WWTP3		Influent	5.4±0.1	ND	ND	6.12±0.01	7.03±0.19	7.42±0.11	3.64
		Effluent	3.91*	3.1*	ND	4.43±0.2	5.38±0.06	5.7±0.02	4.92
WWTP4		Influent	4.05*	4.24±0.38	ND	4.68±0.19	5.35±0.07	7.72±0.05	3.65

		Effluent	ND	3.98*	ND	4.23±0.16	5.03±0.19	5.82±0.03	2.89
WWTP1	MAY	Influent	5.33*	5.24±0.02	ND	6.26±0.07	5.54±0.04	9.09±0.03	9.15
		Effluent	3.88 *	ND	ND	4.96±0.15	ND	4.7±0.19	5.21
WWTP2		Influent	5.89±0.06	5.45±0.02	ND	6.31*	5.79±0.1	8.36±0.05	12.61
		Effluent	4.47±0.12	ND	ND	5.02±0.03	4.89±0.02	5.94±0.02	7.61
WWTP3		Influent	4.85±0.03	5.39±0.2	ND	6.39±0.04	6.39±0.01	8.39±0.01	14.00
		Effluent	ND	4.08±0.24	ND	4.69±0.14	5.38±0.01	5.59±0.07	10.04
WWTP4		Influent	4.69±0.04	4.96±0.11	ND	5.64±0.09	6.78±0.04	8.23±0.01	10.98
		Effluent	ND	ND	ND	4.26±0.45	ND	5.93±0.11	4.00
WWTP1	JUNE	Influent	4.32±0.07	4.85±0.15	ND	4.99±0.14	5.12±0.07	8.49±0.16	12.76
		Effluent	4.2±0.03	4.46±0.14	ND	4.85±0.32	4.36*	5.69±0.03	19.33
WWTP2		Influent	ND	4.66±0.11	ND	3.96±0.15	5.82±0.02	8.72±0.07	2.41
		Effluent	ND	ND	ND	3.66*	ND	5.09*	3.70
WWTP3		Influent	4.32±0.21	5.8±0.04	ND	7.37±0.04	5.63±0.04	8.76±0.09	17.65
		Effluent	ND	ND	ND	4.28±0.08	ND	5.5±0.44	34.80
WWTP4		Influent	4.49±0.39	5.58±0.11	3.28*	4.75±0.22	5.89*	8.37±0.04	3.34
		Effluent	3.63±0.57	4.18±0.23	ND	4.38*	ND	5.82±0.11	5.61
WWTP1	JULY	Influent	4.86±0.14	5.12±0.18	ND	5.25±0.27	4.55±0.06	8.68±0.04	6.03
		Effluent	4.52±0.01	4.35±0.38	ND	5.24±0.01	0±0	5.41±0.35	25.66
WWTP2		Influent	4.3±0.13	5.07±0.04	ND	4.63±0.08	4.77±0.1	8.9±0.04	1.29
		Effluent	4.08±0.29	4.85±0.16	ND	4.17±0.45	ND	6.58±0.11	28.65
WWTP3		Influent	4.1±0.42	5.11±0.2	3.99±0.03	5.44±0.06	5.9*	8.9±0.01	7.86
		Effluent	3.95*	4.04*	ND	4.14±0.33	4.63±0.11	5.65±0.31	8.96
WWTP4		Influent	4.6±0.07	6.01*	ND	4.36±0.3	5.67*	8.99*	4.78
		Effluent	3.7*	3.97*	ND	4.6±0.08	4.47±0.15	6.15±0.05	11.17
WWTP1	AUG	Influent	4.59±0.24	5.78±0.02	ND	4.54±0.03	5.74±0.15	9.1±0.04	6.00
		Effluent	3.36±0.19	ND	ND	ND	ND	5.37±0.3	2.32
WWTP2		Influent	5.02±0.08	5.88*	ND	4.82±0.08	5.76±0.02	8.66±0.09	5.76

		Effluent	ND	4.18*	ND	3.8±0.09	ND	5.76±0.23	8.81
WWTP3		**Influent**	4.52±0.23	5.98±0.13	ND	4.8±0.15	ND	8.33±0.02	9.05
		Effluent	ND	ND	ND	ND	ND	4.56*	7.43
WWTP4		**Influent**	ND	5.55±0.1	ND	ND	5.87±0.01	8.89±0.03	4.14
		Effluent	ND	4.11*	ND	3.96*	4.64±0.08	5.88±0.29	3.42
WWTP1	**SEPT**	**Influent**	3.93±0.35	4.85±0.25	ND	4.4±0.12	4.78±0.03	8.59±0.05	2.04
		Effluent	ND	4.27*	ND	ND	ND	5.1±0.76	5.22
WWTP2		**Influent**	4.41±0.07	4.93±0.12	ND	5.16±0.03	ND	7.94±0.01	3.67
		Effluent	ND	ND	ND	ND	ND	5.57±0.24	3.66
WWTP3		**Influent**	3.23*	4.78±0.11	ND	ND	ND	8.13±0.04	6.82
		Effluent	ND	ND	ND	ND	ND	5.38±0.47	16.02
WWTP4		**Influent**	ND	4.43±0.2	ND	4.19*	4.83±0.02	7.75*	1.89
		Effluent	ND	ND	3.77±0.23	3.58*	ND	6.05*	2.51
WWTP1	**OCT**	**Influent**	5.05±0.15	6.36±0.01	ND	4.39±0.09	5.56*	9.09±0.01	4.88
		Effluent	ND	4.11±0.23	ND	3.67*	4.01*	5.39±0.02	10.24
WWTP2		**Influent**	4.35±0.18	6.08±0.01	ND	4.64±0.04	4.71±0.04	8.51±0.03	2.39
		Effluent	ND	4.23*	ND	2.75*	ND	5.57±0.15	1.97
WWTP3		**Influent**	4.36±0.07	5.65*	ND	3.76*	5.41±0.1	8.5±0.11	4.43
		Effluent	ND	3.98*	ND	ND	ND	4.87±0.43	4.69
WWTP4		**Influent**	4.6±0.19	5.23±0.02	ND	4.47±0.12	4.81±0.2	7.89±0.05	2.76
		Effluent	3.94*	ND	ND	ND	4.54±0.24	6.39±0.05	2.26

Table S3. Physicochemical characterization of influent and effluent wastewater samples.

Abbreviations: MPN, most probable number; COD, chemical oxygen demand; NTU, Nephelometric Turbidity Unit; NA, data no available.

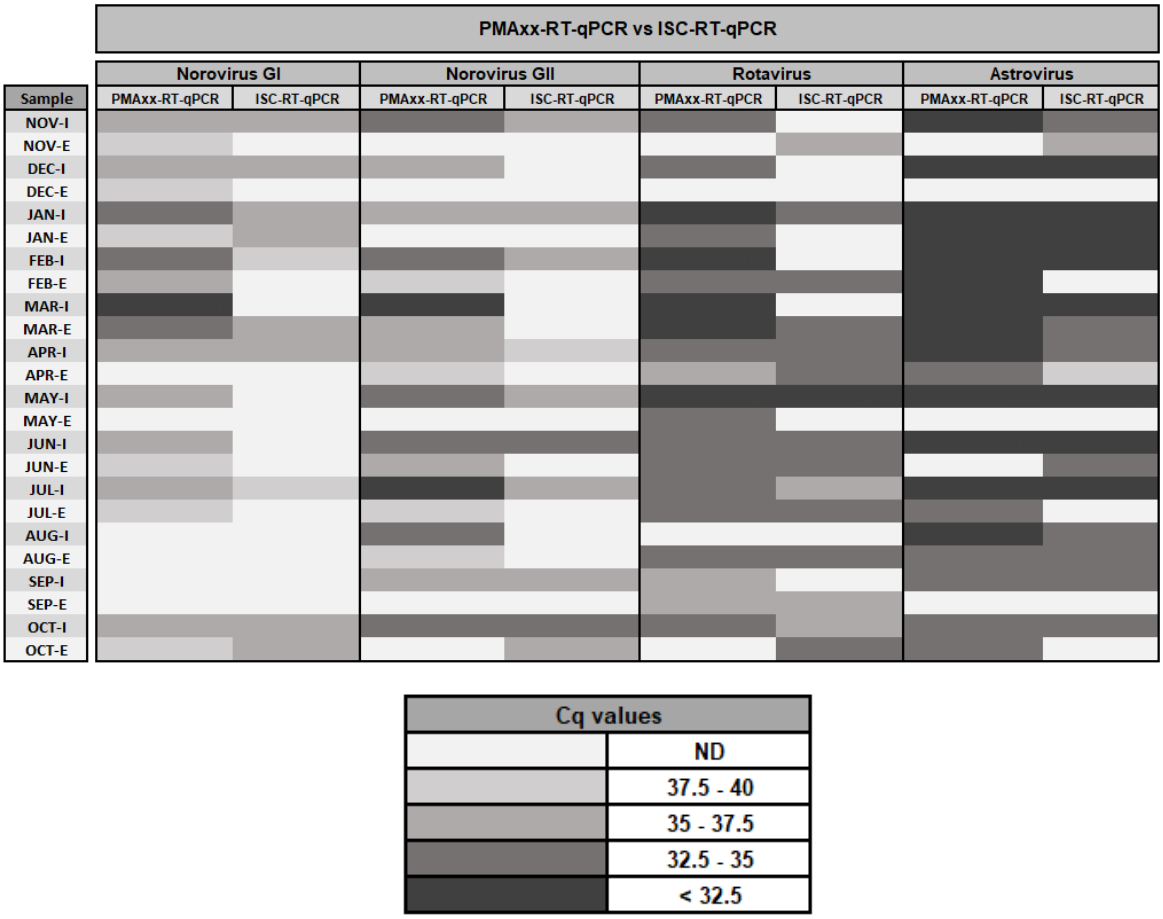
WWTP	Month	Type	*E. coli* (MPN/100mL)	ALKALIMETRIC TITRATION (mg/L $CaCO_3$)	COD (mg/L O_2)	SUSPENDED SOLIDS (mg/L)	TURBIDITY (NTU)	REDOX POTENTIAL (mV)
WWTP1	NOV	Influent	111990	NA	NA	NA	NA	NA
		Effluent	77010	503	30	3	2	162.0
WWTP2		Influent	1935000	NA	NA	NA	NA	NA
		Effluent	22820	278	31	7	3	224.0
WWTP3		Influent	5200000	644	520	260	0	270.40
		Effluent	5	469	18	3	2	23.1
WWTP4		Influent	12997000	614	76	202	37	184.00
		Effluent	155310	589	23	7	1	29.4
WWTP1	DEC	Influent	4611000	579	457	162	44	45.40
		Effluent	3	490	35	4	2	6.2
WWTP2		Influent	129970000	745	682	357	0	54.6
		Effluent	64880	485	59	30	12	49.2
WWTP3		Influent	15531000	544	648	218	88	1.90
		Effluent	0	443	17	5	2	2.2
WWTP4		Influent	6131000	515	41	108	53	3.6
		Effluent	198630	506	22	9	4	3.3
WWTP1	JAN	Influent	3441000	412	708	297	0	47.30
		Effluent	0	292	25	4	2	11.3
WWTP2		Influent	3441000	412	708	297	0	47.3
		Effluent	9804	316	72	30	8	27.2

WWTP3		**Influent**	7170000	673	673	283	0	57.10
		Effluent	2755	498	16	2	1	36.2
WWTP4		**Influent**	2230000	572	139	99	40	37.1
		Effluent	141360	569	27	8	2	44.3
WWTP1	**FEB**	**Influent**	7800000	502	1126	519	95	33.40
		Effluent	6	829	33	10	2	3.1
WWTP2		**Influent**	9207	394	185	261	37	3.7
		Effluent	10760000	500	98	64	27	31.3
WWTP3		**Influent**	8130000	514	589	366	84	11.60
		Effluent	0	367	19	1	1	8.7
WWTP4		**Influent**	2850000	607	95	126	24	32.9
		Effluent	387300	559	34	13	2	34.9
WWTP1	**MAR**	**Influent**	8260000	557	1263	532	0	27.20
		Effluent	2	307	28	4	1	1.2
WWTP2		**Influent**	5120000	433	654	371	0	29.6
		Effluent	504	252	45	17	1	25.2
WWTP3		**Influent**	6690000	539	470	204	0	2.30
		Effluent	1	491	18	7	8	11.5
WWTP4		**Influent**	5380000	529	29	73	38	35.0
		Effluent	111990	464	108	8	2	36.9
WWTP1	**APR**	**Influent**	14210000	66	1597	473	247	3.00
		Effluent	2	48	32	4	2	27.2
WWTP2		**Influent**	18420000	58	430	231	158	42.6
		Effluent	7270	44	32	5	2	27.8
WWTP3		**Influent**	8164000	494	438	402	0	155.10
		Effluent	15	55	73	62	30	158.9
WWTP4		**Influent**	11370000	430	101	119	37	41.0
		Effluent	173290	306	26	9	2	37.8

WWTP1	**MAY**	**Influent**	17326000	478	415	237	0	45.80
		Effluent	6	388	27	4	1	4.8
WWTP2		**Influent**	9804000	508	345	90	75	40.6
		Effluent	1267	331	34	10	4	17.3
WWTP3		**Influent**	19863000	577	1190	393	0	6.40
		Effluent	9	519	59	47	18	33.0
WWTP4		**Influent**	6240000	511	192	164	63	40.3
		Effluent	198630	460	30	6	3	21.1
WWTP1	**JUNE**	**Influent**	19863000	338	234	178	67	25.50
		Effluent	0	337	24	3	3	6.4
WWTP2		**Influent**	5380000	479	671	582	0	30.0
		Effluent	11193	332	29	11	2	19.3
WWTP3		**Influent**	10120000	625	1250	352	206	17.80
		Effluent	47	497	25	5	3	36.5
WWTP4		**Influent**	8860000	401	110	266	0	44.1
		Effluent	189200	475	22	6	0	35.4
WWTP1	**JULY**	**Influent**	7701000	335	228	124	21	63.40
		Effluent	0	256	21	3	1	41.7
WWTP2		**Influent**	3873000	458	315	141	89	24.8
		Effluent	155310	370	33	4	3	30.5
WWTP3		**Influent**	9208000	488	476	312	0	14.20
		Effluent	308	305	28	20	14	10.6
WWTP4		**Influent**	41060000	410	433	291	71	60.2
		Effluent	15650	386	28	7	3	61.4
WWTP1	**AUG**	**Influent**	51720000	574	673	343	0	29.70
		Effluent	115	230	21	2	1	30.2
WWTP2		**Influent**	2950000	473	238	307	0	29.2
		Effluent	198630	705	32	10	2	29.8

WWTP3		**Influent**	86640000	524	631	288	0	0.10
		Effluent	0	398	12	2	1	30.3
WWTP4		**Influent**	155310000	445	623	300	143	37.8
		Effluent	173290	444	28	6	3	35.7
WWTP1	**SEPT**	**Influent**	12997000	638	1982	4560	0	31.90
		Effluent	27550	424	26	3	1	19.1
WWTP2		**Influent**	6488000	498	373	69	32	22.9
		Effluent	46110	251	26	3	1	29.7
WWTP3		**Influent**	26130000	593	1178	271	80	19.50
		Effluent	8	388	20	4	2	38.5
WWTP4		**Influent**	14136000	554	304	140	67	44.1
		Effluent	4950	541	28	7	3	41.9
WWTP1	**OCT**	**Influent**	19863000	525	1047	300	0	25.00
		Effluent	1	347	35	4	1	13.5
WWTP2		**Influent**	15531000	655	543	183	0	55.4
		Effluent	39900	460	28	4	2	23.3
WWTP3		**Influent**	2143000	689	344	263	97	22.90
		Effluent	0	559	28	21	3	
WWTP4		**Influent**	7701000	578	5786	141	58	46.6
		Effluent	920800	528	30	11	4	36.0

Figure S1. Enteric viruses Cq values comparison between PMAxx-RT-qPCR and ISC-RT-qPCR capsid integrity assays on influent and effluent wastewater samples from a single WWTP during a one-year period.



Supplementary material References

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RESULTS

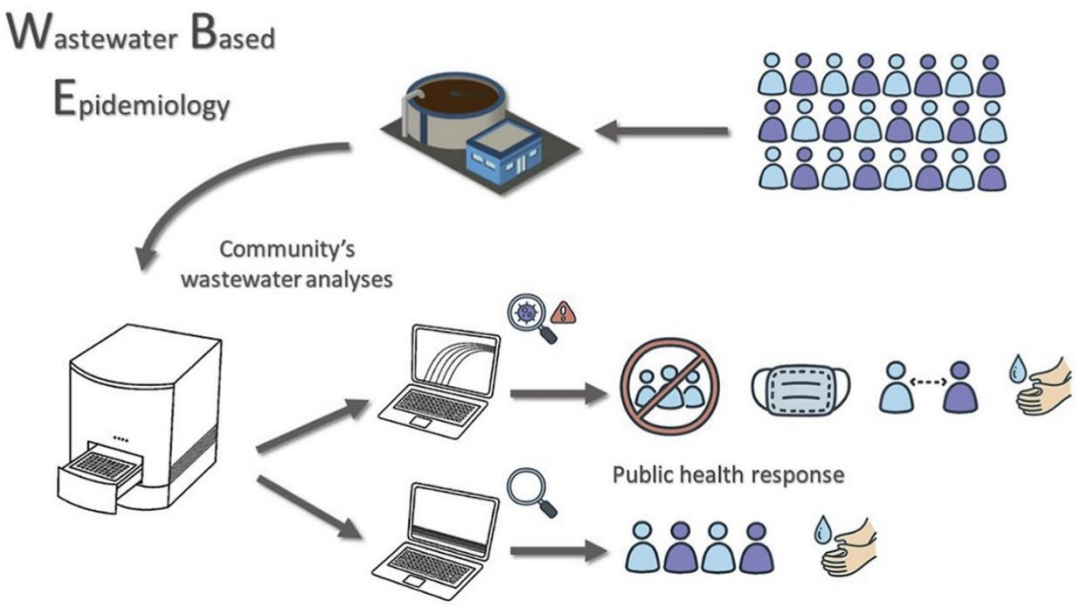
Chapter 4. Implementing a SARS-CoV-2 monitoring system in wastewater: Wastewater Based Epidemiology (WBE) as an early-warning tool for pandemics response management.

Chapter 4. Implementing a SARS-CoV-2 monitoring system in wastewater: Wastewater Based Epidemiology (WBE) as an early-warning tool for pandemics response management.

4.1. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area

This section is an adapted version of the following published research article:

Walter Randazzo, Pilar Truchado, Enric Cuevas-Ferrando, Pedro Simón, Ana Allende, Gloria Sánchez (2020). **SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area.** Water Research, 115942. DOI: https://doi.org/10.1016/j.watres.2020.115942



Highlights

• An adsorption-precipitation concentration method was validated using a porcine coronavirus.

• First detection of SARS-CoV-2 RNA in untreated wastewater in Spain.

• 11% secondary treated water samples tested positive for at least one SARS-CoV-2 RT-qPCR target.

• None of the tertiary effluent samples (n = 12) tested positive for SARS-CoV-2.

• SARS-CoV-2 RNA was detected in wastewater before the first COVID-19 cases were declared by local authorities.

1. Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 200,000 reported COVID-19 cases in Spain resulting in more than 20,800 deaths as of April 21, 2020. Faecal shedding of SARS-CoV-2 RNA from COVID-19 patients has extensively been reported. Therefore, we investigated the occurrence of SARS-CoV-2 RNA in six wastewater treatments plants (WWTPs) serving the major municipalities within the Region of Murcia (Spain), the area with the lowest COVID-19 prevalence within Iberian Peninsula. Firstly, an aluminum hydroxide adsorption-precipitation concentration method was validated using a porcine coronavirus (Porcine Epidemic Diarrhea Virus, PEDV) and mengovirus (MgV). The procedure resulted in average recoveries of 10 ± 3.5% and 10 ± 2.1% in influent water (n = 2) and 3.3 ± 1.6% and 6.2 ± 1.0% in effluent water (n = 2) samples for PEDV and MgV, respectively. Then, the method was used to monitor the occurrence of SARS-CoV-2 from March 12 to April 14, 2020 in influent, secondary and tertiary effluent water samples. By using the real-time RT-PCR (RT-qPCR) Diagnostic Panel validated by US CDC that targets three regions of the virus nucleocapsid (N) gene, we estimated quantification of SARS-CoV-2 RNA titers in untreated wastewater samples of 5.4 ± 0.2 log10 genomic copies/L on average. Two secondary water samples resulted positive (2 out of 18) and all tertiary water samples tested as negative (0 out 12). These environmental surveillance data were compared to declared COVID-19 cases at municipality level, revealing that members of the community were shedding SARS-CoV-2 RNA in their stool even before the first

cases were reported by local or national authorities in many of the cities where wastewaters have been sampled. The detection of SARS-CoV-2 in wastewater in early stages of the spread of COVID-19 highlights the relevance of this strategy as an early indicator of the infection within a specific population. At this point, this environmental surveillance could be implemented by municipalities right away as a tool, designed to help authorities to coordinate the exit strategy to gradually lift its coronavirus lockdown.

Keywords

Environmental surveillance; Influent water; Reclaimed water; Concentration protocol; RNA virus; Coronavirus.

2. Introduction

Coronaviruses (CoVs) are a family of viruses pathogenic for humans and animals associated to respiratory and gastro-intestinal infections. CoVs used to be considered as minor human pathogens as they were responsible of common cold or mild respiratory infections in immunocompetent people (Channappanavar and Perlman, 2017). Nonetheless, the emergence of novel and highly pathogenic zoonotic diseases caused by CoVs such as Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) and most recently SARS-CoV-2 brings to light questions to be addressed to guide public health response.

CoVs are mainly transmitted through respiratory droplets (Meselson, 2020). However, as for SARS and MERS, SARS-CoV-2 RNA has been detected in stool samples from patients exhibiting symptoms of COVID-19 and from asymptomatic carriers (He et al., 2020; Pan et al., 2020; Wölfel et al., 2020; Young et al., 2020; Zhang et al., 2020). The duration of viral shedding has been observed to vary among patients with means of 14–21 days (Y. Wu et al., 2020b; Xu et al., 2020). As well as the magnitude of shedding varies from 102 up to 108 RNA copies per gram (Lescure et al., 2020; Pan et al., 2020; Wölfel et al., 2020).

Infectious viruses deriving from fecal and urine specimen have reportedly been cultured in Vero E6 cells (Sun et al., 2020; W. Wang et al., 2020b). In addition, gastric, duodenal, and rectal epithelial cells are infected by SARS-CoV-2 and the release of the infectious virions to the gastrointestinal tract supports the possible fecal-oral transmission route (Xiao et al., 2020). Even

though the possibility of faecal-oral transmission has been hypothesized, the role of secretions in the spreading of the disease is not clarified yet (W. Wang et al., 2020b; Y. Wu et al., 2020b; Xu et al., 2020; Yeo et al., 2020).

Wastewater monitoring has been a successful strategy pursued to track chemical and biological markers of human activity including illicit drugs consumption, pharmaceuticals use/abuse, water pollution, and occurrence of antimicrobial resistance genes (Choi et al., 2018; de Oliveira et al., 2020; Lorenzo and Picó, 2019; Mercan et al., 2019). Viral diseases have been also surveilled by the detection of genetic material into wastewater as for enteric viruses (Hellmer et al., 2014; Prevost et al., 2015; Santiso-Bellón et al., 2020), re-emerging zoonotic hepatitis E virus (Cuevas-Ferrando et al., 2020; Miura et al., 2016), and poliovirus during the global eradication programme (Asghar et al., 2014).

Currently, various studies detected SARS-CoV-2 RNA in wastewater worldwide (Ahmed et al., 2020; La Rosa et al., 2020; Lodder and de Roda Husman, 2020; Medema et al., 2020; Rimoldi et al., 2020; F. Wu et al., 2020a; Wurtzer et al., 2020), and wastewater testing has been suggested as a non-invasive early-warning tool for monitoring the status and trend of COVID-19 infection and as an instrument for tuning public health response (Daughton, 2020; Mallapaty, 2020; Naddeo and Liu, 2020). Under current circumstance, this environmental surveillance could be implemented in wastewater treatment plants as a tool designed to help authorities to coordinate the exit strategy to gradually lift its coronavirus lockdown.

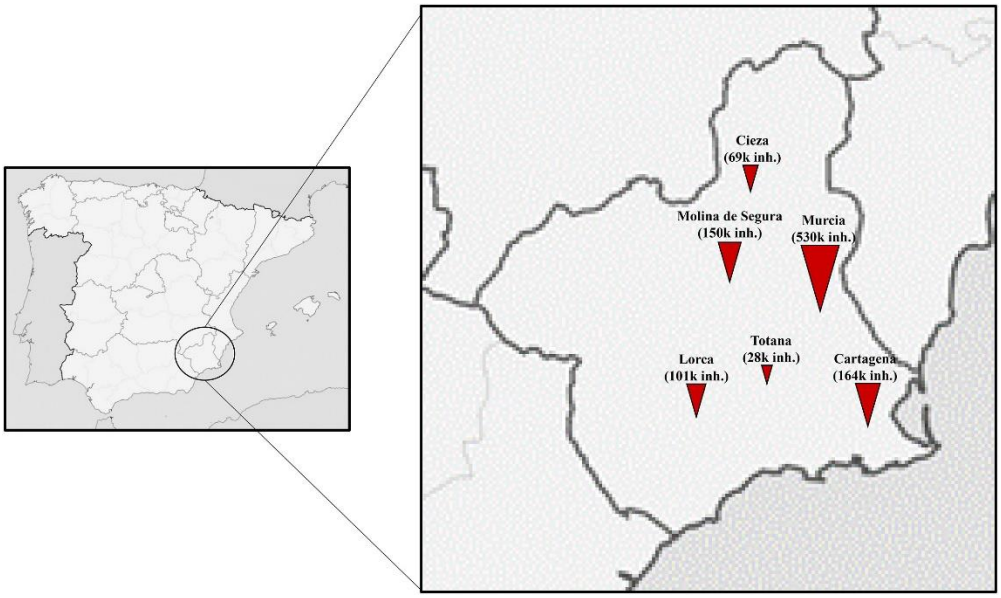
Here, we report the first detection of SARS-CoV-2 RNA in untreated wastewater samples in Spain collected from six different wastewater treatment plants (WWTPs) in Murcia, the lowest prevalence area in Iberian Peninsula. Additionally, the efficacy of the tertiary treatments implemented in the WWPTs against SARS-CoV-2 has been confirmed. The outcomes of the environmental surveillance reflect the epidemiological data in a low COVID-19 diagnosed cases setting, thus supporting the need of developing and implementing advanced models for wastewater-based epidemiology (WBE).

3. Materials and methods

a. Sampling sites and samples collection

Influent, secondary and tertiary treated effluent water samples were collected from six WWTPs located in the main cities of the Region of Murcia, Spain (Fig. 1). Technical data on WWTPs are provided in Table 1.

Fig. 1. Maps of the sampling location. Symbols represents WWTPs and are sized according to the number of equivalent inhabitants (inh.).



A total of 42 influent, and 18 secondary and 12 tertiary treated effluent water samples were collected from 12 March to 14 April 2020 and investigated for the occurrence of SARS-CoV-2 RNA. All samples were grabbed early in the morning (7–12am) by collecting 500–1000 mL of water in sterile HDPE plastic containers (Labbox Labware, Spain). Collected samples were transferred on ice to the laboratory, kept refrigerated at 4 °C and concentrated within 24 h. To this end, subsamples of 200 mL were processed as detailed hereafter.

Table 1. Data on population and operating characteristics of WWTPs in the area of study.

	Served Population[a]	Population Equivalent[b]	Capacity (m^3/y)[c]		Reclamation processes	Reuse
			Designed	Current[d]		
Murcia	370,893	530,499	36,500,000	36,952,999	Activated sludge (A2O process), Disinfection, NaClO	Public domain
Cartagena	175,870	163,969	12,775,000	8,625,103	Activated sludge, Disinfection	Irrigation
Molina de Segura	67,455	150,545	9,125,000	5,699,930	Activated sludge, Decantation, Coagulation, Flocculation, Sand filtration, Disinfection, UV, NaClO	Irrigation
Lorca	73,057	101,161	7,300,000	3,366,919	Activated sludge, Coagulation, Flocculation, Sand filtration, Disinfection, UV, NaClO	Irrigation
Cieza	33,744	69,502	3,650,000	2,338,673	Activated sludge (Extended aeration), Disinfection, Coagulation, Flocculation, Sand filtration, Disinfection, UV	Irrigation
Totana	29,113	28,289	2,190,000	1,440,463	Activated sludge (Extended aeration), Disinfection, UV	Irrigation

[a] Population connected to the wastewater treatment facility.

[b] Calculated based on the organic biodegradable load having a five-day biochemical oxygen demand (BOD5) of 60 g of oxygen per day.

[c] m3/y, water flow expressed as volume per year.

[d] Average water flow observed during the period of study.

b. Wastewater and effluent water concentration

The porcine epidemic diarrhea virus (PEDV) strain CV777, an enveloped virus member of the Coronaviridae family, genus Alphacoronavirus, and etiological agent of porcine epidemic diarrhea (PED), was preliminary used to evaluate the water concentration protocol together with the mengovirus (MgV) vMC0 (CECT 100000), a non-enveloped member of the Picornaviridae designated in the ISO 15216-1, 2017 standard method as process control.

The concentration method consisted in an aluminum hydroxide adsorption-precipitation protocol previously described for concentrating enteric viruses from wastewater and effluent water (AAVV, 2011; Cuevas-Ferrando et al., 2020; Randazzo et al., 2019). The validation was carried out by using biobanked influent (n = 2) and effluent water samples (n = 2) collected in July and October 2019 and stored at −80 °C until processed. In brief, 200 mL of sample was transferred in 250 mL PPCO centrifuge bottles (Thermo Fisher Scientific, Rochester, US) and artificially inoculated with PEDV and MgV. Then pH was adjusted to 6.0 and Al(OH)3 precipitate formed by adding 1 part 0.9N AlCl3 (Acros organics, Geel, Belgium) solution to 100 parts of sample. The pH was readjusted to 6.0 and sample mixed using an orbital shaker at 150 rpm for 15 min at room temperature. Then, viruses were concentrated by centrifugation at 1,700×g for 20 min in a RC-5B Sorvall centrifuge with SS-34 rotor. The pellet was resuspended in 10 mL of 3% beef extract pH 7.4, transferred in 50 mL PPCO centrifuge tubes and shaken for 10 min at 150 rpm. Concentrate was recovered by centrifugation at 1,900×g for 30 min in a RC-5B Sorvall centrifuge with F14S rotor and pellet resuspended in 1 mL of PBS. Alternatively, ST16R Sorvall centrifuge (Thermo Fisher Scientific, Rochester, US) with a TX-1000 ROTOR for 225 mL PPCO centrifuge bottles was used for the two concentration steps following the conditions previously indicated.

All wastewater and effluent water samples included in this study were processed as described and MgV (5 log10 PCR units, PCRU) was spiked as process control.

c. Viral extraction, detection and quantification

Viral RNA was extracted from concentrates using the NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions with some modifications. Briefly, 150 µL of the concentrated sample was mixed with 25 µL of Plant RNA Isolation Aid (Thermo Fisher Scientific, Vilnius, Lithuania) and 600 µL of lysis buffer from the NucleoSpin virus kit and subjected to pulse-vortexing for 1 min. Then, the homogenate was centrifuged for 5 min at 10,000×g to remove the debris. The supernatant was subsequently processed according to the manufacturer's instructions and eluted in 100 µL of RNAse free dH2O.

Viral RNA was detected by TaqMan real-time RT-PCR (RT-qPCR) on LightCycler 480 instrument (Roche Diagnostics, Germany) for all reactions. MgV RNA was quantified by using UltraSense One-Step kit (Invitrogen, SA, US) and the RT-qPCR assay as in ISO 15216–1:2017 (Costafreda et al., 2006; ISO 15216-1, 2017). Reaction mix (10 µL) consisted of 2.00 µL 5X Reaction Mix, 0.50 µL 20X Bovine Serum Albumin, 0.20 µL ROX Reference Dye, 0.50 µL Enzyme Mix, 0.90 pmol/µL Mengo 209 REV primer, 0.5 pmol/µL Mengo 110 FW primer and 0.25 pmol/µL Mengo FAM probe. The cycling parameters were as RT at 55 °C for 1 h, preheating at 95 °C for 5 min and 45 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min and 65 °C for 1 min. Undiluted and ten-fold diluted MgV RNA was tested to check for RT-qPCR inhibitors.

PEDV RNA was detected by using One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio, USA) and the TaqMan RT-qPCR assay described by (Zhou et al., 2017). Reaction mix (10 µL) consisted of 5.00 µL 2X One Step RT-PCR Buffer III, 0.20 µL PrimeScript RT enzyme Mix II, 0.20 µL TaKaRa Ex Taq HS, 0.20 µL ROX, 0.50 µL REV primer (10 µM), 0.50 µL FW primer (10 µM), 0.50 µL FAM labelled TaqMan probe (10 µM). The thermal cycling conditions were as RT at 45 °C for 15 min, preheating at 95 °C for 5 min and 45 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. SARS-CoV-2 RNA was detected by using One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) and the RT-qPCR diagnostic panel assays validated by the US Centers for Disease Control and Prevention (CDC, 2020). The first version of the kit with three sets of oligonucleotide primers and probes was used to target three different SARS-CoV-2 regions of the nucleocapsid (N) gene. The sets of primers and probe (2019-nCoV RUO Kit) as well as the positive control (2019-nCoV_N_Positive Control, 2 × 105 genome copies (gc)/µL) were provided by IDT (Integrated DNA Technologies, Leuven, Belgium). Reaction mix (10 µL) consisted of 5.00 µL 2X One Step RT-PCR Buffer III, 0.20 µL PrimeScript RT

enzyme Mix II, 0.20 µL TaKaRa Ex Taq HS, 0.75 µL for each sets of primers and probe. The thermal cycling conditions were as RT at 50 °C for 10 min, preheating at 95 °C for 3 min and 45 cycles of amplification at 95 °C for 3 s and 55 °C for 30 s. Each RNA was analyzed in duplicate and every RT-qPCR assay included negative (nuclease-free water) and positive controls.

Biobanked samples (n = 4) collected in October 2019, before the first COVID-19 case was documented, were used as relevant negative control to exclude false positive reactions.

SARS-CoV-2 RNA was quantified as gc by plotting the quantification cycles (Ct) to an external standard curve built with 10-fold serial dilution of a quantified plasmid control (IDT). Calibration curves for N1 (y = −3.3774x + 41.515, R2 = 0.95), N2 (y = −3.7752x + 43.951, R2 = 0.989), and N3 (y = -3.6006x + 43.142, R2 = 0.99) showed a linear dynamic range between 5 × 10 and 5 × 104. The limit of detection (LOD) resulted as 50 gc per reaction with Ct values of 37.05 ± 0.77, 38.12 ± 0.24 and 37.29 ± 1.48 for N1, N2 and N3, respectively. The theoretical limits of quantification of the overall method resulted as 4.45, 4.91, and 4.75 log10 gc/L for N1, N2 and N3, respectively.

MgV and PEDV RNA were quantified by plotting the Cts to external standard curves generated by serial end-point dilution method using RNA extracted from purified cell culture suspensions. Quantification were referred as PCRU. Standard curve showed a linear dynamic range between 10 and 107 and between 10 and 105 for MgV (y = −3.603x + 38.02, R2 = 0.99) and PEDV (y = −3.8281x+36.81, R2 = 0.98), respectively.

MgV recovery rates were calculated and used as quality assurance parameters according to ISO 15216–1:2017 (ISO 15216-1, 2017).

4. Results and discussion

a. Performance of the concentration methods

The aluminum hydroxide adsorption-precipitation method was tested by spiking influent and effluent samples with MgV and PEDV. On average, MgV was recovered at ranges of 11 ± 2.1% in influent and 6.2 ± 1.0% in effluent water. PEDV was recovered at ranges of 11 ± 3.5% in influent and 3.3 ± 1.6% in effluent water. Notably, not significant differences ($p > 0.05$) were detected between recovery rates in influent waters. This finding implies that a non-

enveloped virus may be used as process control for coronavirus detection in influent waters upon method validation. In contrast, significant differences ($p < 0.05$) were reported between PEDV and MgV recoveries in effluent waters.

These results are in line with the MgV recoveries reported for enteric viruses concentration in water samples by the same aluminum-based method (Cuevas-Ferrando et al., 2020; Randazzo et al., 2019) and higher than the 1% as the quality assurance parameter indicated for bottled water into ISO 15216–1:2017 (ISO 15216-1, 2017).

Similarly, MgV was successfully used as recovery control for hepatitis E virus concentration from influent and effluent water samples (5–13%) by applying a polyethylene glycol (PEG) precipitation method (Miura et al., 2016). A similar PEG-based protocol was recently used to recover SARS-CoV-2 from wastewater, although recovery control was not included in the study (F. Wu et al., 2020a).

Moreover, filtration through 10 kDa Centricon® Plus-70 centrifugal device successfully recovered SARS-CoV-2 in wastewater with recovery efficiencies of F-specific RNA phages of 73% (Medema et al., 2020). However, concentration by electropositive membrane should be further evaluated given a SARS-CoV recovery from wastewater of 1% (Wang et al., 2005).

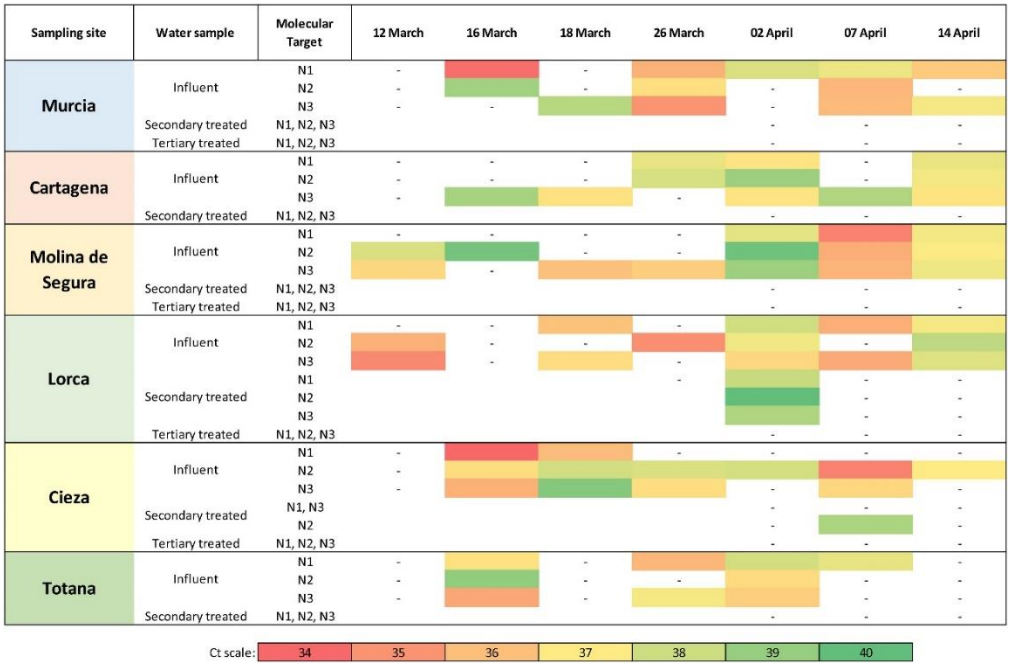
Rigorous limits of detection should be established by spiking SARS-CoV-2 cell-culture adapted strain or positive COVID-19 fecal samples in influent and effluent wastewater samples to be concentrated following the aluminum hydroxide adsorption-precipitation method. Nonetheless, the need of a BSL3 laboratory facility to handle SARS-CoV-2 represents the main limitation of this experiment.

b. SARS-CoV-2 titers in wastewater and effluent water

A total of 42 influent, and 18 secondary and 12 tertiary treated effluent water samples were collected from 12 March to 14 April 2020 and investigated for the occurrence of SARS-CoV-2 RNA. Samples were considered positive for Ct below 40 (as in Medema et al., 2020 and F. Wu et al., 2020a) and titrated by using the quantified plasmid control for each of the RT-qPCR targets. As expected, biobanked samples collected in October 2019, before the first COVID-19 case was documented, tested negative for all the three RT-qPCR assays thus excluding false positive reactions. The 83% (35 positive samples out of 42) influent samples and the 11% (2 out of 18) secondary treated water

samples were tested positive for at least one SARS-CoV-2 RT-qPCR target. None of the tertiary effluent samples (0 out of 12) tested positive for any of the SARS-CoV-2 RT-qPCR target (Fig. 2). A relevant number of influent water samples (12%) showed Ct ranging between 37 and 40, even though lower Ct of 34–37 were observed (29%).

Fig. 2. Mean amplification cycles of SARS-CoV-2 RNA in influent, secondary and tertiary effluent waters in monitored WWTPs within Murcia Region (Spain). Results are reported for each of the three regions of the virus nucleocapsid (N) gene according to the first version of the Real-Time RT-PCR Diagnostic Panel by US CDC. Abbreviations: , negative; white boxes, not tested.



In influent samples, a poor positive correlation among RT-qPCR assays was detected, being 0.5, 0.3, and 0.6 the resulting coefficients between N1 and N2, N1 and N3, N2 and N3, respectively. The total number of RT-qPCR determinations was 84 for each target. For N1, 23 results showed Ct below 37 out of 33 positive samples (70%), for N2 18 out of 31 (58%), and for N3 28 out of 36 (78%). In all samples, MgV recoveries were above 1% (11 ± 15%). MgV recovery for each sample and Ct values for each SARS-CoV-2 target are reported in Table S1 in Supplementary Material.

On average, SARS-CoV-2 RNA titers of 5.1 ± 0.3, 5.5 ± 0.2, and 5.5 ± 0.3 log10 gc/L were quantified in wastewater by using N1, N2 and N3 primer/probe

mixes, respectively. Titers of 4 and 5 to more than 6 log10 gc/L have been reported in Massachusetts and France, respectively (F. Wu et al., 2020a; Wurtzer et al., 2020).

A secondary effluent sample resulted positive for N2 and quantified as 5.4 log10 gc/L. An additional secondary effluent sample was positive for the three molecular targets and below the limit of quantification.

Detection of SARS-CoV-2 RNA in influent water has been reported worldwide (Ahmed et al., 2020; La Rosa et al., 2020; Lodder and de Roda Husman, 2020; Medema et al., 2020; Wu et al., 2020a), and only one study tested treated wastewater that resulted positive (Paris) (Wurtzer et al., 2020). We observed discrepancies among RT-qPCR N1, N2 and N3 assays for several water samples in agreement to a previous report (Medema et al., 2020). This could be due to the different analytical sensitivity among the assays as well as the detection of possible false positive samples by RT-qPCR N3 in low concentrated clinical samples (Jung et al., 2020; Vogels et al., 2020). The latter possibility has been solved by excluding the N3 primers/probe set from the US CDC 2019-nCoV RT-qPCR diagnostic panel in its last revision (March, 30) (CDC a, CDC b, n.d.). In addition, a partial inhibitory effect of the matrix is not to be completely excluded despite the controls included in the assays. A more sensitive estimation of SARS-CoV-2 loads in wastewater should be studied by digital RT-qPCR (dRT-qPCR). dRT-qPCR could be used to quantify samples with low viral loads as reported for norovirus in wastewater (Monteiro and Santos, 2017) and SARS-CoV-2 in clinical samples (Dong et al., 2020; Suo et al., 2020), even though it may not be the best practical and economically sustainable option for environmental surveillance (Abachin et al., 2018).

Even though the SARS-CoV-2 RNA detection in wastewater is functional for WBE purposes, the risk for human health associated to the water cycle is still under debate as infectivity of viral particles in sewage and faeces remain to be confirmed as well as its potential fecal-oral transmission. A pre-print report suggests that the risk of infection from wastewater and river is negligible given the failure in cell culturing SARS-CoV-2 from water samples despite the high number of RNA copies (Rimoldi et al., 2020).

In spite of the high concentration of viral RNA in specimen and the evidence of gastrointestinal infection (Xiao et al., 2020), infectious viruses from stools have been isolated in one study (W. Wang et al., 2020b) while another attempt resulted without success (Wölfel et al., 2020).

The potential transmission of SARS-CoV-2 via wastewater has not been proven (CDC a, CDC b, n.d.; WHO, 2020) and it seems unlikely given the poor stability of viable SARS-CoV-2 in wastewater (Rimoldi et al., 2020; J. Wang et al., 2020a) that resembles some previous studies made with representative coronaviruses (Gundy et al., 2008) and enveloped surrogates (Casanova and Weaver, 2015). As well, the elevated sensitivity of human pathogenic coronaviruses to environmental conditions (Chin et al., 2020; Darnell et al., 2004; Darnell and Taylor, 2006) and disinfectants (Chin et al., 2020; J. Wang et al., 2020a) suggests a poor risk of transmission via wastewater, even though formal risk analysis needs to be performed (Haas, 2020).

c. Environmental surveillance

Epidemiological data on COVID-19 in the Murcia Region have been retrieved from the publically available repository of the "Servicio de epidemiologia" of the "Consejería de Salud de la Región de Murcia" (available at http://www.murciasalud.es/principal.php) (Table 2) and plotted to the SARS-CoV-2 RNA mean loads as detected by three RT-qPCR assays (Fig. 3).

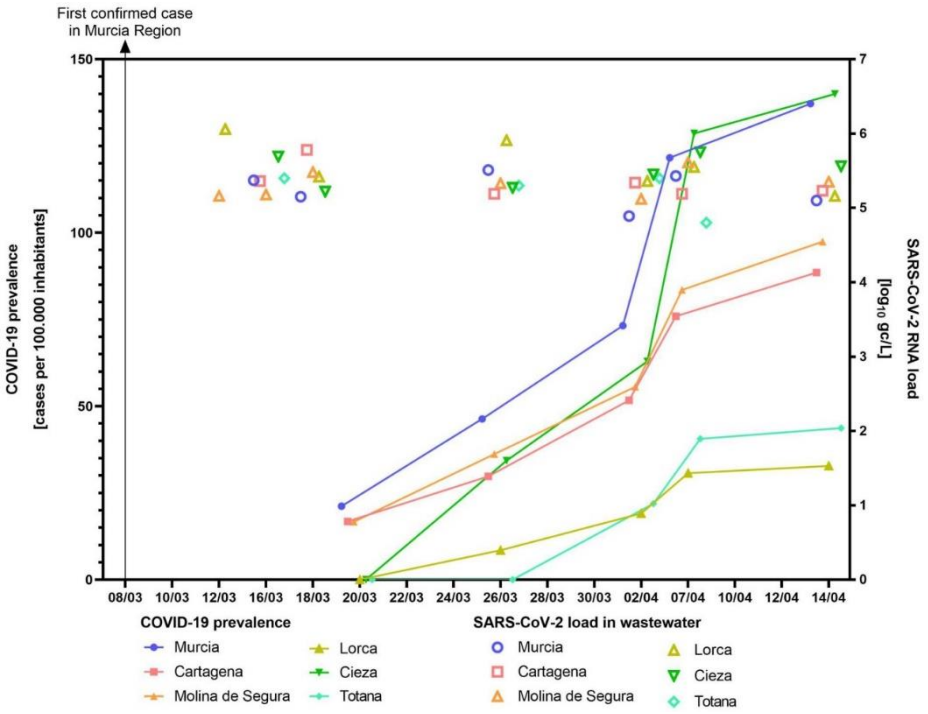
Table 2. Epidemiological data[a] summary of COVID-19 cases in the area of study.

	20/03/2020		25/03/2020		30/03/2020		08/04/2020		15/04/2020	
	Cases	Prevalence[b]	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence
Murcia	96	21.18	210	46.33	332	73.2	551	121.6	622	137.2
Cartagena	36	16.76	64	29.79	111	51.7	163	75.9	190	88.5
Molina de Segura	12	16.69	26	36.17	40	55.6	60	83.5	70	97.4
Lorca	–	–	8	8.47	18	19.1	29	30.7	31	32.8
Cieza	–	–	12	34.30	22	62.9	45	128.6	49	140.0
Totana	–	–	–	–	7	21.9	13	40.6	14	43.7

[a] Data retrieved from the public repository of the "Servicio de epidemiologia" of the "Consejería de Salud de la Región de Murcia" (available at http://www.murciasalud.es/principal.php).

[b] Prevalence, percentage of diagnosed cases per 100.000 inhabitants.

Fig. 3. Epidemiological surveillance of COVID-19 by wastewater SARS-CoV-2 RT-qPCR in six municipalities.



In general, RT-qPCR amplification signals have been detected in wastewaters when cases were diagnosed within the municipality. Positive wastewater samples have been detected with at least two out of three RT-qPCR assays in low prevalence municipalities as in Murcia (96 cases, 21.18 cases per 100,000 inhabitants), Cartagena (36 cases, 16.76) and Molina de Segura (12 cases, 16.69). Of note, positive wastewater samples were detected 12–16 days before COVID-19 cases were declared in Lorca, Cieza and Totana municipalities.

A similar study conducted in Paris (France) demonstrated the detection of viral genome before the exponential phase of the epidemic (Wurtzer et al., 2020). However, our results indicate that SARS-CoV-2 can be detected weeks before the first confirmed case. The early detection of SARS-CoV-2 RNA in wastewater could have alerted about the imminent danger, giving a valuate time to the managers to coordinate and implement actions to slow the spread of the disease. Therefore, our outcomes support that WBE could be used as an early warning tool to monitor the status of COVID-19 infection within a community.

On the other hand, we believe that this environmental surveillance could be used as an instrument to drive the right decisions to reduce the risk of lifting restrictions too early. For instance, a key question is how to reduce the risk of a "second wave" and/or recurring local outbreaks. Massive population tests are the first choice, but in their absence, wastewater monitorization of SARS-CoV-2 RNA can give a reliable picture of the current situation. Our wastewater data do not quantitatively resemble the prevalence of COVID-19 confirmed cases. To this end, a quantitative model that includes and corrects all the variables affecting these wastewater surveillance data would be useful for a better interpretation. For instance, not all COVID-19 positive patients excrete SARS-CoV-2 RNA in faeces, and when it occurs, the titers and the duration of shedding vary among individuals and across time (He et al., 2020; Pan et al., 2020; Wölfel et al., 2020; Xu et al., 2020). On the other hand, the real number of positive cases within the Murcia Region remains unknown because of the large number of mild or asymptomatic carriers that have not been included in epidemiological statistics.

These aspects together with environmental variables (e.g., rainfall events, temperature, hydraulic retention time in sewers) increase the uncertainties linked to the correlation between SARS-CoV-2 RNA detection in wastewater samples and the prevalence of COVID-19 that could be explored by using complex models.

5. Conclusion

Overall, wastewater surveillance and WBE may represent a complementary approach to estimate the presence and even the prevalence of COVID-19 in communities. This represents an effective tool that needs to be further explored in order to direct public health response, especially in cases of limited capacity for clinical testing.

6. Supplementary material

Table S1. Analytical details of concentration and RT-qPCR assays per each water sample.

Sampling site	Sample	Sampling date (dd/mm)	Concentrated volume (mL)	MgV recovery (%)	N1 [Ct ± SD]	N2 [Ct ± SD]	N3 [Ct ± SD]
Cartagena	Influent	12-Mar	2.50	12.37	-	-	-
Cieza	Influent	12-Mar	3.00	25.38	-	-	-
Lorca	Influent	12-Mar	5.50	21.43	-	-	35.20 ± 0.09
Molina	Influent	12-Mar	1.50	8.29	-	-	36.45
Murcia	Influent	12-Mar	3.50	4.45	-	-	-
Totana	Influent	12-Mar	3.50	47.45	-	-	-
Cartagena	Influent	16-Mar	3.50	16.50	-	-	37.76
Cieza	Influent	16-Mar	3.50	32.99	34.68 ± 0.22	36.53 ± 0.63	35.83 ± 0.86
Lorca	Influent	16-Mar	5.50	61.64	-	-	-
Molina	Influent	16-Mar	3.00	73.13	-	38.31	-
Murcia	Influent	16-Mar	2.50	4.50	34.77	37.82	-
Totana	Influent	16-Mar	3.50	17.20	36.61	38.00	35.68
Cartagena	Influent	18-Mar	7.00	17.30	-	-	36.61
Cieza	Influent	18-Mar	3.00	7.46	36.01 ± 0.01	37.27 ± 0.70	38.14
Lorca	Influent	18-Mar	2.50	57.76	36.12 ± 0.35	-	36.52 ± 0.29
Molina	Influent	18-Mar	2.50	25.93	-	-	36.09
Murcia	Influent	18-Mar	3.00	10.40	-	-	37.58
Totana	Influent	18-Mar	3.50	19.90	-	-	-
Cartagena	Influent	26-Mar	3.00	3.54	37.02	37.21	-
Cieza	Influent	26-Mar	3.00	7.04	-	37.16	36.54
Lorca	Influent	26-Mar	3.00	1.35	-	35.30	-
Molina	Influent	26-Mar	2.00	2.68	-	-	36.29
Murcia	Influent	26-Mar	3.00	3.02	35.85 ± 0.01	36.56 ± 0.04	35.37
Totana	Influent	26-Mar	3.00	16.02	35.92	-	36.85
Cartagena	Influent	2-Apr	3.75	2.88	36.64 ± 0.89	37.87	36.64 ± 0.20
Cartagena	Secondary treated	2-Apr	3.60	7.97	-	-	-
Cieza	Influent	2-Apr	3.00	1.75	-	37.25	-
Cieza	Secondary treated	2-Apr	3.00	1.95	-	-	-
Cieza	Tertiary treated	2-Apr	3.50	1.19	-	-	-
Lorca	Influent	2-Apr	4.00	7.79	37.28 ± 0.14	36.91	36.45 ± 1.05
Lorca	Secondary treated	2-Apr	3.75	7.79	37.39 ± 0.39	38.54	37.66 ± 0.13
Lorca	Tertiary treated	2-Apr	4.00	1.08	-	-	-
Molina	Influent	2-Apr	3.50	3.32	37.05 ± 0.37	38.39 ± 0.53	37.86 ± 1.53
Molina	Secondary treated	2-Apr	3.50	2.98	-	-	-
Molina	Tertiary treated	2-Apr	3.50	1.41	-	-	-
Murcia	Influent	2-Apr	3.00	2.34	37.16	-	-
Murcia	Secondary treated	2-Apr	3.75	11.43	-	-	-
Murcia	Tertiary treated	2-Apr	3.00	1.90	-	-	-
Totana	Influent	2-Apr	3.50	41.62	37.25	36.48	36.29

Totana	Influent	2-Apr	3.50	41.62	-	-	-
Totana	Secondary treated	2-Apr	3.75	1.08	-	-	-
Cartagena	Influent	7-Apr	3.50	1.28	-	-	37.65
Cartagena	Secondary treated	7-Apr	2.20	4.61	-	-	-
Cieza	Influent	7-Apr	3.25	1.98	-	35.10	36.45
Cieza	Secondary treated	7-Apr	3.50	11.80	-	37.70	-
Cieza	Tertiary treated	7-Apr	3.50	20.41	-	-	
Lorca	Influent	7-Apr	3.50	1.14	35.80	-	35.71
Lorca	Secondary treated	7-Apr	2.80	5.85	-	-	-
Lorca	Tertiary treated	7-Apr	2.50	6.57	-	-	-
Molina	Influent	7-Apr	3.00	3.22	35.09 ± 0.62	35.78	35.87 ± 0.41
Molina	Secondary treated	7-Apr	2.50	3.42	-	-	-
Molina	Tertiary treated	7-Apr	2.50	1.69	-	-	-
Murcia	Influent	7-Apr	2.70	1.01	36.97	35.93	35.97 ± 0.52
Murcia	Secondary treated	7-Apr	3.15	8.34	-	-	-
Murcia	Tertiary treated	7-Apr	3.50	10.99	-	40.00	-
Totana	Influent	7-Apr	2.25	6.63	37.04	-	-
Totana	Secondary treated	7-Apr	3.00	7.59	-	-	-
Cartagena	Influent	14-Apr	2.75	1.67	36.99 ± 0.00	36.89 ± 0.41	36.65
Cartagena	Secondary treated	14-Apr	2.75	4.85	-	-	-
Cieza	Influent	14-Apr	3.00	15.10	-	36.75 ± 0.06	-
Cieza	Secondary treated	14-Apr	3.00	5.30	-	-	-
Cieza	Tertiary treated	14-Apr	4.00	4.71	-	-	-
Lorca	Influent	14-Apr	2.75	2.82	36.85 ± 0.23	37.50 ± 0.71	37.13
Lorca	Secondary treated	14-Apr	3.00	4.55	-	-	-
Lorca	Tertiary treated	14-Apr	2.50	3.20	-	-	-
Molina	Influent	14-Apr	3.50	3.98	36.90 ± 0.49	36.80 ± 0.18	36.94 ± 0.42
Molina	Secondary treated	14-Apr	4.50	5.51	-	-	-
Molina	Tertiary treated	14-Apr	3.50	4.14	-	-	-
Murcia	Influent	14-Apr	2.25	1.00	36.22 ± 0.72	-	36.87
Murcia	Secondary treated	14-Apr	2.75	2.41	-	-	-
Murcia	Tertiary treated	14-Apr	2.50	2.80	-	-	-
Totana	Influent	14-Apr	2.00	12.42	-	-	-
Totana	Secondary treated	14-Apr	3.00	13.28	-	-	-

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4.2. Spatial and temporal distribution of SARS-CoV-2 diversity circulating in wastewater

This section is an adapted version of the following published research article:

AlbaPérez-Cataluña, Álvaro Chiner-Oms, Enric Cuevas-Ferrando, Azahara Díaz-Reolid, Irene Falcó, Walter Randazzo, Inés Girón-Guzmán, Ana Allende, María A. Bracho, Iñaki Comas, Gloria Sánchez (2022). **Spatial and temporal distribution of SARS-CoV-2 diversity circulating in wastewater.** Water Research, 118007

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Highlights

• Spatial and temporal analysis of SARS-CoV-2 sequences from Spanish wastewaters.

• Presence of amino acid substitutions in the spike protein not previously described in Spain.

• Detection of amino acid substitutions in the spike protein even months before their detection in clinical samples.

• SARS-CoV-2 genomics in wastewater as a complementary tool for WBE.

1. Abstract

Wastewater-based epidemiology (WBE) has proven to be an effective tool for epidemiological surveillance of SARS-CoV-2 during the current COVID-19 pandemic. Furthermore, combining WBE together with high-throughput sequencing techniques can be useful for the analysis of SARS-CoV-2 viral diversity present in a given sample. The present study focuses on the genomic analysis of SARS-CoV-2 in 76 sewage samples collected during the three epidemiological waves that occurred in Spain from 14 wastewater treatment plants distributed throughout the country. The results obtained demonstrate that the metagenomic analysis of SARS-CoV-2 in wastewater allows the detection of mutations that define the B.1.1.7 lineage and the ability of the technique to anticipate the detection of certain mutations before they are detected in clinical samples. The study proves the usefulness of sewage sequencing to track Variants of Concern that can complement clinical testing to help in decision-making and in the analysis of the evolution of the pandemic.

Keywords

SARS-CoV-2; Wastewater; Genome sequencing; Spike mutations; Variants of concern; Variants of interest.

1. Introduction

The family Coronaviridae is a family of enveloped RNA viruses generally associated with mild respiratory and gastrointestinal infections (Shang et al., 2020). Nevertheless, in recent decades new and highly pathogenic zoonotic coronavirus (CoVs) have emerged such as the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003), the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Zaki et al., 2012) and, most recently, SARS-CoV-2 (Zhu et al., 2020) which has resulted in the Coronavirus Disease 2019 (COVID-19) pandemic. Transmission of SARS-CoV-2 occurs mainly through aerosols or respiratory secretions (Chan et al., 2020) but it has also been found that, due to its replication capacity in the gastrointestinal tract (Xiao et al., 2020), it is excreted in feces and urine, as was previously reported for its counterparts SARS-CoV and MERS-CoV. For this reason, it has been possible to detect the genetic material of the virus in the feces of not only symptomatic, but also asymptomatic people (Polo et al., 2020). These findings have led to the use of wastewater monitoring for SARS-CoV-2. As for other pathogens, the use of Wastewater-Based Epidemiology (WBE) has proven to be a very useful tool as an early detection warning system, allowing trend-estimations as well as establishing correlations between different epidemiological indicators (Bivins et al., 2020; Medema et al., 2020; Randazzo et al., 2020b, 2020a). One of the reasons for the success of WBE is that wastewater samples are a non-invasive and inexpensive source of information to investigate the spread of SARS-CoV-2 within a community. Moreover, it provides real-time information on the circulating lineages of SARS-CoV-2, which is essential for the development of vaccines and drugs. This is particularly relevant in view of the current situation where the world's population is being vaccinated against SARS-CoV-2 and where, due to the appearance of emerging lineages, vaccine effectiveness might be compromised (Zhou et al., 2021).

Massive parallel sequencing techniques applied to sewage samples allow us to analyze a large number of SARS-CoV-2 genomes, including those present in symptomatic and asymptomatic persons. Through the analysis of sequences, it is possible to detect low-frequency variants (LFV) and to infer which lineages are circulating at a certain time and place (Bar-Or et al., 2021; Crits-Christoph et al., 2021; Dharmadhikari et al., 2021; Herold et al., 2021; Izquierdo-Lara et al., 2021; La Rosa et al., 2021; Nemudryi et al., 2020; Rios et al., 2021). Additionally, genomic analyses may allow to detect the entry of described lineages or Variants of Concern (VOCs) into populations, as well as the appearance of emerging lineages, to characterize new outbreaks, and to aid

in viral strains tracking (Bar-Or et al., 2021; Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021; La Rosa et al., 2021; Nemudryi et al., 2020; Rios et al., 2021). These studies also evidenced that improvement on sequencing techniques must be performed in order to reduce error rates, as the case of Nanopore sequencing (Nemudryi et al., 2020). Despite these limitations, the published works showed that genomic analysis of SARS-CoV-2 in wastewater should be used as a complementary tool in epidemiological surveillance. This aspect has grown in significance because during the spread of SARS-CoV-2, different mutations (i.e. D614G, Δ69/70, N501Y, E484K, K417N) present in VOCs (i.e. B.1.1.7, B.1.351, B.1.617.2, and B.1.1.28.1) have emerged (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/), which have a considerable impact on transmissibility, infection severity (Singh et al., 2021), or immunity. These characteristics, if they occur, can aggravate the epidemiological situation in certain areas, so the detection of new lineages and the appearance of VOCs in any specific population is crucial to overcome the current pandemic situation and control the spread of the virus. Variants of interest (VOI) (for example, B.1.427) have also been defined which are currently under investigation and must be monitored to ensure a prompt response should they pose a greater threat to the population. The usefulness of these techniques is evident from the fact that the European Commission published, on March 17, 2021, recommendations for the establishment of SARS-CoV-2 surveillance in wastewater, highlighting the importance of SARS-CoV-2 sequencing in wastewater as a tool for the detection of VOC and VOI (https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=uriserv:OJ.L_.2021.098.01.0003.01.ENG). The aim of this study was to analyze SARS-CoV-2 genomes in wastewater through high-throughput sequencing in order to monitor the emergence of mutations, lineages or the detection of signature mutations of VOCs and VOIs.

2. Materials and methods

a. Sample processing

In the framework of SARS-CoV-2 wastewater monitoring in Spain, grab samples were collected from 14 treatment plants located in different parts of the Spanish territory, with equivalent inhabitant values ranging from 60,600 to 1900,800. The samples taken between April 2020 and January 2021 encompass the three waves that have affected the country. The first wave

occurred between March and April of 2020, the second wave in November 2020, and the third wave between January and February 2021. For each sample, 200 mL of wastewater samples were artificially inoculated with porcine epidemic diarrhea virus (PEDV) as process control with a final concentration of 4.5 log (PCRU/L), and concentrated following an aluminum-based adsorption precipitation method (AAVV, 2018; Pérez-Cataluña et al., 2021; Randazzo et al., 2020b). Then, 200 mL of wastewater was adjusted to pH 6.0. Precipitation by Al(OH)3 was carried out by mixing 1 part of 0.9 N AlCl3 per 100 parts of sample. Next, the solution was mixed at 150 rpm for 15 min, centrifuged at 1700 × g for 20 min, and the resulting pellet was resuspended in 10 mL of 3% beef extract (pH 7.4) then stirred at 150 rpm for 10 min at room temperature (RT). Finally, the suspension was centrifuged at 1900 × g for 30 min and the pellet resuspended in 1 mL of phosphate buffered saline solution (PBS, pH 7.4). After this, concentrated samples were stored at −80 °C until analysis.

b. Nucleic acid extraction and SARS-CoV-2 RT-qPCR quantification

Nucleic acid extraction from wastewater concentrates was performed using an automated method with the Maxwell RSC Pure Food GMO and authentication kit (Promega) with slight modifications (Pérez-Cataluña et al., 2021). Firstly, 300 µL of concentrated samples were mixed with 400 µL of cetyltrimethyl ammonium bromide (CTAB) and 40 µL of proteinase K solution. The mixed sample was incubated at 60 °C for 10 min and centrifuged for 10 min at 16,000 × g. Next, the resulting supernatant was transferred to the loading cartridge and 300 µL of lysis buffer added. The cartridge was then loaded in the Maxwell® RSC Instrument (Promega) using the "Maxwell RSC Viral total Nucleic Acid" running program for the nucleic acid extraction. The obtained RNA was eluted in 100 µL nuclease-free water. Negative controls were included by using nuclease-free water instead of concentrated sample.

SARS-CoV-2 nucleic acid detection was performed by RT-qPCR using One Step PrimeScriptTM RT-PCR Kit (Perfect Real Time) (Takara Bio, USA) targeting a genomic region of the nucleocapsid gene (N1 region) using primers, probes and conditions previously described (CDC, 2020). The complete genomic RNA of SARS-CoV-2 (ATCC VR-1986D) and nuclease free water were used as positive and negative controls, respectively.

c. SARS-CoV-2 genome sequencing and analysis

Samples with RT-qPCR cycle threshold (Ct) values below 36 were selected for sequencing analysis. Genomic sequencing of SARS-CoV-2 present in selected wastewater samples was carried out following ARTIC protocol version 3 for retrotranscription and amplification by multiplex PCR (Quick, 2020; https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye). Sequencing libraries were built using the Nextera Flex kit (Illumina) and sequenced on Illumina MiSeq platform by paired-end reads (2 × 200).

Raw reads were cleaned for adaptors and low quality nucleotides by using cutadapt software (Martin, 2011) and reformat.sh from bbmap (sourceforge.net/projects/bbmap/), respectively. Nucleotides with Phred score lower than 30 were discarded. Clean reads were aligned to the genome of SARS-CoV-2 isolate Wuhan-Hu-1 (MN908947.3) using the Burrows-Wheeler Aligner v0.7.17-r1188 with default parameters (Li and Durbin, 2009) and indexed by samtools (Li et al., 2009). For the analysis of genomic coverage for each sample, only nucleotides with at least 20X depth were taken into account. Nucleotide substitutions and deletions regarding SARS-CoV-2 isolate Wuhan-Hu-1 genome (MN908947.3) were detected with the aligned reads using mpileup from samtools (Li, 2011) and the command variants of ivar software (Grubaugh et al., 2019). For the assumption of one nucleotide polymorphism, at least a 50X depth of the alternative nucleotide and quality score higher than 30 were used as cutoff. Alignments were manually curated to avoid nucleotide substitutions that corresponded to incorrectly trimmed adaptors (Nemudryi et al., 2020). Information about SARS-CoV-2 mutation distribution worldwide was obtained from outbreak.info (Mullen et al., 2020).

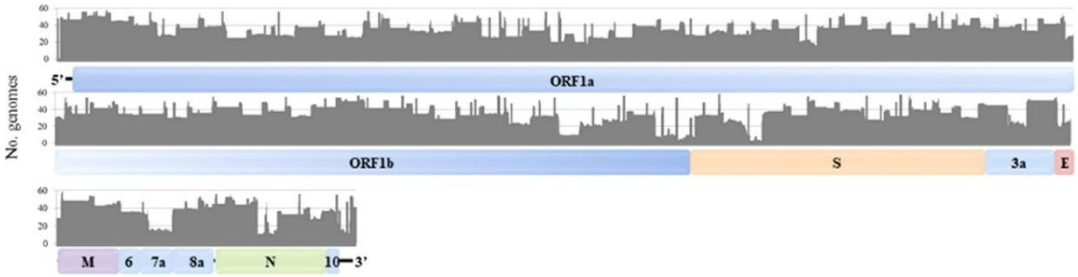
3. Results and discussion

a. SARS-CoV-2 quantification and genome coverage

A total of 76 sewage samples positive for SARS-CoV-2 by RT-qPCR (Ct < 36) collected throughout the three epidemiological waves were sequenced during this study (Supplementary Fig. S1). Samples were grouped in three regions: north (2 WWTPs, n = 8), center (7 WWTPs, n = 39), and south (5 WWTPs, n = 29). Results showed Ct values of SARS-CoV-2 target N1 ranged from 26.59 to 34.75 (Table S1). From the 76 sequenced samples, 11 (14.5%) showed percentages of 20X coverage values higher than 90% (Figs. 1 and S2), and a

mean genomic percentage of coverage of 50.1 ± 30.6%. In order to study the potential correlation between viral loads and genome coverage in wastewater samples, correlation analyses between RT-qPCR outputs (genome copies (gc) per liter) versus genome coverage were carried out for each individual sample. No correlations were found for the analyzed target, as occurred in the study of Izquierdo-Lara et al. (2021) for Illumina reads. Fig. 1 shows the number of samples that covered a certain nucleotide position with depths higher than 20X among the samples with genomic coverage greater than 20% (n = 59). However, some areas were only covered by less than 20 samples (< 33.90%), as is the case of two regions at the end of ORF 1b (nucleotides 21,456–21,467 and several regions between nucleotides 21,162 and 21,600), the regions of the S gene from nucleotides 22,303 to 22,342 and from 22,364 to 22,523, most of the ORF7a (nucleotides 27,529–27,790), and the central region of the N gene (nucleotides 28,773–28,853 and 28,901–28,993) (Fig. 1).

Fig. 1. Representation of the number of samples that covered each nucleotide of the SARS-CoV-2 isolate Wuhan-Hu-1 genome (MN908947.3) with coverage values higher than 20X.



b. Overview of detected nucleotide substitutions and deletions

Sequence analysis showed a total of 627 nucleotide substitutions and 20 deletions (Table 1) in comparison with the reference genome of SARS-CoV-2 isolate Wuhan-Hu-1 (MN908947.3). Among detected nucleotide substitutions: 248 were found in ORF1a polyprotein; 171 in ORF1b; 71 in the spike glycoprotein; 32 in ORF3a; 29 in the membrane glycoprotein; 20 in ORF8; 39 in the nucleocapsid gene; 31 in intergenic regions; 3 in ORF10; and one in the envelope protein, ORF6, and ORF7a each (Table 1, Fig. 2). Regarding deletions, a total of 8 deletions were found in samples of the first and second waves: 5 of them in the ORF1a region (Δ21–23, Δ82–84, Δ84–86, Δ141–143,

and Δ682); one in the spike glycoprotein (Δ385); and two in the ORF3a (Δ80 and Δ11–20). Two of these deletions were found in two samples: ORF1a:Δ141–143 in samples N1–18–2020 and C3–40–2020; and ORF3a:Δ11–20 in samples N1–18–2020 and N2–37–2020. None of these deletions were previously reported according to GISAID database after searching each deletion in the outbreak.info database. In samples from the third wave, a total of 12 deletions were detected: three in ORF1a (Δ141–143 found in two samples of first and second waves and in three samples of different locations from the third wave (C4–2–2021, C4–3–2021, and C5–4–2021), Δ2037, and Δ3675–3677 found in three samples (C2–1–2021, C4–1–2021, and C5–2–2021))), and nucleocapsid gene (Δ266–273, Δ352–356, and Δ392); and two in ORF1b (Δ176 and Δ1111), spike glycoprotein (Δ69/70 and Δ144, both found in samples S1–2–2021 and C4–2–2021), and ORF3a (Δ234 and Δ259/262). The percentage of non-synonymous substitutions ranged from 45.5% (in membrane glycoprotein) to 100% (in ORF7a and ORF10) in samples from the first and second waves, while in samples from the third wave this percentages ranged from 28.0% (in membrane glycoprotein) to 82.4% (in ORF8) (Table 1).

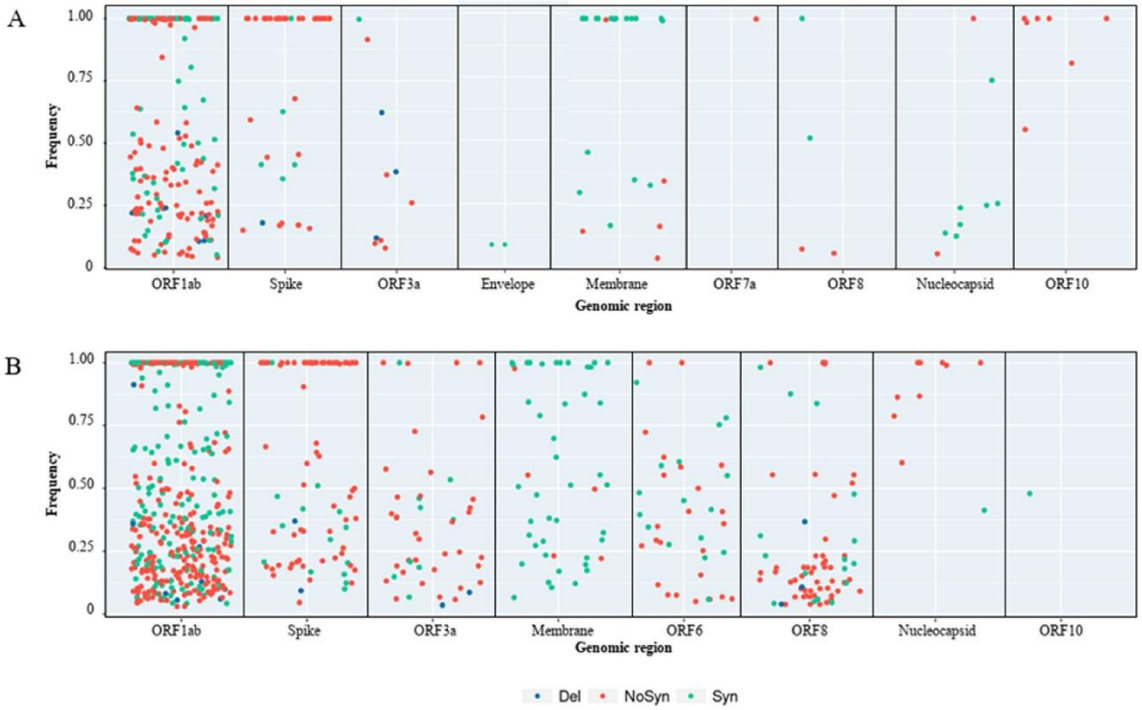
Table 1. Overview of the nucleotide substitutions and deletions detected in SARS-CoV-2 genomes from wastewater samples (n = 76) as compared to the SARS-CoV-2 isolate Wuhan-Hu-1 reference genome (MN908947.3). NA, not applicable.

Wave		5′ UTR	ORF1a polyprotein	ORF1b polyprotein	Surface glycoprotein	ORF3a protein	Intergenic region	Envelope protein	Membrane glycoprotein	ORF6 protein	ORF7a protein	Intergenic region	ORF8 protein	Nucleocapsid phosphoprotein	ORF10 protein
1st and 2nd [(a)]	Synonymous	NA	32	15	6	1	NA	1	6	0	0	NA	1	6	0
	Non synonymous	NA	52	43	14	4	NA	0	5	0	1	NA	2	2	1
	Deletions	NA	5	0	1	2	NA	0	0	0	0	NA	0	0	0
	Total	10	89	58	21	7	1	1	11	0	1	1	3	8	1
3rd [(b)]	Synonymous	NA	53	33	12	7	NA	0	13	1	0	NA	3	11	1
	Non synonymous	NA	103	78	36	16	NA	0	5	0	0	NA	14	17	1
	Deletions	NA	3	2	2	2	NA	0	0	0	0	NA	0	3	0
	Total	16	159	113	50	25	2	0	18	1	0	1	17	31	2
	Total	26	248	171	71	32	3	1	29	1	1	2	20	39	3

(a) 1st wave: Samples from week 15 to 19 of 2020, n = 5; 2nd wave: Samples from week 34 to 42 of 2020, n = 35.

(b) 3rd wave: Samples from week 50 of 2020 to week 8 of 2021, n = 36.

Fig. 2. Frequency of the different nucleotide substitutions and deletions obtained for samples of each epidemiological wave grouped by genomic region. A, first and second waves; B, third wave.



Some of these nucleotide substitutions and deletions were present along with the homologous nucleotide of SARS-CoV-2 isolate Wuhan-Hu-1 genome (Fig. 2) evidencing the presence of multiple genomes in wastewaters. Mean values of the frequency of these nucleotide polymorphisms were 70±35% for synonymous substitutions, 56±38% for non-synonymous substitutions and 27±18% for deletions in samples from the first and second waves (Fig. 2A). These values in the third wave samples were 53±34%, 43±34%, and 19±22% for synonymous substitutions, non-synonymous substitutions, and deletions, respectively (Fig. 2B).

c. Nucleotide substitutions and deletions in the spike glycoprotein gene

Table 2 shows the non-synonymous nucleotide substitutions (n = 49) and deletions (n = 3) found in the spike glycoprotein gene. Among these polymorphisms, 18 of them were not previously described in genomes obtained from Spanish sequences, according to the database available at https://outbreak.info/ (Mullen et al., 2020). However, two of these nucleotide substitutions (amino acid substitutions G404V and G648V) have been found at low frequencies among the reads obtained in the sequencing of Spanish genomes from clinical samples. These results evidence the ability of this technique to detect mutations that are in low percentage in the viral population and from different lineages.

Interestingly, some of these amino acid substitutions in the spike protein were found in sewage at the same time or even weeks or months before their appearance in genomes from clinical samples. For example, among nucleotide substitutions that have been detected in Spain in clinical samples, two spike mutations (G639S and V642G) were found in waters around the same time that they appeared in clinical genomes, while spike mutations A648V was found in waters 6 weeks before, and mutations S884F, G404V, and A372T were found in waters between 4 and 5 months before their detection in clinical genomes. It should be noted that, in the case of G404V, its first detection occurred at very low percentages of sequencing reads in some clinically obtained genomes (n = 2), and its appearance at higher frequencies in one clinical genome was 5 months later. Additionally, for these genomic mutations, the number of clinical cases was very low, ranging from 1 to 6 cases. Moreover, mutations A893T, L1152S, and N1173K had not been detected in Spanish clinical genomes but their detection in other countries occurred after detection in Spanish wastewater, more specifically 3, 4, and 8 months before, respectively. These results, along with those obtained by other authors who found genomes and single nucleotide polymorphisms (SNPs) widely described in the clinical samples (Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021), show that high-throughput sequencing of SARS-CoV-2 in wastewater is a very useful complementary tool for studies and decision-making related to the epidemiology of the virus.

Table 2. Non-synonymous nucleotide substitutions and deletions detected in the spike glycoprotein region as compared to the SARS-CoV-2 isolate Wuhan-Hu-1 reference genome (MN908947.3). Reference (ref) and alternative (alt) depth relate to the percentage of the total depth that corresponded to the nucleotide present in the reference genome MN908947.3 and the alternative nucleotide, respectively. Mixed samples related to the number of samples showing nucleotides according to reference and alternative sequence. Light gray, region of the receptor binding domain; dark gray, region of the S1/S2 cleavage region. NA, not applicable.

23604	1	1	3	33%	67%	P681H	Yes (14,717)	P.3, Av.1, B.1.1.338, Xa, B.1.474, B.1.1.519, B.1.1.318, B.1.1.7, B.1.575.1, B.1.530, B.1.1.336, B.1.1.351, B.1.620, B.1.621, B.1.575, B.1.1.207, B.1.623, B.1.1.522, B.1.469
23612	1	1	3	88%	12%	A684S	Yes (4)	B.1.160.32
23628	1	1	3	81%	19%	S689N	No	< 0.5%
23709	1	1	3	37%	63%	T716I	Yes (14,502)	B.1.214.3, Xa, B.1.214.4, B.1.1.7, B.1.575.1, B.1.214.2, B.1.575
23851	1	1	3	51%	49%	L763F	No	B.1.160.27
24213	1	1	2	83%	17%	S884F	Yes (7)	B.1.1.122, B.1.351.1, B.1.177.74, B.1.538, B.1.1.513, B.1.1.322, B.1.517.
24227	1	0	3	0%	100%	G889C	No	B.1.340
24239	1	1	2	84%	16%	A893T	No	B.1.604
24375	1	1	3	79%	21%	L938P	No	< 0.5%
24479	1	1	3	67%	33%	I973V	No	< 0.5%
24506	2	2	3	See Figure 3		S982A	Yes (14,091)	Xa, B.1.1.7, B.1.411, B.1.1
24654	1	1	3	81%	19%	E1031V	No	< 0.5%
24655	1	1	3	81%	19%	E1031D	No	< 0.5%
24764	1	0	3	10%	90%	V1068F	Yes (30)	B.1.36.12, B.1.1.447, W.1, D.2, B.1.159, B.1.139, C.26, B.1.1.362, B.1.9.4, B.1.36.28, B.1.160.16
24812	1	1	3	78%	22%	D1084Y	Yes (123)	B.1.1.269, A.15, B.1.1.319, C.9, C.29, B.1.1.411, B.4, R.2, B.1.326, B.1.595, B.1.560, B.1.1.273, B.1.1.416, B.1.9, B.1.189, B.1.1.354, B.1.623, B.6
24872	1	1	3	69%	31%	V1104L	Yes (45)	L.4, B.1.1.341, B.1.160.32, B.1.1.299, B.1.1.228, B.1.177.75, B.1.287, B.1.1.324, B.1.177.89, B.1.178, B.1.1.219, B.1.177.88, B.1.1.433, A.19
24914	2	1	3	See Figure 3		D1118H	Yes (14,088)	Xa, B.1.1.7, B.1.620, B.1.1.34, B.1.411, B.1.1.462, B.1.1.213, B.1.1.378, B.1.1.194, B.1.533, B.1.170, B.1.1.135, P.1.1, B.1.1.375, B.1.1.319, B.1.570
25017	1	1	2	85%	15%	L1152S	No	< 0.5%
25047	1	1	2	83%	17%	P1162R	Yes (16)	Y.1, B.1.177.52, B.1.9
25049	1	1	3	82%	18%	D1163Y	Yes (226)	B.1.177, B.1.406, B.1.1.332, B.53, B.1.36.20, B.1.240.2, B.1.1.396, B.1.291, B.6.3
25081	1	0	2	0.00%	100.00%	N1173K	No	< 0.5%
25088	2	2	3	58±12%	41±12%	V1176F	Yes (672)	P.1, P.2, P.3, B.1.1.28, B.1.1.332, B.1.324, B.1.1.378, B.1.177.75, B.1.466, B.1.1.382, B.1.369, B.1.566, B.1.1.184, B.1.177.53, B.1.1.218, B.1.545, B.1.1.333, N.1
25244	1	1	3	82%	18%	V1228L	Yes (67)	B.1.258.15, B.1.177.66, L.3, B.1.181, B.1.1.512, B.1.36.21, B.1.1.112, B.1.390, B.1.596, B.1.1.424, B.1.258.7, C.7, B.1.236, B.1.570, B.1.393, B.1.426, B.1.533, B.4.1, N.3

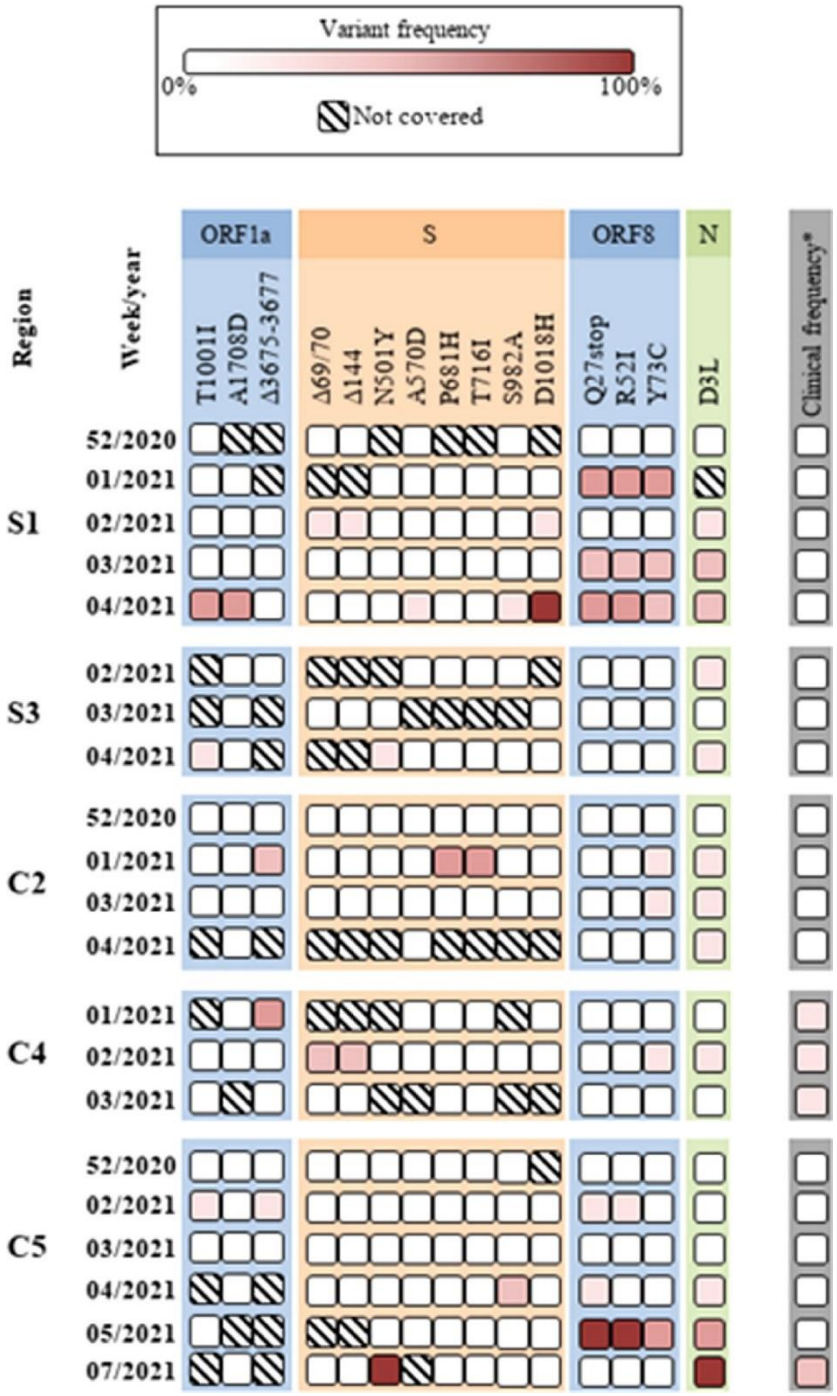
[a]Nucleotide position in SARS-CoV-2 isolate Wuhan-Hu-1 genome (MN908947.3).
[b]Lineage, according to PANGO lineages, that carries the mutation (with frequencies higher than 1%) as stated in outbreak.info.

d. Identification of B.1.1.7 (VOC 202,012/01) mutation signatures

The highly transmissible B.1.1.7 lineage of SARS-CoV-2 contains 16 characteristic non-synonym nucleotide substitutions and deletions (Rambaut et al., 2020) and was first detected in Spain in week 52 of 2020 in different Spanish regions (Madrid, Basque Country, and the Balearic islands).

The characteristic mutations described in the genome of the B.1.1.7 lineage were searched for in our sequencing data. These mutations corresponded to 2 nucleotide substitutions and one deletion in ORF1a, 6 nucleotide substitutions and 2 deletions in spike gene, 3 nucleotide substitutions in ORF8, and one nucleotide substitution in N gene (Fig. 3). Amino acid substitutions S235F of nucleocapsid protein was not shown because it was absent or not covered. Samples with Ct values below 36 for N1 were analyzed, starting from the week 52 of 2020 up to week 7 in the case of samples from region C5. Among the analyzed samples, only samples from regions S1, S3, C3, C4, and C5 showed characteristic mutations of the B.1.1.7 lineage. None of the samples showed all the 18 markers that were searched for, at the same time. The highest presence and frequency of mutations was found in nucleocapsid (detected in 60% of the samples) and in ORF8 region (detected in 45% of the samples), and the lowest in ORF1a. Interestingly, the three characteristic mutations in ORF8 (Q27stop, R52I, and Y73C) were detected together in 4 of the 9 samples. Only one sample, S1–4–2021, showed 9 out of 15 characteristic mutations. The deletion of spike amino acids 69 and 70 (S:Δ69/70) was found in 2 samples from different geographical regions, that were present along with deletion ΔY144. Although some of these mutations can belong to different lineages (Table 2), their absence in samples from the first and second waves denotes the introduction of new mutations that could be related with the lineage B.1.1.7. This idea is reinforced by the fact that some mutations, such as the three present in ORF8, appeared together in some samples. Additionally, as occurred with the detection of new amino acid substitutions in the spike gene (Section 3.3), some of these mutations associated to the lineage B.1.1.7. appeared even 4 weeks before their detection in clinical samples, according to the results obtained by S-gene target failure (SGTF) marker or detection of N501Y amino acid substitution (Fig. 3). This was the case for samples from regions S1, S3, and C2, where detection of the lineage B.1.1.7 in clinical samples occurred as of the fifth week.

Fig. 3. Frequency of nucleotide substitutions and deletions related with B.1.1.7 linage according to the sampling week. *Clinical frequency based on data obtained from the Spanish Ministry of Health according to the results obtained by S-gene target failure (SGTF) marker or detection of N501Y mutation.



4. Conclusions

SARS-CoV-2 has created a pandemic scenario unprecedented in modern times. The rapid spread of this virus together with the appearance of emerging linages has also mobilized the scientific community like never before. Its detection in wastewater has been very helpful for the epidemiological study in large populations and is currently being implemented worldwide. However, few studies using mass sequencing have been published.

• The present study describes the mutations found in SARS-CoV-2 genomes isolated from wastewater in 14 different regions of Spain. This is the first study carried out in Spain that analyzes the diversity of SARS-CoV-2 present in wastewater in the three epidemiological waves which occurred between 2020 and 2021.

• These results confirm the potential of sewage sequencing to detect new mutations and lineages of SARS-CoV-2, which is of utmost relevance for the monitoring efforts of emerging vaccine-escape SARS-CoV-2 mutants in the forthcoming post-vaccination era.

• Genomic sequencing of viruses found in wastewater provides complementary results to those of clinical laboratories, as has been demonstrated in various ways such as the confirmation of the initial detection of low number of reads on genomes from clinical specimens that was later confirmed in wastewater samples; the detection of amino acid substitutions in the spike protein weeks or months before their discovery in clinical samples; or the known amino acid substitutions in the spike protein detected for the first time in Spain.

• This technique provides complementary information for SARS-CoV-2 surveillance, allowing both the control of lineages including VOC and VOI already described and the detection and control of new emerging lineages.

• This data supports the hypothesis that the study of wastewater using high-throughput sequencing techniques is a useful and effective tool that can be implemented worldwide in support of public health for the epidemiological control of SARS-CoV-2.

5. Supplementary materials

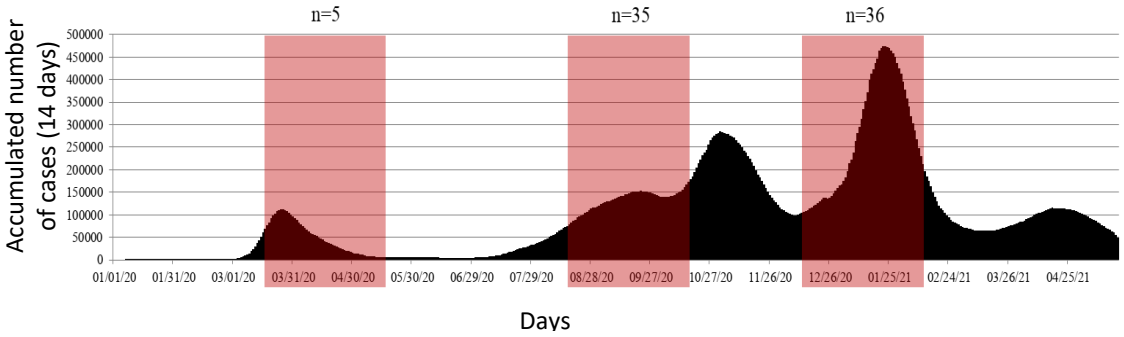
Supplementary table S1. Summary of the analyzed samples and results obtained for detection of SARS-CoV-2 by RT-qPCR targeting N1 region of the nucleocapsid gene, number of aligned sequences to the genome of SARS-CoV-2 isolate Wuhan-Hu-1 (MN908947.3), genome coverage (higher than 20X), mean depth of the sample sequencing, and number of mutations detected.

Region	*Sample*	*Wave*	*Sampling date*	*Ct value (N1)*	*Aligned seqs*	*20x coverage (%)*	*Mean depth (nt)*	*No. Mutations*
C1	*C1-37-2020*	***2***	*09/08/20*	*30.41*	*208555*	*66.61%*	*766.60*	*9*
	C1-38a-2020	***2***	*09/15/20*	*31.35*	*24638*	*3.03%*	*71.17*	*0*
	C1-38b-2020	***2***	*09/15/20*	*31.01*	*100902*	*43.90%*	*292.72*	*2*
	C1-39a-2020	***2***	*09/22/20*	*30.98*	*83995*	*39.15%*	*176.42*	*4*
	C1-39b-2020	***2***	*09/22/20*	*31.48*	*69883*	*18.26%*	*293.42*	*5*
	C2-36-2020	***2***	*09/01/20*	*31.71*	*360159*	*94.35%*	*1255.40*	*29*
C2	*C2-37a-2020*	***2***	*09/09/20*	*32.98*	*220088*	*56.15%*	*636.50*	*15*
	C2-37b-2020	***2***	*09/09/20*	*31.29*	*13607*	*1.31%*	*70.75*	*0*
	C2-52-2020	***3***	*12/22/20*	*30.59*	*142526*	*69.27%*	*440.27*	*14*
	C2-1a-2021	***3***	*01/07/21*	*30.98*	*265692*	*93.22%*	*970.58*	*43*
	C2-1b-2021	***3***	*01/07/21*	*31.50*	*227866*	*90.88%*	*853.45*	*35*
	C2-3-2021	***3***	*01/19/21*	*30.61*	*293231*	*91.74%*	*987.70*	*54*
	C2-4-2021	***3***	*01/26/21*	*31.75*	*159599*	*42.34%*	*652.52*	*20*
C3	*C3-40a-2020*	***2***	*10/01/20*	*32.07*	*191152*	*42.98%*	*625.96*	*11*
	C3-40b-2020	***2***	*10/01/20*	*30.98*	*41282*	*3.03%*	*123.22*	*0*
	C3-41-2020	***2***	*10/08/20*	*30.12*	*46246*	*8.64%*	*132.48*	*0*
C4	*C4-35-2020*	***2***	*08/25/20*	*31.81*	*218711*	*36.89%*	*638.74*	*10*
	C4-36-2020	***2***	*09/01/20*	*31.99*	*22540*	*1.72%*	*98.29*	*0*
	C4-37-2020	***2***	*09/08/20*	*31.29*	*267476*	*82.98%*	*759.03*	*25*
	C4-52-2020	***3***	*12/22/20*	*33.02*	*173796*	*33.11%*	*432.28*	*10*
	C4-1-2021	***3***	*01/07/21*	*30.66*	*203989*	*68.26%*	*627.14*	*9*

	C4-2-2021	3	01/12/21	30.26	245116	83.63%	759.73	46
	C4-3-2021	3	01/19/21	32.11	291782	50.17%	679.46	16
C5	C5-15a-2020	1	04/06/20	34.53	315764	38.37%	1942.57	2
	C5-15b-2020	1	04/06/20	34.75	383717	44.68%	1882.54	8
	C5-15c-2020	1	04/06/20	34.67	310331	26.33%	1671.01	0
	C5-52-2020	3	12/22/20	31.06	295202	82.14%	1002.60	43
	C5-1-2021	3	01/07/21	30.93	104891	9.40%	774.43	0
	C5-2-2021	3	01/12/21	29.54	373194	97.00%	1488.39	88
	C5-3-2021	3	01/19/21	29.55	236800	87.95%	743.41	38
	C5-4-2021	3	01/26/21	31.07	222975	84.35%	758.20	43
	C5-5-2021	3	02/02/21	31.72	219329	74.90%	697.50	38
	C5-6-2021	3	02/09/21	32.77	174812	59.50%	516.05	16
	C5-7-2021	3	02/16/21	33.29	292113	58.10%	887.52	24
	C5-8-2021	3	02/23/21	31.65	134337	33.20%	515.24	7
C6	C6-40-2020	2	09/29/20	26.59	155313	59.45%	523.76	18
	C6-41-2020	2	10/06/20	32.45	59764	5.66%	145.20	0
	C6-42-2020	2	10/13/20	33.36	125885	60.64%	363.06	14
C7	C7-34-2020	2	08/17/20	29.04	100701	43.83%	297.80	7
N1	N1-18-2020	1	04/30/20	32.43	359304	93.06%	1566.53	33
	N1-19-2020	1	05/05/20	33.08	355888	42.99%	2003.25	5
N2	N2-36-2020	2	09/02/20	30.80	466349	94.80%	1663.94	34
	N2-37-2020	2	09/09/20	30.40	412126	96.81%	1401.06	31
	N2-38-2020	2	09/16/20	30.72	521352	88.77%	1504.50	20
	N2-50-2020	3	12/09/20	32.49	233709	42.21%	761.09	8
	N2-3-2021	3	01/20/21	32.58	258743	40.70%	737.18	14
	N2-4-2021	3	01/27/21	32.94	148558	29.67%	632.26	4
S1	S1-36-2020	2	08/30/20	32.58	216965	66.38%	712.84	16
	S1-37-2020	2	09/06/20	32.99	55398	8.45%	140.17	1
	S1-38-2020	2	09/13/20	32.59	261766	73.60%	748.27	14
	S1-52-2020	3	12/20/20	31.66	141646	44.94%	556.13	8
	S1-1-2021	3	01/03/21	32.36	207768	51.10%	776.47	15

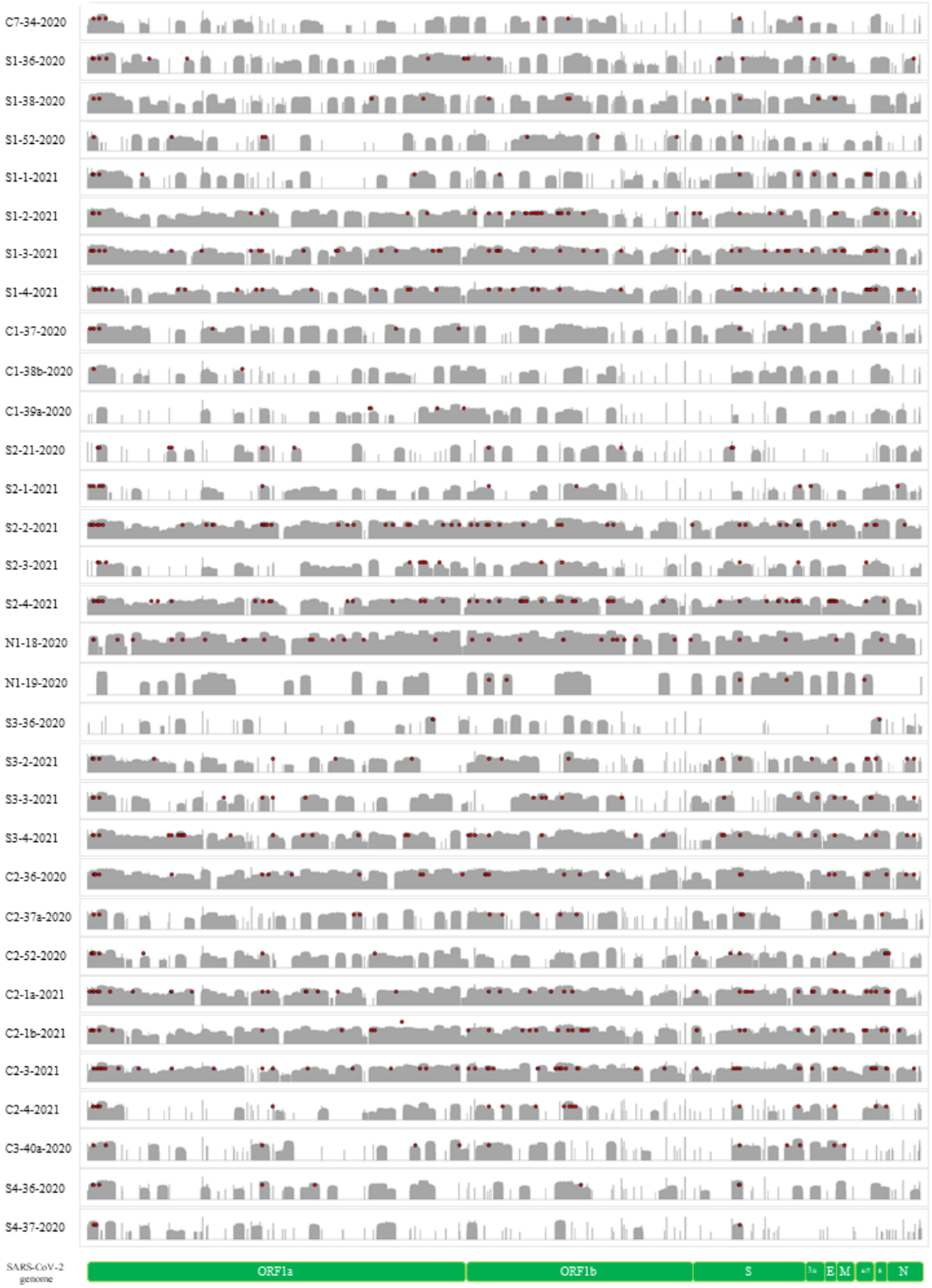
	S1-2-2021	***3***	*01/10/21*	*31.02*	*214827*	*85.11%*	*776.22*	*41*
	S1-3-2021	***3***	*01/17/21*	*30.05*	*251904*	*91.64%*	*887.97*	*48*
	S1-4-2021	***3***	*01/24/21*	*30.53*	*137608*	*89.58%*	*486.58*	*45*
S2	*S2-37-2020*	***2***	*09/09/20*	*33.26*	*299258*	*13.59%*	*345.55*	*0*
	S2-51-2020	***3***	*12/16/20*	*32.08*	*125723*	*32.67%*	*588.48*	*10*
	S2-1-2021	***3***	*01/06/21*	*28.07*	*72647*	*47.16%*	*318.98*	*10*
	S2-2-2021	***3***	*01/13/21*	*28.58*	*299761*	*94.40%*	*1232.23*	*51*
	S2-3-2021	***3***	*01/20/21*	*29.41*	*60709*	*59.34%*	*256.05*	*13*
	S2-4-2021	***3***	*01/27/21*	*30.24*	*227544*	*92.06%*	*891.81*	*51*
S3	*S3-36-2020*	***2***	*09/03/20*	*30.74*	*99272*	*21.99%*	*266.82*	*3*
	S3-37-2020	***2***	*09/10/20*	*30.96*	*8860*	*11.77%*	*69.15*	*0*
	S3-40-2020	***2***	*10/01/20*	*30.45*	*46731*	*1.85%*	*110.95*	*0*
	S3-41-2020	***2***	*10/06/20*	*30.13*	*27869*	*1.90%*	*82.47*	*0*
	S3-52-2020	***3***	*12/22/20*	*32.32*	*66707*	*6.39%*	*764.75*	*0*
	S3-1-2021	***3***	*01/05/21*	*29.28*	*79077*	*17.23%*	*531.79*	*6*
	S3-2-2021	***3***	*01/12/21*	*28.81*	*139429*	*61.50%*	*575.35*	*22*
	S3-3-2021	***3***	*01/19/21*	*28.05*	*202320*	*60.84%*	*917.74*	*20*
	S3-4-2021	***3***	*01/26/21*	*29.16*	*233778*	*87.01%*	*865.35*	*38*
S4	*S4-36-2020*	***2***	*09/01/20*	*31.64*	*129416*	*43.00%*	*449.91*	*8*
	S4-37-2020	***2***	*09/08/20*	*32.67*	*149389*	*24.77%*	*356.15*	*3*
S5	*S5-37-2020*	***2***	*09/07/20*	*31.79*	*140941*	*32.45%*	*467.41*	*5*
	S5-38-2020	***2***	*09/14/20*	*31.58*	*237982*	*43.48%*	*549.82*	*12*
	S5-40-2020	***2***	*09/28/20*	*31.49*	*123873*	*18.09%*	*233.10*	*5*
	S5-42-2020	***2***	*10/12/20*	*31.18*	*93288*	*10.25%*	*254.33*	*0*

Supplementary figure S1. Distribution of the samples used in this study along the three epidemic waves of SARS-CoV-2 occurred in Spain between 2020 and 2021.



Supplementary Figure S2. Representation of the genome coverage (>20X) in logaritmic scale (max 4.5 log) reached by samples that covered A) more than 20% or B) less than 18% of the SARS-CoV-2 isolate Wuhan-Hu-1 genome (MN908947.3).

A




C4-35-2020
C4-37-2020
C4-52-2020
C4-1-2021
C4-2-2021
C4-3-2021
C5-15a-2020
C5-15b-2020
C5-15c-2020
C5-52-2020
C5-2-2021
C5-3-2021
C5-4-2021
C5-5-2021
C5-6-2021
C5-7-2021
C5-8-2021
C6-40-2020
C6-42-2020
S5-37-2020
S5-38-2020
N2-36-2020
N2-37-2020
N2-38-2020
N2-50-2020
N2-3-2021
N2-4-2021
SARS-CoV-2 genome
ORF1a
ORF1b
S
3a
E
M
6/7
8
N

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RESULTS

Chapter 5. Evaluation of different procedures for coronaviruses' nucleic acid detection in water matrices.

Chapter 5. Evaluation of different procedures for coronaviruses' nucleic acid detection in water matrices.

5.1. Comparing analytical methods to detect SARS-CoV-2 in wastewater

This section is an adapted version of the following published

research article:

AlbaPérez-Cataluña, Enric Cuevas-Ferrando, Walter Randazzo, Irene Falcó, Ana Allende, Gloria Sánchez (2021). **Comparing analytical methods to detect SARS-CoV-2 in wastewater**. Science of The Total Environment, Volume 758, 143870, ISSN 0048-9697.

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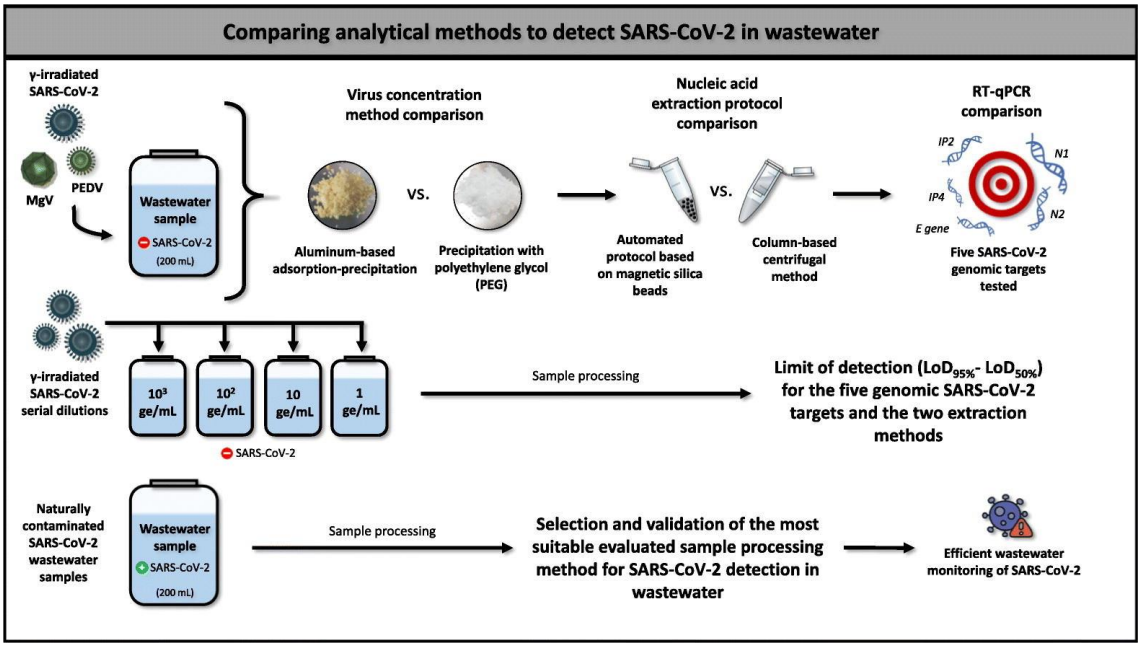


Highlights

• Gamma-irradiated SARS-CoV-2 was used to assess method performance.

• Different methods were compared for SARS-CoV-2 WBE surveillance.

• Tested methods showed no significant differences for SARS-CoV-2 recovery from wastewater.

• Automated nucleic acid extraction showed lower LoD95% than column-based method.

• Different sensitivity of RT-qPCR assays was observed.

1. Abstract

Wastewater based epidemiology (WBE) has emerged as a reliable strategy to assess the coronavirus disease 2019 (COVID-19) pandemic. Recent publications suggest that SARS-CoV-2 detection in wastewater is technically feasible; however, many different protocols are available and most of the methods applied have not been properly validated. To this end, different procedures to concentrate and extract inactivated SARS-CoV-2 and surrogates were initially evaluated. Urban wastewater seeded with gamma-irradiated SARS-CoV-2, porcine epidemic diarrhea virus (PEDV), and mengovirus (MgV) was used to test the concentration efficiency of an aluminum-based adsorption-precipitation method and a polyethylene glycol (PEG) precipitation protocol. Moreover, two different RNA extraction methods were compared in this study: a commercial manual spin column centrifugation kit and an automated protocol based on magnetic silica beads. Overall, the evaluated concentration methods did not impact the recovery of gamma-irradiated SARS-CoV-2 nor MgV, while extraction methods showed significant differences for PEDV. Mean recovery rates of 42.9 ± 9.5%, 27.5 ± 14.3% and 9.0 ± 2.2% were obtained for gamma-irradiated SARS-CoV-2, PEDV and MgV, respectively. Limits of detection (LoD95%) for five genomic SARS-CoV-2 targets (N1, N2, gene E, IP2 and IP4) ranged from 1.56 log genome equivalents (ge)/mL (N1) to 2.22 log ge/mL (IP4) when automated system was used; while values ranging between 2.08 (N1) and 2.34 (E) log ge/mL were observed when using column-based extraction method. Different targets were also evaluated in naturally contaminated wastewater samples with 91.2%, 85.3%, 70.6%, 79.4% and 73.5% positivity, for N1, N2, E, IP2 and IP4, respectively. Our

benchmarked comparison study suggests that the aluminum precipitation method coupled with the automated nucleic extraction represents a method of acceptable sensitivity to provide readily results of interest for SARS-CoV-2 WBE surveillance.

Keywords

SARS-CoV-2Porcine epidemic diarrhea virusPolyethylene glycol precipitationAluminum-based adsorption-precipitationWastewater based epidemiologyRT-qPCR

Abbreviations

COVID-19, Coronavirus disease 2019; EC, European Commission; MgV, Mengovirus; PEDV, Porcine Epidemic Diarrhea Virus; PEG, polyethylene glycol; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; WBE, wastewater-based epidemiology; WHO, World Health Organization

2. Introduction

The use of wastewater as a tool for epidemiology tracking, known as wastewater-based epidemiology (WBE), has a long history of use in public health, particularly for human enteric viruses (Asghar et al., 2014; Cuevas-Ferrando et al., 2020; Hellmér et al., 2014; Miura et al., 2016; Prevost et al., 2015; Santiso-Bellón et al., 2020). In the midst of the ongoing COVID-19 pandemic, WBE is being implemented globally for the detection of SARS-CoV-2 RNA shed into wastewater, sewers, and sludge (Ahmed et al., 2020a; Bivins et al., 2020; Guerrero-Latorre et al., 2020; Haramoto et al., 2020; Kumar et al., 2020; La Rosa et al., 2020; Lodder and de Roda Husman, 2020; Medema et al., 2020; Prado et al., 2020; Randazzo et al., 2020a, Randazzo et al., 2020b; Rimoldi et al., 2020; Sherchan et al., 2020; Wang et al., 2020; Wu et al., 2020). All these studies have been implemented in research contexts; nevertheless, different countries are currently implementing wastewater surveillance into their national or regional COVID-19 monitoring programs for early warning of SARS-CoV-2 community spread and disease outbreaks (WHO, 2020). Additionally, WBE has the potential to be applied in high-risk settings such as nursing homes and hospital or in low-resource settings (WHO, 2020). As recently stated by WHO, WBE research should be seen as an important public health objective to advance knowledge about COVID-19, however, many technical issues still need to be addressed (Ahmed et al., 2020b; Polo et al., 2020; Rusiñol et al., 2020; WHO, 2020). In an attempt to coordinate current knowledge and data gaps, the European Commission (EC) created a Pan-European Umbrella Study to better understand the limitations and challenges of this approach including the development of a roadmap for a systemic rollout of complementing ongoing national and regional surveillances in a unique approach (EC, 2020). One of the problems highlighted by these collaborative studies is the need of standardized procedures, spanning from sampling to data analysis. In this sense, viral concentration and nucleic acid extraction methods are two critical steps for the analysis of viruses in wastewater and quality controls must be accurately defined. To our knowledge, three studies have compared different concentration methods

using SARS-CoV-2 surrogates (Ahmed et al., 2020c; Jafferali et al., 2020; Rusiñol et al., 2020). However, the analytical performances of SARS-CoV-2 concentration, extraction, and detection procedures tested alongside are not yet characterized for wastewater samples. Thus, the aim of this work was to evaluate different concentration methods, nucleic acid extraction procedures, and quantitative RT-qPCR assays to efficiently detect SARS-CoV-2 in wastewater using gamma-irradiated SARS-CoV-2, porcine epidemic diarrhea virus (PEDV) as coronavirus model, and mengovirus as non-enveloped counterpart. Importantly, limits of detection were established using gamma-irradiated SARS-CoV-2 particles and five different RT-qPCR assays targeting various genetic fragments. We finally validate the selected RT-qPCR assays in wastewater samples collected during the COVID-19 pandemic in different regions of Spain.

3. Materials and methods

3.1. Concentration methods

An aluminum-based adsorption-precipitation and a polyethylene-glycol (PEG) precipitation methods were compared to assess their analytical performance and thus their suitability in concentrating SARS-CoV-2 from wastewater. To this end, 200 mL of grab wastewater samples (n = 8) that previously tested negative for SARS-CoV-2 (Randazzo et al., 2020a) were inoculated with 105 genome equivalents (ge) gamma-irradiated (5 × 106 RADs) SARS-CoV-2 (Bei Resources; NR-52287), 106 PCR units (PCRU) PEDV strain CV777, an enveloped virus member of the Coronaviridae family and surrogate for SARS-CoV-2; and, 106 PCRU mengovirus (MgV) vMC0 (CECT 100000), a non-enveloped member of the Picornaviridae designated in the ISO 15216-1:2017 standard method as process control. The PEDV cytopathogenic CV777 strain (Friedrich-Loeffler-Insitut, Greifswald, Germany) and MgV vMC0 were propagated in Vero and HeLa cell monolayers, respectively (Puente et al., 2020). Two hundred milliliters of seeded wastewater samples (n = 4) were concentrated through aluminum-based adsorption-precipitation (Randazzo et al., 2019, Randazzo et al., 2020a, Randazzo et al., 2020b). A final concentrate was then formed by centrifugation at 1900 ×g for 30 min and the resulting pellet was resuspended in 1 mL of PBS, pH 7.4. Alternatively, 200 mL of seeded wastewater samples (n = 4) were concentrated through precipitation with 20% polyethylene glycol (PEG) 8000 (PanReac, Spain) and resuspended in 1 mL of PBS, pH 7.4. Briefly, 25 mL of Tris Glycine-Beef Extract buffer (TGEB) pH 9.5 were added to each sample and incubated in agitation at 300 rpm for 2 h at 4 °C. After incubation,

samples were centrifuged at 2500 ×g for 10 min at 4 °C. Supernatant was adjusted to pH 7.0–7.2. PEG and NaCl were added to a final concentration of 20% and 0.3 M, respectively, and mixed gently. Sample was incubated in agitation overnight at 4 °C and then centrifuged at 3500 ×g for 30 min at 4 °C. Pellet was resuspended in 1 mL of PBS and concentrated samples stored at −80 °C for further analysis.

For both concentration methods, recovery controls were prepared by spiking each virus at the concentration detailed above in 1 mL of PBS. For each sample, the percentage recovery was calculated dividing the viral titer of concentrated sample by the titer of the recovery control.

3.2. Viral extraction, detection and quantification

3.2.1. Nucleic acid extraction

Viral extraction from wastewater concentrates was carried out comparing a manual column-based commercial kit and an automated instrument relying on magnetic beads for nucleic acid purification.

Manual nucleic acid extraction was performed from 150 µL of concentrated sample using the Nucleospin RNA virus Kit (referred as MN) (Macherey-Nagel GmbH & Co., Germany) following the manufacturer's protocol together with an initial pre-treatment step with Plant RNA Isolation Aid (Ambion, USA) (Cuevas-Ferrando et al., 2020; Randazzo et al., 2020a, Randazzo et al., 2020b). In parallel, Maxwell® RSC Instrument (Promega, Spain) was used for automated nucleic acid isolation using the Maxwell RSC Pure Food GMO and authentication kit (Promega) (referred as Max). Some modifications of the original provider's protocol were established based on preliminary laboratory results during a method optimization step (data not shown). Finally, 400 µL of cetyltrimethyl ammonium bromide (CTAB) and 40 µL of proteinase K solution (both provided with the kit) were added to 300 µL of concentrated water samples, the mix was then incubated at 60 °C for 10 min and centrifuged for 10 min at 16,000 ×g. Then, the supernatant was transferred to the loading cartridge along with 300 µL of lysis buffer. The cartridge was loaded in the Maxwell® RSC Instrument and the extraction performed by selecting the “Maxwell RSC Viral total Nucleic Acid” running program in the instrument software. For both manual and automated extractions, RNA was finally eluted in 100 µL nuclease-free water. Negative controls constituted by nuclease-free water instead of concentrated sample were included in both extraction methods.

3.2.2. Viral detection and quantification

Viral detection of SARS-CoV-2, PEDV, and MgV was performed by RT-qPCR using One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio, USA). SARS-CoV-2 detection was performed targeting N1 region of the nucleocapsid gene (CDC, 2020) while membrane gene (M) specific primers were used for PEDV detection as described by Puente et al., 2020. For mengovirus, detection was carried out using primers and probe described in ISO 15216-1:2017. Reaction mixes, thermal cycling conditions and sequences for primers and probes are shown in Tables S1, S2 and S3, respectively. All RT-qPCR assays were performed in duplicate on a LightCycler 480 instrument (Roche Diagnostics, Germany). Positive (genomic RNA) and negative (nuclease-free water) controls were always included. Standard curves for PEDV, MgV and SARS-CoV-2 quantifications were performed using the genomic RNA of each virus with serial 10-fold dilutions in triplicate. Differences between methods were statistically analyzed using Saphiro test for normal distribution and T-student for mean comparison ($p < 0.05$). Influence of concentration and extraction methods was analyzed using multifactorial ANOVA for each virus ($p < 0.05$).

3.3. SARS-CoV-2 detection limit in wastewater

The limits of detection at 95% and 50% confidence intervals (LoD95% and LoD50%, respectively) were obtained by detecting gamma-irradiated SARS-CoV-2 (Bei Resources, NR-52287) ten-fold serially diluted from 1.7 × 103 to 1.7 ge/mL and seeded in 200 mL of wastewater samples tested negative for SARS-CoV-2. Samples were also spiked with PEDV (107 gc/mL) as process control. Viral particles were concentrated by the aluminum-based adsorption-precipitation method and RNA extracted using both RNA extraction protocols as described above. Experiments were performed in triplicate by concentrating three independent samples for each inoculation level. LoD95% and LoD50% were calculated according to Wilrich and Wilrich (2009).

To determine SARS-CoV-2 detection limits, five different targets were used: N1 and N2 regions of the nucleocapsid gene, the envelope gene (E), and regions IP2 and IP4 of the RNA-dependent RNA polymerase gene (RdRp). The amplification of the N1 region was conducted as previously described. Region N2 detection was fulfilled using primers and probes available at the diagnostic panel assays 2019-nCoV RUO Kit from the US CDC (CDC, 2020). Detection of

gene E was performed using primers and probes described by Corman et al. (2020) (Table S1 and S2). To amplify and quantify IP2 target, One Step PrimeScript™ III RT-PCR kit (Takara Bio) was used.

3.4. RT-qPCR comparison in naturally contaminated wastewater samples

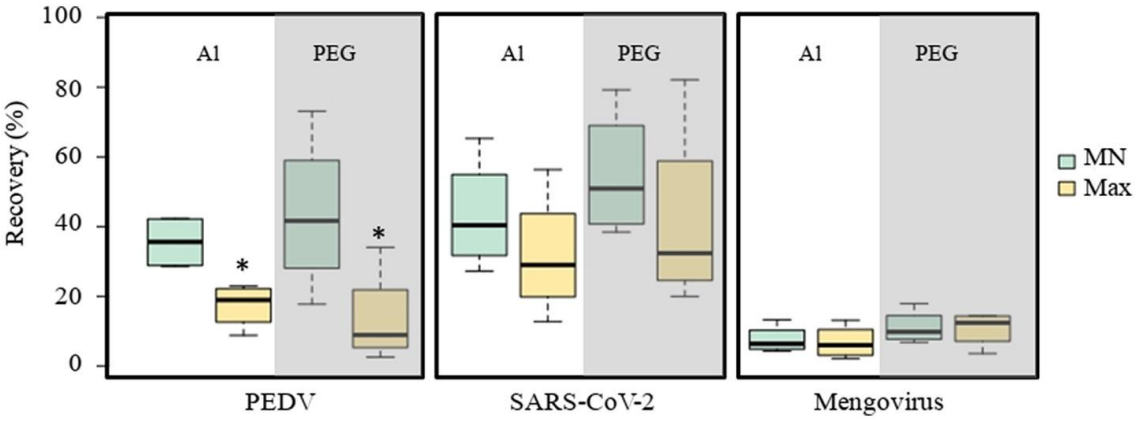
A total of 34 influent wastewater samples collected in different regions of Spain were analyzed for the detection and quantification of SARS-CoV-2. Samples were concentrated using the aluminum-based adsorption-precipitation method as described before. Detection of SARS-CoV-2 was conducted through the analysis of five aforementioned SARS-CoV-2 genome targets (Tables S1, S2, and S3). Each reaction was performed in duplicate. Genomic RNA of SARS-CoV-2 (ATCC VR-1986D) and nuclease free water were used as positive and negative controls, respectively. Viral quantifications were calculated by using two different standard curves for N1, N2 and E genes. The standard curves were built by using N1, N2 (2019-nCoV_N_Positive Control from CDC, IDT Catalog No. 10006625) and E gene (2019-nCoV_E Positive Control from Charité/Berlin, IDT Catalog No. 10006896) plasmids and a complete genomic RNA of SARS-CoV-2 (ATCC VR-1986D).

4. Results and discussion

4.1. Concentration and extraction method comparison

Fig. 1 shows the viral recovery rates of eight wastewater samples tested negative for SARS-CoV-2 spiked with gamma-irradiated SARS-CoV-2, PEDV and MgV, subjected to two different concentration and two nucleic acid extraction methods (Fig. 1). To determine the presence of potential inhibitors, a 10-fold dilution of each sample was also analyzed by RT-qPCR for the three targeted viruses. RT-qPCR results showed that no significant inhibitions occurred (data not shown).

Figure 1. Virus recovery (%) in wastewater samples using the aluminum-based adsorption-precipitation (Al) and polyethylene glycol (PEG) precipitation methods and two RNA extraction assays (MN and Max). SARS-CoV-2 detection was performed using N1 target. Abbreviations: MN, NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co.); Max, Maxwell RSC (Promega). * $p < 0.05$ in comparison with the Al-MN protocol.



SARS-CoV-2 mean recoveries ranged from 30.2 ± 17.7% (Al-Max) to 52.8 ± 18.2% (PEG-MN). In the case of MgV, mean recoveries were lower and showed less variability than those obtained for SARS-CoV-2, ranging from 6.8 ± 4.8% (Al-Max) to 11.1 ± 4.9% (PEG-MN). Despite the observed differences, mean recoveries of SARS-CoV-2 and MgV were not significantly different among the tested concentration and extraction methods. In light of those results, recoveries of SARS-CoV-2 and MgV would not be significantly affected by any combination of concentration and extraction methods tested in this study (Fig. 1).

PEDV showed a global mean recovery of 27.5 ± 14.3%, with values ranging from 2.6% (PEG-Max) to 73% (PEG-MN). Results obtained with PEG concentration showed high variability (coefficient of variation (CV) of 82.99%) in comparison with the aluminum method (CV of 44.16%). Significant differences ($p < 0.05$) were observed for Al-MN with Al-Max (p-value = 0.012) and PEG-Max (p-value = 0.043) (Fig. 1). These results highlight the suitability of tested methods for the analysis of enveloped viruses in wastewater.

Ahmed et al. (2020c) recently reported similar mean recoveries ranging from 26.7 to 65.7% using murine hepatitis virus as surrogate for SARS-CoV-2 concentrated from wastewater by ultracentrifugation, filtration and flocculation methods. Interestingly, the authors report mean recovery of 44.0

± 27.7% for PEG flocculation that is similar to the recovery 43.5 ± 22.8% obtained for PEDV using PEG and MN in this study. In the study of Gonzalez et al. (2020), recovery percentages of bovine coronavirus were 5.5% and 4.8% when using InnovaPrep and electronegative filtration methods for viral concentration. These recovery values were more in concordance with the ones obtained in our study for the non-enveloped MgV. From what we know, this is the first study that used gamma-irradiated SARS-CoV-2 for methods assessment and comparison. However, taking into account that these protocols are intended to be used for early SARS-CoV-2 monitoring, in which the readily availability of results is crucial to set a timely public health response up, the choice of a suitable analytical method should be based on the bench work time needed for each procedure, alongside its sensitivity. Since the PEG protocol includes an overnight incubation step, unlike the aluminum-based adsorption-precipitation method (total time less than 2 h), we selected the aluminum protocol for further comparisons.

4.2. SARS-CoV-2 detection limit in wastewater

Detection limits of five SARS-CoV-2 genome targets in wastewater were evaluated through the analysis of serial diluted spiked samples. For the detection of IP2 target, no amplification was obtained when using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (RR064) due to the presence of inhibitors. Therefore, the One Step PrimeScript™ III RT-PCR kit (RR600) was used since is claimed to be highly resistant to a wide variety of inhibitory substances. Limit of detection values (LoD95% and LoD50%) obtained for each gene target processed with the two extraction protocols analyzed in this study are shown in Table 1. LoD95% values for MN protocol ranged from 2.08 to 2.34 log ge/mL; while Max protocol showed values between 1.56 and 2.22 log ge/mL. These results suggest that Maxwell RSC instrument coupled with Maxwell RSC Pure Food GMO and authentication kit are slight more sensitive than the MN protocol. However, given the large demand for commercial RNA extraction kits and the shortages of provisions, different suitable alternatives are worthy to further evaluate.

Table 1. Detection ratio and limit of detection (LoD95% and LoD50%) of gamma-irradiated SARS-CoV-2 viral particles in wastewater using the aluminum-precipitation protocol. Viral RNA was extracted using two different nucleic acid extraction protocols and detected by targeting N1, N2, E, IP2 and IP4 genomic fragments. Abbreviations: MN, NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co.); Max, Maxwell RSC (Promega).

Method	Target	Levels of inoculated gamma-irradiated SARS-CoV-2 (ge/mL)				$LoD_{95\%}$ (ge/mL)[a]	$LoD_{50\%}$ (ge/mL)[a]
		1700	170	17	1.7		
MN	N1	6/6	6/6	2/6	0/6	2.08	1.44
	N2	6/6	6/6	2/6	0/6	2.08	1.44
	E	6/6	6/6	0/6	0/6	2.34	1.71
	IP2	6/6	6/6	1/6	0/6	2.22	1.58
	IP4	6/6	6/6	1/6	0/6	2.22	1.58
Max	N1	6/6	6/6	5/6	0/6	1.56	0.92
	N2	6/6	6/6	4/6	0/6	1.74	1.10
	E	6/6	6/6	3/6	0/6	1.91	1.28
	IP2	6/6	6/6	4/6	0/6	1.74	1.10
	IP4	6/6	6/6	1/6	0/6	2.22	1.58

[a] Calculated according to Wilrich and Wilrich (2009).

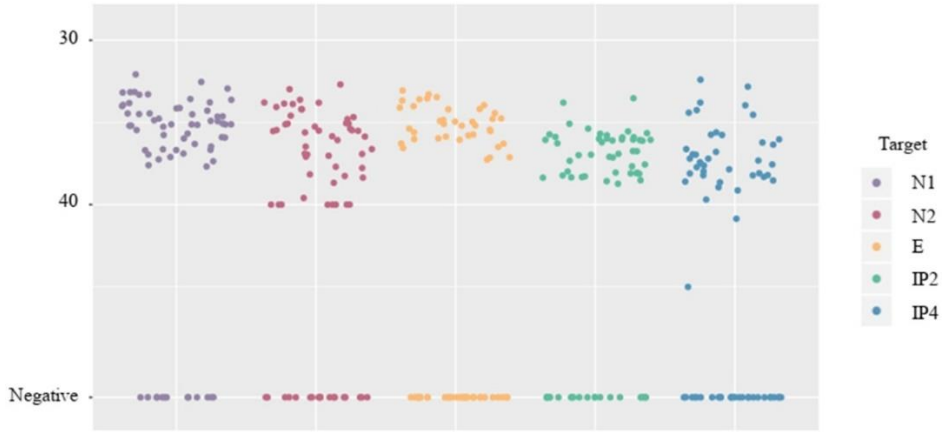
Validation of the two extractions methods was performed in wastewater samples naturally contaminated with SARS-CoV-2 using N1 target. As occurred with spiked samples, slightly better results were obtained when automated system was used (Supplementary Table S4). Regarding the LoD95% values, E gene with MN (2.34 log ge/mL) and IP4 gene with Max (2.22 log ge/mL) showed the highest detection values. Randazzo et al. (2020a) established the theoretical LoD95% as 1.45 and 1.91 log gc/mL for N1 and N2, respectively. By spiking gamma-irradiated SARS-CoV-2 we are now able to establish rigorous

LoD95% values for N1 and N2, being of 2.08 log ge/mL with MN for both genes, and 1.56 for N1 and 1.74 log ge/mL for N2 with Max. In line, Cuevas-Ferrando et al. (2020), applying the aluminum concentration method combined with the MN extraction, reported LoD95% of 2.46 log gc/mL for HEV in wastewater.

4.3. Bias of RT-qPCR assays for SARS-CoV-2 detection in wastewater samples

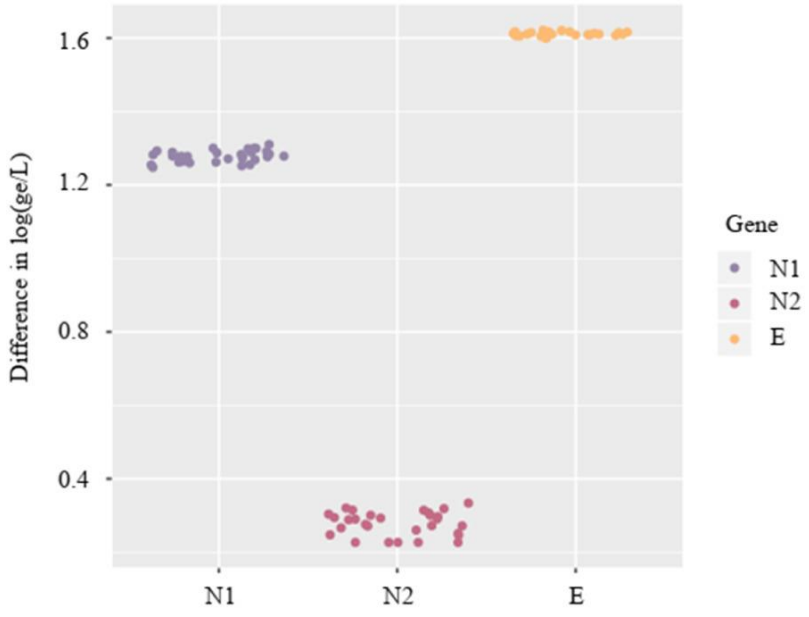
Naturally contaminated wastewater samples were analyzed by targeting five different SARS-CoV-2 genomic fragments (N1, N2, E, IP2 and IP4) to evaluate the sensitivity of each RT-qPCR assay. As shown in Fig. 2, several differences were found in the detection of SARS-CoV-2 depending on the target used. The percentage of positive samples for each target resulted as 91.2%, 85.3%, 70.6%, 79.4% and 73.5%, for N1, N2, E, IP2 and IP4, respectively. These results evidenced the variability that can be obtained in positive samples depending on the primer set used. Additionally, reproducibility of SARS-CoV-2 detection varied within each genomic target. For example, for 10 out of 24 samples that tested positive for gene E, no fluorescence was detected in any of its technical replicates. In this sense, N1 was the target with the highest percentage of positive replicates (77%) in line of results previously reported (Muenchhoff et al., 2020). The lower sensitivity to detect gamma-irradiated SARS-CoV-2 by targeting gene E could be due to the presence of mutations in the primer binding site that would hamper amplification and, therefore, its detection (Artesi et al., 2020).

Figure 2. Distribution of cycle threshold values for N1, N2, E, IP2 and IP4 genomic targets of SARS-CoV-2 detected in wastewater samples (n = 34 samples).



For viral quantification of N1, N2 and gene E, standard curves were built using complete genomic RNA of SARS-CoV-2 (ATCC VR-1986D) and synthetic plasmids developed for genes N and E (Supp. Fig. S1). Fig. 3 shows the difference in the logarithm of gc/L obtained with each standard curve for each gene. Mean values of these differences were 1.27 ± 0.01, 0.27 ± 0.03, and 1.61 ± 0.01 log (gc/L) for N1, N2, and E, respectively. This overestimation was produced by the plasmid quantification as described previously (Lin et al., 2011). Quantification bias was observed depending on the reference material used, which is very important when comparing quantification data from different studies. Thus, the use of genomic RNA of SARS-CoV-2 as standard material should be recommended. Moreover, given the final WBE aim, that is the estimation of the number of infected people in a given community, this bias needs to be accurately assessed before the introduction of the quantification values into predictive epidemiological models.

Figure 3. Differences in SARS-CoV-2 RNA concentrations (log10 gc/L) in wastewaters (n = 34) calculated according to standard curves generated by using genomic RNA (ATCC VR-1986D) and synthetic plasmids (IDT 10006625 for N1 and N2; IDT 10006896 for E gene).



5. Conclusions

The introduction of SARS-CoV-2 and its spread to the pandemic status have put all countries on alert and WBE has been readily implemented as an early warning tool for outbreaks. In most of the countries, wastewater surveillance for monitoring COVID-19 started very hastily, even before the scientific community could have robust data on the optimal methodologies. In fact, procedures used for viral detection have been little studied and standardization is still needed. This study benchmarked two concentration methods and two nucleic acid extraction methods widely used in environmental virology. Results obtained in this study reveal the variability obtained depending on the surrogate used as process control to validate the analyses, the extraction method, and the molecular target used for SARS-CoV-2 detection. These are critical decision which will affect the sensitivity of the analyses. On the other hand, despite the difference on sample processing time, both aluminum and PEG concentration methods can be indiscriminately used, as they did not show significant differences. However, the reduced time needed for the concentration of the samples using the aluminum-based adsorption-precipitation method, makes it the preferred method for this step. In contrast, a different sensitivity of the RT-qPCR assay has been observed suggesting that the selection of the molecular target for detection is crucial. The findings of this study expand the knowledge on the analytical procedures and its efficiencies for SARS-CoV-2 detection in wastewater constituting a step forward for the global implementation of COVID-19 WBE.

6. Supplementary material

Figure S1. Standard curves for targets N1, N2 and gene E performed with 10-fold dilutions (100-106 gc/reaction) of the genomic RNA (ATCC VR-1986D) and the synthetic plasmids of genes N (IDT 10006625) and E (IDT 10006896). For each gene and standard material, slope and R2 are shown.

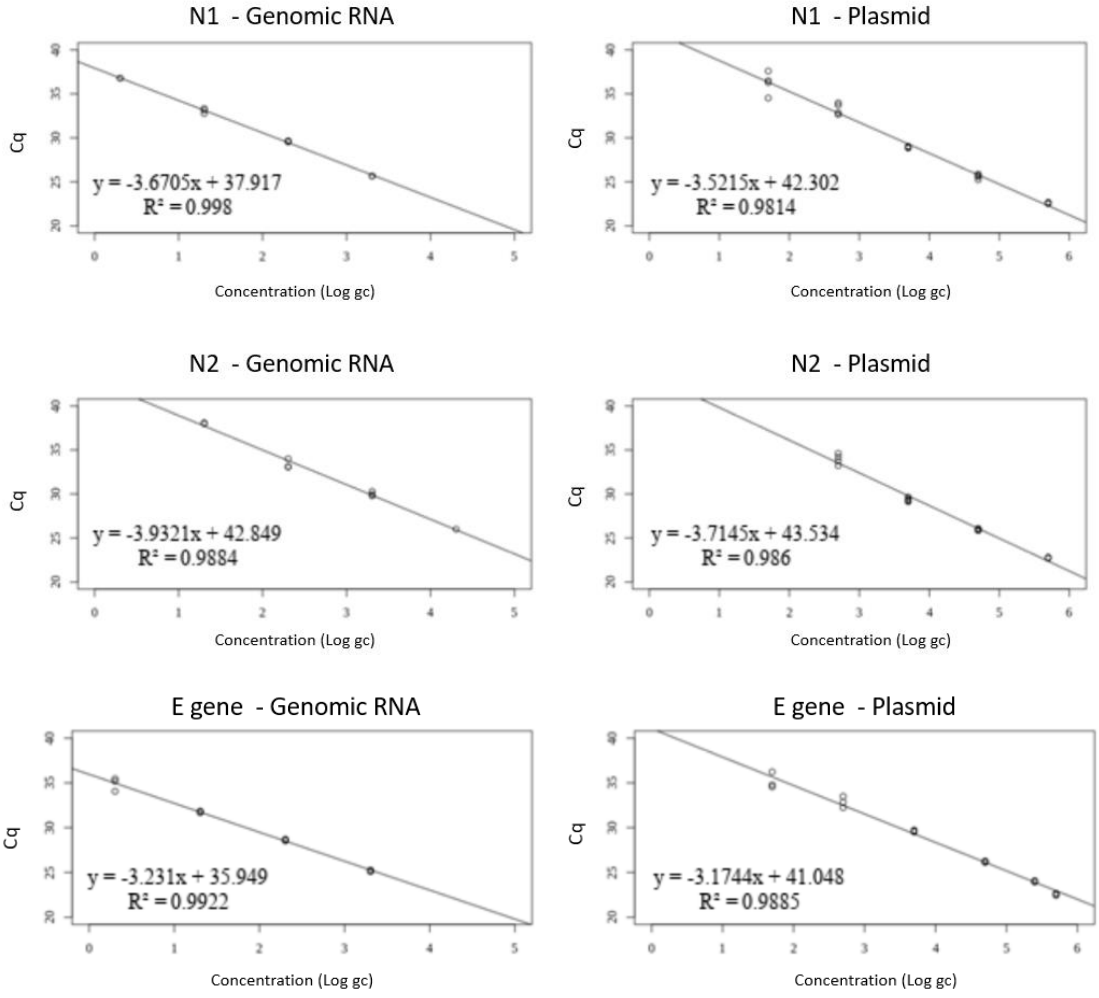


Table S1. Reaction mix volumes (in µl) used for the detection of SARS-CoV-2 by RT-qPCR. Reagents: Buffer, 2X One Step RT-PCR Buffer III; Enzyme 1, Takara Ex Taq HS (5u/ µL); Enzyme 2, PrimeScript RT enzyme Mix II. Total RNA volume of 2.5 µl. Water was added to a final reaction volume of 10 µl.

Target	Buffer	Enzyme 1	Enzyme 2	Forward primer	Reverse primer	Probe
N1, N2	5	0.2	0.2	0.75[a]		
E	5	0.2	0.2	0.5[a]		
IP4	5	0.2	0.2	0.4[b]	0.4[b]	0.2[b]
MgV, PEDV	5	0.2	0.2	0.5[b]	0.5[b]	0.5[b]

[a] Primers at 400nM final concentration; Probes at 200nM final concentration. Primers and probes premixed in commercial kits from IDT Technologies.

[b] From stock with an initial concentration of 10 µM.

Table S2. Thermal amplification conditions for the RT-qPCR used in the study for the detection

of SARS-CoV-2, MgV and PEDV.

Target	Retrotranscription	Activation	Denaturation	Annealing	Cycles
N1, N2	50°C, 15 min	95°C, 2 min	95°C, 3 sec	55°C, 30 sec	45
IP2, IP4, E	55°C, 20 min	95°C, 3 min	95°C, 15 sec	58°C, 30 sec	50
MgV, PEDV	55°C, 15 min	95°C, 5 min	95°C, 5 sec	60°C, 10 sec 65°C, 10 sec	45

Table S3. Primers and probes used for the detection of SARS-CoV-2, PEDV and MgV.

Virus	Target	Name	Sequences (5' – 3')	Amplicon size (bp)	Reference
SARS-CoV-2	N1	USCDC_N1_F	GACCCCAAAATCAGCGAAAT	72	
		USCDC_N1_P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
		USCDC_N1_R	TCTGGTTACTGCCAGTTGAATCTG		
	N2	USCDC_N2_F	TTACAAACATTGGCCGCAAA	67	
		USCDC_N2_P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1		
		USCDC_N2_R	GCGCGACATTCCGAAGAA		
	E	Charité_E_F	ACAGGTACGTTAATAGTTAATAGCGT	113	
		Charité_E_P	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ		Corman et al., 2020
		Charité_E_R	ATATTGCAGCAGTACGCACACA		
	IP2	Pasteur_IP2_F	ATGAGCTTAGTCCTGTTG	108	
		Pasteur_IP2_P	FAM-AGATGTCTTGTGCTGCCGGTA-BHQ1		
		Pasteur_IP2_R	CTCCCTTTGTTGTGTTGT		
	IP4	Pasteur_IP4_F	GGTAACTGGTATGATTTCG	107	
		Pasteur_IP4_P	FAM-TCATACAAACCACGCCAGG-BHQ1		
		Pasteur_IP4_R	CTGGTCAAGGTTAATATAGG		
PEDV	M gene	PEDV_forward	CAGGACACATTCTTGGTGGTCTT	140	Zhou et al., 2017
		PEDV_probe	FAM-ACGCGCTTCTCACTAC-MGBNFQ		
		PEDV_reverse	CAAGCAATGTACCACTAAGGAGTGTT		
MgV	5' NCR	Mengo 110	GCGGGTCCTGCCGAAAGT	100	ISO 15216-1:2017
		Mengo probe	FAM-ATCACATTACTGGCCGAAGC-MGBNFQ		
		Mengo 209	GAAGTAACATATAGACAGACGCACAC		

Table S4. Detection by RT-qPCR of SARS-CoV-2 in sewage samples targeting N1 region. Abbreviations: MN, NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co.); Max, Maxwell RSC (Promega); Ct, RT-qPCR cycle threshold.

Sample	MN (Ct)		Maxwell (Ct)	
Sample 1	36.50	35.77	34.53	35.18
Sample 2	-	-	-	36.14
Sample 3	-	-	32.34	32.02
Sample 4	34.95	35.54	36.29	35.76
Sample 5	37.05	-	35.78	35.71
Sample 6	-	36.61	34.96	35.96
Sample 7	39.31	39.42	33.99	34.62
Sample 8	40.00	40.00	37.14	38.8
Sample 9	38.32	-	37.04	-
Sample 10	36.93	36.83	36.77	35.7
Sample 11	39.14	-	37.65	36.84

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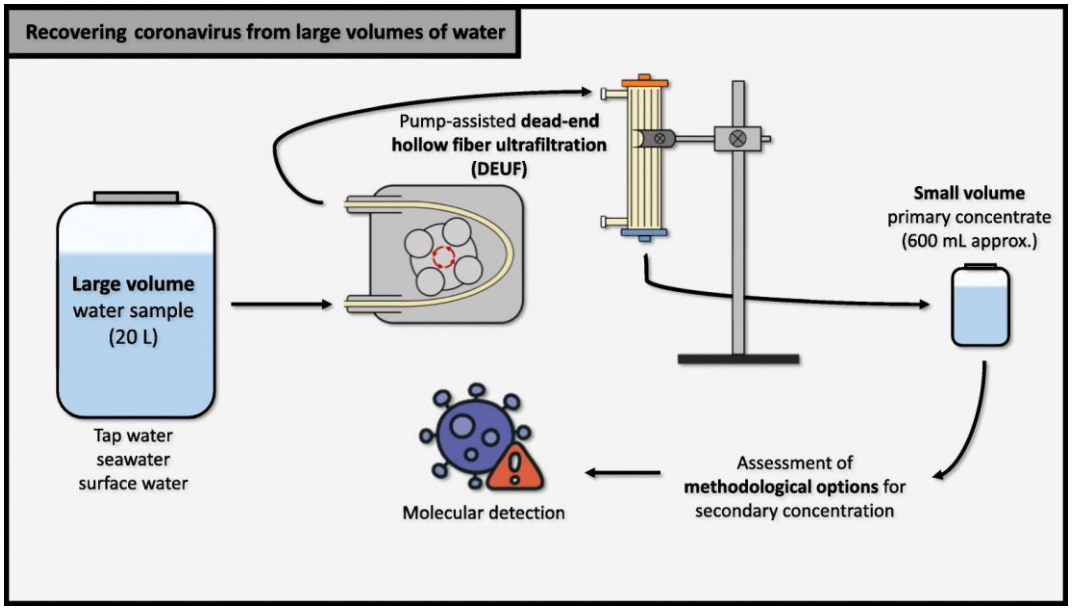
5.2. Recovering coronavirus from large volumes of water

This section is an adapted version of the following published research article:

Enric Cuevas-Ferrando, AlbaPérez-Cataluña, Ana Allende, Susana Guix, Walter Randazzo, Gloria Sánchez (2020). **Recovering coronavirus from large volumes of water.** Science of The Total Environment,143101. DOI: https://doi.org/10.1016/j.scitotenv.2020.143101.



Highlights

• Suitable options to investigate coronavirus in tap water, seawater and surface water were assessed.

• DEUF coupled with PEG-precipitation and SENS-kit better recovered PEDV in tap water.

• High and low centrifugation speeds do not differ in recovering PEDV and mengovirus from seawater.

• Co-concentration of inhibitory substances may occur in seawater and surface water.

1. Abstract

The need for monitoring tools to better control the ongoing coronavirus disease (COVID-19) pandemic is extremely urgent and the contamination of water resources by excreted viral particles poses alarming questions to be answered. As a first step to overcome technical limitations in monitoring SARS-CoV-2 along the water cycle, we assessed the analytical performance of a dead end hollow fiber ultrafiltration coupled to different options for secondary concentrations to concentrate viral particles from large volume of spiked tap water, seawater and surface water together with two quantitative RT-qPCR detection kits. Spiking the porcine epidemic diarrhea virus (PEDV), an enveloped virus surrogate for SARS-CoV-2, together with the mengovirus, we demonstrated that PEG-precipitation and SENS-kit better recovered PEDV (13.10 ± 0.66%) from tap water, while centrifugal filtration resulted the best option to recover mengovirus regardless of the detection kit used. No statistical significant differences were found when comparing high (10,000 ×g) and low (3500 ×g) centrifugation speeds for the secondary PEG- based concentration of spiked seawater, while considerable inhibition was observed for both viruses detected by NoInh-kit assay. Similarly, the co-concentration of PCR inhibitors and viral particles was observed in surface waters detected with either SENS-kit or NoInh-kit and RNA dilution was needed to achieve acceptable recoveries at the expenses of the overall sensitivity of the method. These methodologies represent suitable options to investigate SARS-CoV-2 occurrence in different water resources and allow to conduct on site sampling of large volume of water.

Keywords

Coronavirus; Tap water; Surface water; Seawater; Concentration; RT-qPCR.

2. Introduction

The access to safe and clean water is a universal human right (United Nations, 2010), that has been further questioned by the ongoing coronavirus disease (COVID-19) pandemic. While severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of COVID-19, is mainly a respiratory pathogen, the detection of virus particles in stool supports the hypothesis that fecal-oral transmission may occur (Yeo et al., 2020). Despite this conjecture has not been elucidated yet, wastewater-based epidemiology (WBE) has been implemented worldwide for tracking the pandemic within a given community and for gaining preparedness for future SARS-CoV-2 local outbreaks (Bivins et al., 2020; Farkas et al., 2020; Haramoto et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Randazzo et al., 2020a; Westhaus et al., 2021; WHO, 2020a). Special interests have been also given to the presence of SARS-CoV-2 in effluent wastewater and recreational waters such as river water and seawater to assess public health risks (Cahill and Morris, 2020; Guerrero-Latorre et al., 2020; Liu et al., 2020). A colossal number of laboratories have been involved in wastewater monitoring programs worldwide, being the lack of standard methods the main bottleneck for implementing WBE nation- and world-wide. In this sense, it has been imperative to assess the analytical performances of concentration methods for SARS-CoV-2 in different types of water, as protocols validated for common viral human pathogens such as enteric viruses may not succeed in well-recovering enveloped viruses (Ahmed et al., 2020; Randazzo et al., 2020b). To a larger extent, the controversial debate on the fate of SARS-CoV-2 along the water cycle brought to light the need for the development of robust methods for concentrating enveloped viruses from large volume of water in order to investigate natural water resources such as tap, reclaimed, surface, drinking and sea-waters. In fact, current methods used to concentrate viruses from wastewater are not feasible for larger volumes because of (i) the low viral titers; (ii) the co-concentration of PCR inhibitors (e.g., salt); (iii) the presence of suspended solids, and (iv) the logistics and costs of delivering water samples to laboratories. However, whether the existing methods already validated for concentrating enteric viruses from large volumes are also suitable for enveloped viruses, and therefore used to investigate SARS-CoV-2 contamination in water resources, is unknown.

3. Methods

We assessed the analytical performance of a Dead End Hollow Fiber Ultrafiltration (DEUF) concentration and two quantitative RT-qPCR detection kits with the final aim of developing a tool of interest for studying the potential SARS-CoV-2 contamination of different types of water.

To this end, we concentrated tap, surface and seawaters spiked with porcine epidemic diarrhea virus (PEDV, strain CV777), an enveloped virus member of the Coronaviridae family, and mengovirus (CECT 100000, strain vMC0), a non-enveloped member of the Picornaviridae, used as process controls to evaluate the procedures for concentrating large volume of water. PEDV and mengovirus viral stocks were obtained from Vero and HeLa cells culture infected suspensions, respectively (Puente et al., 2020).

All water samples used in this study were of blinded origin and collected in April–May 2020. Specifically, a large volume (20L) of tap water (n = 2), seawater (n = 2) and surface water (n = 2) was collected as a simple grab sample and transferred to the laboratory within 6 h to be subsequently processed. All water samples were spiked with 107 PEDV genomic copies (gc) and 108 mengovirus gc and primary concentrated by DEUF as detailed by Cuevas-Ferrando et al. (2020). Different options were evaluated for secondary concentrations depending on the type of water: (i) a polyethylene glycol (PEG) precipitation and a centrifuge filtration with Centricon Plus-70 devices with a 30 kDa cutoff NMWL membrane (Merck Millipore Ltd.) for tap water; (ii) a PEG precipitation at 10,000 ×g or 3500 ×g for seawater; (iii) a PEG precipitation at 3500 ×g for secondary concentrating surface waters. RNA extraction from concentrates was carried out using the NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co.), including a purification step with Plant RNA Isolation Aid (Ambion). For RNA detection, two commercially available kits were compared. Specifically, One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio, USA) (referred as SENS-kit) and One Step PrimeScript™ III RT-PCR Kit (Takara Bio, USA) (referred as NoInh-kit) were used. The first kit is claimed to provide a sensitive detection of very small amounts of RNA, while the latter is highly resistant to a wide variety of inhibitory substances. For all assays, undiluted, 10- and 50-fold diluted RNA were tested to check for RT-qPCR inhibitors. Details on RT-qPCR and quantification have been reported by Randazzo et al., 2020a, Randazzo et al., 2020b. The percent virus recovery (r) was calculated as follows:

The effects of the variables considered in this study (concentration method, RT-qPCR kit, virus, dilution) were separately tested for each type of water

sample (tap water, seawater, surface water) by the analysis of variance (ANOVA) followed by the Tukey's HSD as post hoc test to obtain homogenous groups. A P value <0.05 was deemed significant.

4. Results

We defined PEDV and mengovirus recovery yields as the performance characteristic for the viral concentration of spiked tap water, seawater and surface water (Fig. 1, Fig. 2, Fig. 3). Different modifications for the concentration method specific for each type of water were assessed along with two RT-qPCR quantification assays.

Figure 1. Median recoveries (%) and standard deviations of spiked porcine epidemic diarrhea virus (PEDV) and mengovirus (MgV) in tap water primary concentrated by dead-end hollow fiber ultrafiltration followed by a secondary concentration procedure based on a centrifuge filtration or, alternatively, on a polyethylene glycol (PEG) precipitation. Letters denote homogeneous groups according to the analysis of variance (ANOVA) and Tukey's HSD post hoc test ($p < 0.05$).

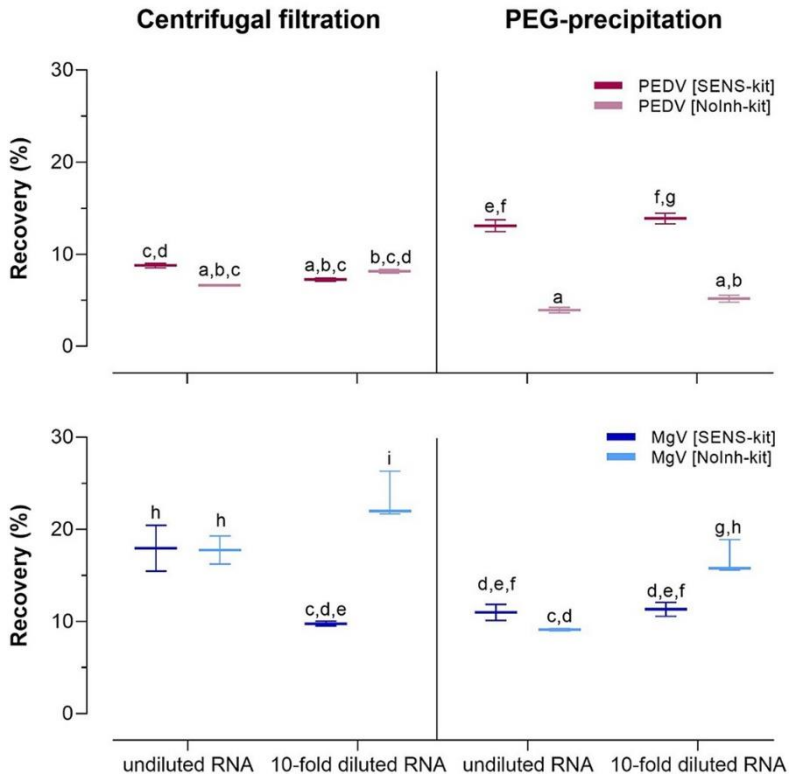


Figure 2. Median recoveries (%) and standard deviations of spiked porcine epidemic diarrhea virus (PEDV) and mengovirus (MgV) in seawater primary concentrated by dead end hollow fiber ultrafiltration followed by a secondary concentration procedure based on polyethylene glycol (PEG) precipitation using a high- (10,000 ×g) or low- (3500 ×g) speed centrifugation.

Letters denote homogeneous groups according to the analysis of variance (ANOVA) and Tukey's HSD post hoc test ($p < 0.05$).

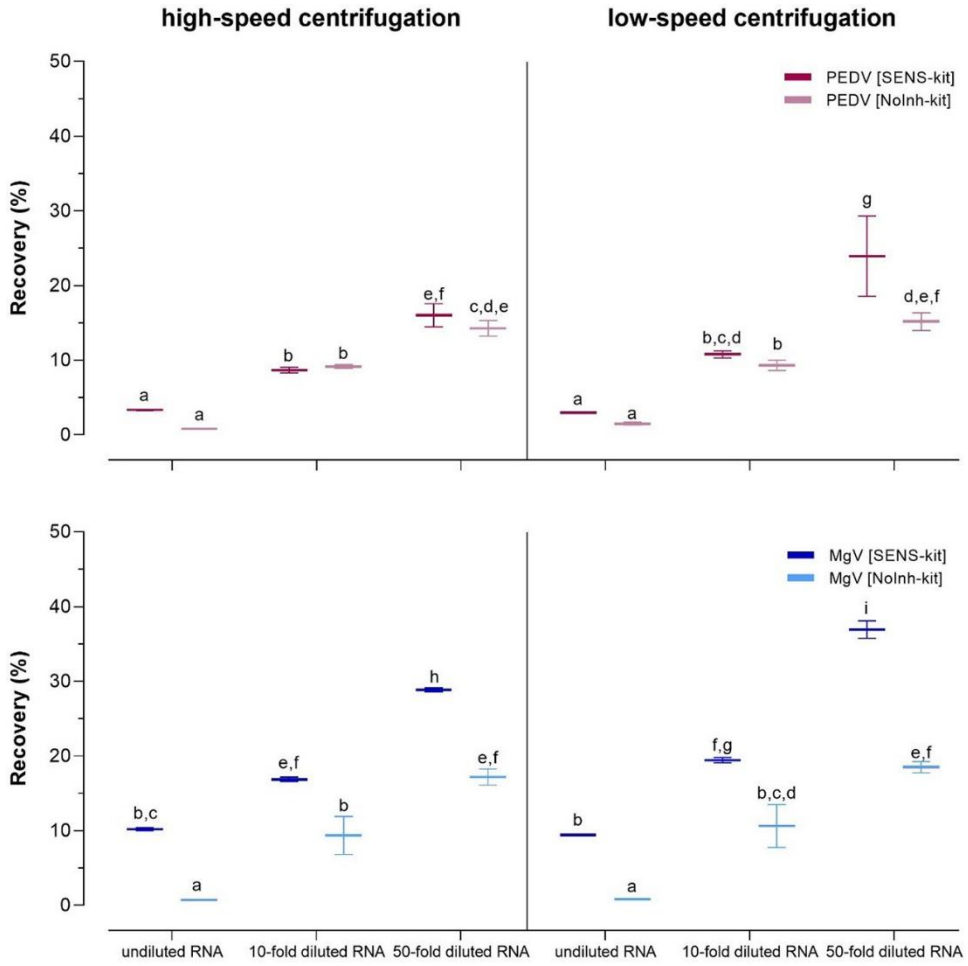
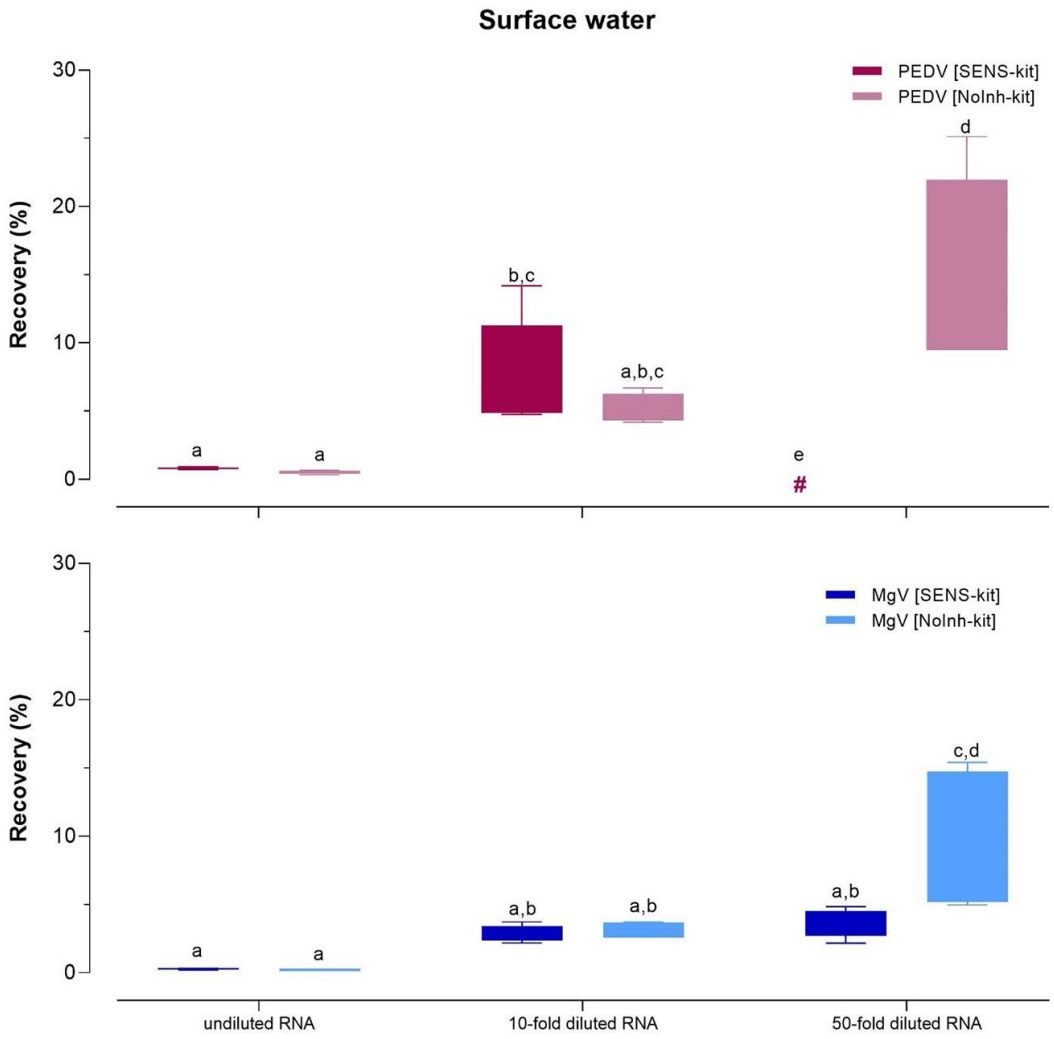


Figure 3. Median recoveries (%) and standard deviations of spiked porcine epidemic diarrhea virus (PEDV) and mengovirus (MgV) in surface water primary concentrated by dead-end hollow fiber ultrafiltration followed by a secondary concentration procedure based on polyethylene glycol (PEG) precipitation using a low- (3500 ×g) speed centrifugation.

#, negative.

Letters denote homogeneous groups according to the analysis of variance (ANOVA) and Tukey's HSD post hoc test ($p < 0.05$).



In tap water, significant differences were observed between centrifugal filtration and PEG precipitation, but not between the SENS-kit and the NoInh-kit. PEDV showed higher recoveries when secondary concentrated by PEG-precipitation and detected by SENS-kit (13.10 ± 0.66%), while lower recoveries of 3.94 ± 0.28% were yielded by NoInh-kit. On the contrary, mengovirus was better recovered with centrifugal filtration regardless of the detection kit used, being the recovery rates of 17.95 ± 2.50% for SENS-kit and 17.76 ± 1.52 for NoInh-kit (Fig. 1). As it could be expected, no significant PCR inhibitions were detected for both PEDV and mengovirus by using either SENS-kit or NoInh-kit in concentrated tap water.

The options evaluated for the secondary concentration of spiked seawater samples showed no statistical significant differences between the centrifugation speeds (10,000 ×g and 3500 ×g), while considerable inhibition was observed for both viruses detected by NoInh-kit assay. Specifically, centrifugation at high (10,000 ×g) and low (3500 ×g) speed recovered 3.36 ± 0.10% and 2.98 ± 0.05% of PEDV, and 10.19 ± 0.19% and 9.45 ± 0.12% of mengovirus, respectively, detecting undiluted RNA with SENS-kit (Fig. 2). On the contrary, when viral detection was carried out by NoInh-kit on undiluted RNAs, recoveries of 0.76 ± 0.00% and 0.84 ± 0.03% for PEDV, and 0.81 ± 0.07% and 1.52 ± 0.17% for mengovirus were yielded at high and low speed, respectively. This indicates the presence of PCR inhibitions that was confirmed by the higher recovery rates achieved by diluting the RNAs by 10-fold and 50-fold (Fig. 2).

Similarly, the co-concentration of inhibitors and viral particles was observed in surface waters detected either with SENS-kit or NoInh-kit. The recovery rates of undiluted RNA resulted as low as 0.82 ± 0.06% and 0.51 ± 0.12% for PEDV and 0.29 ± 0.05% and 0.22 ± 0.02% for mengovirus with SENS-kit or NoInh-kit, respectively. Again, by diluting viral RNA by 10-fold and 50-fold, the recoveries rates resulted higher than 2.89% in all cases (Fig. 3).

5. Discussion

SARS-CoV-2 has been detected in effluent waters from wastewater treatments plants (Randazzo et al., 2020b), and in surface water polluted with wastewater (Guerrero-Latorre et al., 2020; Rimoldi et al., 2020), highlighting the need for protocols to non-sewage testing (Cahill and Morris, 2020; WHO, 2020a).

The present study reports the analytical performances of several modifications of a DEUF method to concentrate viruses from large volumes of tap water, seawater and surface waters of interest for studying the potential contamination of water resources by SARS-CoV-2. Until recently, studies to assess the efficiency of concentration methods in water matrices mostly involved nonenveloped virus, such as human enteric viruses (reviewed by Bofill-Mas and Rusiñol, 2020; Haramoto et al., 2018; Ikner et al., 2012; Matrajt et al., 2018), even the need to investigate enveloped viruses along the water cycle was already raised following SARS, MERS, Ebola and avian influenzas outbreaks (Wigginton et al., 2015). This farseeing call for validated analytical tools lays its reason on the structural and biochemical differences between nonenveloped and enveloped viruses questioning that methods developed for the former would not fit for concentrating the latter. Interestingly, PEG precipitation has been applied as a secondary concentration step to recover enveloped viruses from large volume of water (reviewed by Bofill-Mas and Rusiñol, 2020), and an optimized procedure based on glass wool primary concentration detected naturally occurring alphacoronavirus in surface water in Saudi Arabia (Blanco et al., 2019).

Moreover, to assess the sensitivity of RT-qPCR assays to inhibitors, we compared two quantitative detection kits: one claimed to be optimized for low RNA amounts (SENS-kit) and a second specified to be highly resistant to a wide variety of inhibitory substances (NoInh-kit). Despite the use of a contaminants/inhibitors removal product before RNA extraction (Plant RNA Isolation Aid), we observed a different sensitivity of RT-qPCR assays to co-concentrated inhibitory substances, being the SENS-kit less prone to such limiting factor. Nucleic acid dilution is a well-known approach to evaluate the presence of inhibitors in complex matrices (ISO 15216-1:2017; McKee et al., 2015), however, the sensitivity of the assay decreases according to the dilution factor applied. In our study, we had to dilute up to 50-fold the RNA concentrated from surface water and seawater samples to overcome PCR inhibition effects. This resulted in exceeding the detection limit of the assay in some cases (Fig. 3). Recent studies aimed to detect SARS-CoV-2 in river water reported none to minimal inhibitors carryover in samples concentrated from 1 to 5 l (Guerrero-Latorre et al., 2020; Haramoto et al., 2020; Rimoldi et al., 2020).

Despite the approaches applied to evaluate possible RT-qPCR inhibitions (e.g., RNA dilution, internal or external amplification controls), the concentration methods used and the nature of water sampled, the feasibility of a given method finally relies on its sensitivity. This latter is mostly correlated to the

volume of the concentrated sample but also to the co-concentration of inhibitors. These factors could explain the reason of the divergent proneness to inhibition found in our study, in which large volumes of water samples (20l) were concentrated.

Assessing a secondary concentration method for tap water, we found that PEG-precipitation resulted the best option for concentrating PEDV, an enveloped virus suggested as SARS-CoV-2 surrogate, while the centrifugal filtration was observed to better recover mengovirus, a non-enveloped virus included in the ISO 15216-1:2017 as process control to detect human enteric viruses. In contrast, high and low centrifugation speeds did not significantly differ in recovering both spiked viruses from seawater. These findings are of importance because of the shortage of provision of the centrifugal filtration units currently occurring in European market, which are linked to the current pandemic situation. Similarly, by using either high or low centrifugation speeds, a larger number of laboratories could be involved in seawater monitoring programmes, even those equipped with simple bench centrifuges.

In general, data on water reservoirs contaminated by human enteric viruses are limited (Haramoto et al., 2018), and to date, no evidences of SARS-CoV-2 occurrence in natural water resources have been reported. In line, WHO and CDC agree in defining the risk associated with contracting SARS-CoV-2 via water sources as low (CDC, 2020; WHO, 2020b). However, in the midst of the current pandemic, chances of SARS-CoV-2 transmission routes cannot be excluded, especially in densely populated areas with poor sanitization systems or when overflows occur (Bhowmick et al., 2020; Heller et al., 2020).

Further research is required for monitoring the potential SARS-CoV-2 contamination of downstream waters used for irrigation or recreational purposes, as well as drinking water resources in settings with limited availability of water, sanitation and hygiene (Street et al., 2020).

To this end, large volume of water has to be sampled and concentration methods need to be validated by either using SARS-CoV-2 spiked or naturally contaminated waters, along with the determination of the limit of detection. Meanwhile, the assessed methodologies represent suitable options to investigate SARS-CoV-2 occurrence in different water resources and allow to conduct on site sampling of large volume of water.

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RESULTS

Chapter 6. Implementing rapid molecular methods to infer SARS-CoV-2 infectivity.

Chapter 6. Implementing rapid molecular methods to infer SARS-CoV-2 infectivity.

6.1. Platinum chloride-based viability RT-qPCR for SARS-CoV-2 detection in complex samples

This section is an adapted version of the following published research article:

Enric Cuevas-Ferrando, Walter Randazzo, Alba Pérez-Cataluña, Irene Falcó, David Navarro, Sandra Martin-Latil, Azahara Díaz-Reolid, Inés Girón-Guzmán, Ana Allende & Gloria Sánchez (2021). **Platinum chloride-based viability RT-qPCR for SARS-CoV-2 detection in complex samples**. Scientific Reports, 18120

DOI: https://doi.org/10.1038/s41598-021-97700-x

1. Abstract

Isolation, contact tracing and restrictions on social movement are being globally implemented to prevent and control onward spread of SARS-CoV-2, even though the infection risk modelled on RNA detection by RT-qPCR remains biased as viral shedding and infectivity are not discerned. Thus, we aimed to develop a rapid viability RT-qPCR procedure to infer SARS-CoV-2 infectivity in clinical specimens and environmental samples. We screened monoazide dyes and platinum compounds as viability molecular markers on five SARS-CoV-2 RNA targets. A platinum chloride-based viability RT-qPCR was then optimized using genomic RNA, and inactivated SARS-CoV-2 particles inoculated in buffer, stool, and urine. Our results were finally validated in nasopharyngeal swabs from persons who tested positive for COVID-19 and in wastewater samples positive for SARS-CoV-2 RNA. We established a rapid viability RT-qPCR that selectively detects potentially infectious SARS-CoV-2 particles in complex matrices. In particular, the confirmed positivity of nasopharyngeal swabs following the viability procedure suggests their potential infectivity, while the complete prevention of amplification in wastewater indicated either non-infectious particles or free RNA. The viability RT-qPCR approach provides a more accurate ascertainment of the infectious viruses detection and it may complement analyses to foster risk-based investigations for the prevention and control of new or re-occurring outbreaks with a broad application spectrum.

2. Introduction

The rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to an unprecedented global health and economic crisis. SARS-CoV-2 belongs to the Coronaviridae family, which includes enveloped RNA viruses causing respiratory, enteric, and systemic infections in a wide range of hosts, including humans and animals. Human coronaviruses have been traditionally considered responsible for endemic infections causing common cold symptoms, as in the cases of HKU1, 229E, OC43, and NL63 viruses, while more recently Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV produced more severe epidemics in the Arabian Peninsula and in Asia. COVID-19 symptoms range from mild to severe, in which severe pneumonia and respiratory distress syndrome can lead to death. However, a

significant number of infected people are asymptomatic, making the epidemiological control even more challenging.

SARS-CoV-2 is an airborne human pathogen primarily transmitted through droplets and aerosols, even though its detection in urine and faecal specimens raised the hypothesis of the possible fecal-oral transmission further sustained by the successful viral replication in cell culture (Guo et al., 2021; Meselson, 2020). To control SARS-CoV-2 spread, extreme containment measures have been enforced worldwide along with several epidemiological surveillance strategies, which include tracing confirmed and suspected cases by clinical testing (e.g., SARS-CoV-2 nucleic acid or antigen tests on nasal or oral swabs or saliva samples), and monitoring community transmission by wastewater analysis (known as Wastewater Based Epidemiology, WBE) (A Bivins et al., 2020).

In this context, several molecular assays based on real-time reverse transcriptase polymerase chain reaction (RT-qPCR) have been developed to detect and quantify SARS-CoV-2 RNA in clinical and environmental samples. For instance, a test-based strategy (at least two consecutive negative RT-qPCR tests) has been widely adopted as a general public health guidance for release from (self-) isolation, reincorporation into the workplace, and patient transferral. However, COVID-19 patients can continue to shed viral RNA well beyond clinical recovery and persistent positive RT-qPCR does not necessarily indicate infectiousness (Owusu et al., 2021). In line, surveillance of SARS-CoV-2 by RT-qPCR in wastewaters is currently used as an effective tool to predict the prevalence of COVID-19 in communities however SARS-CoV-2 transmission through wastewater has not been demonstrated.

Besides being a rapid, easy-to-use, and cost-effective technique, RT-qPCR informs on the presence of viral RNA that does not correlate with infectivity, yet such testing is still being used as a surrogate marker of infectivity (Atkinson and Petersen, 2020; Krupp et al., 2020; Romero-Gómez et al., 2021; Widders et al., 2020). On the contrary, viral replication in permissive cell line(s) is widely used to assess viral infectivity, and it has been readily available for SARS-CoV-2. Conversely, the facility requirements needed to handle SARS-CoV-2 infectious materials (biosafety level 3 laboratory, BSL-3), in addition to the low sensitivity and long turnaround time for results, typically from three to ten days, limited its extensive implementation for both clinical diagnosis and environmental risk assessment (Ogando et al., 2020).

Recently, novel molecular techniques, referred to as capsid integrity or viability qPCR assays incorporating viability markers such as monoazide dyes

and metal compounds into qPCR-based methods, have been demonstrated to selectively remove false-positive qPCR signals deriving from free nucleic acids and virions with damaged capsids, finally allowing an estimation on viral infectivity (Leifels et al., 2020). However, the application of such techniques for enveloped viruses has not fully elucidated as it has failed for avian influenza virus (IAV) and infectious laryngotracheitis virus (ILTV) while it has recently been optimized for porcine epidemic diarrhea coronavirus (Bindari et al., 2020; Graiver et al., 2010; Puente et al., 2020) and applied in environmental samples for SARS-CoV-2.

3. Results

Initial assessment of viability markers and RT-qPCR assays

With regard to the viability markers tested, platinum compounds were better at preventing PCR amplification of SARS-CoV-2 genomic RNA suspension than monoazide dyes, regardless of the RT-qPCR target (Fig. 1). Compared to untreated RNA, significant differences were detected for $PtCl_4$ and CDDP treated samples in all the five RT-qPCR targets tested. While $PtCl_4$ completely prevented the RNA amplification for all replicates, it occurred in 5 out of 20 CDDP treated replicates targeting N1, N2 and IP4. Among photoactivatable dyes, 50 µM PMAxx offered the best performance as it removed the signal in 8 replicates showing statistically significant differences for E gene, IP2 and IP4. An additional assay tested 100 µM PMAxx on SARS-CoV-2 genomic RNA without any improvement of the results with respect to 50 µM PMAxx concentration (data not shown). EMA and PEMAx completely removed RT-qPCR signals in 2 out of 20 replicates. Given these preliminary results, we further assessed PMAxx and $PtCl_4$ effect on SARS-CoV-2 gamma- (ca. 8.50×10^5 gc/mL corresponding to 140 TCID50/mL) and heat inactivated (ca. 1.88×105 gc/mL corresponding to 80 TCID50/mL) viral particles by using N1 as the most sensitive RT-qPCR assay among all the compared targets. PMAxx at 50 µM minimally reduced the PCR signals by 2.82 and 3.17 Cts with respect to the gamma- and heat inactivated controls, while the superior ability of $PtCl_4$ was confirmed for both gamma- and heat inactivated SARS-CoV-2 viral particles (Fig. 2). A final concentration of 1.0 mM $PtCl_4$ was needed to consistently prevent the amplification of inactivated viruses by viability RT-qPCR. Thus, we further applied the $PtCl_4$ viability RT-qPCR to high concentrated gamma-inactivated viral suspensions (ca. 8.50×10^6 gc/mL). Results showed that 0.5

and 1.0 mM $PtCl_4$reduced by 3.4 and 6.8 Cts compared to the control. Although significant statistical differences were detected for all treatments regardless of the concentration of the metal compound, only 2.0 mM $PtCl_4$showed to consistently prevent signal amplification (only one positive out of 8 replicates, Ct = 39.11).

Figure 1. Performance of monoazide photoactivatable dyes and platinum compounds on SARS-CoV-2 genomic RNA assessed by targeting five different RNA regions. Dashed grey line represents RT-qPCR theoretical limit of detection for N1, N2 and gen E; dotted grey line represents RT-qPCR theoretical limit of detection for IP2 and IP4. Asterisks indicate significant difference from untreated control for each molecular target: *p < 0.01; **p < 0.001; ***p < 0.0001; ns, not significant.

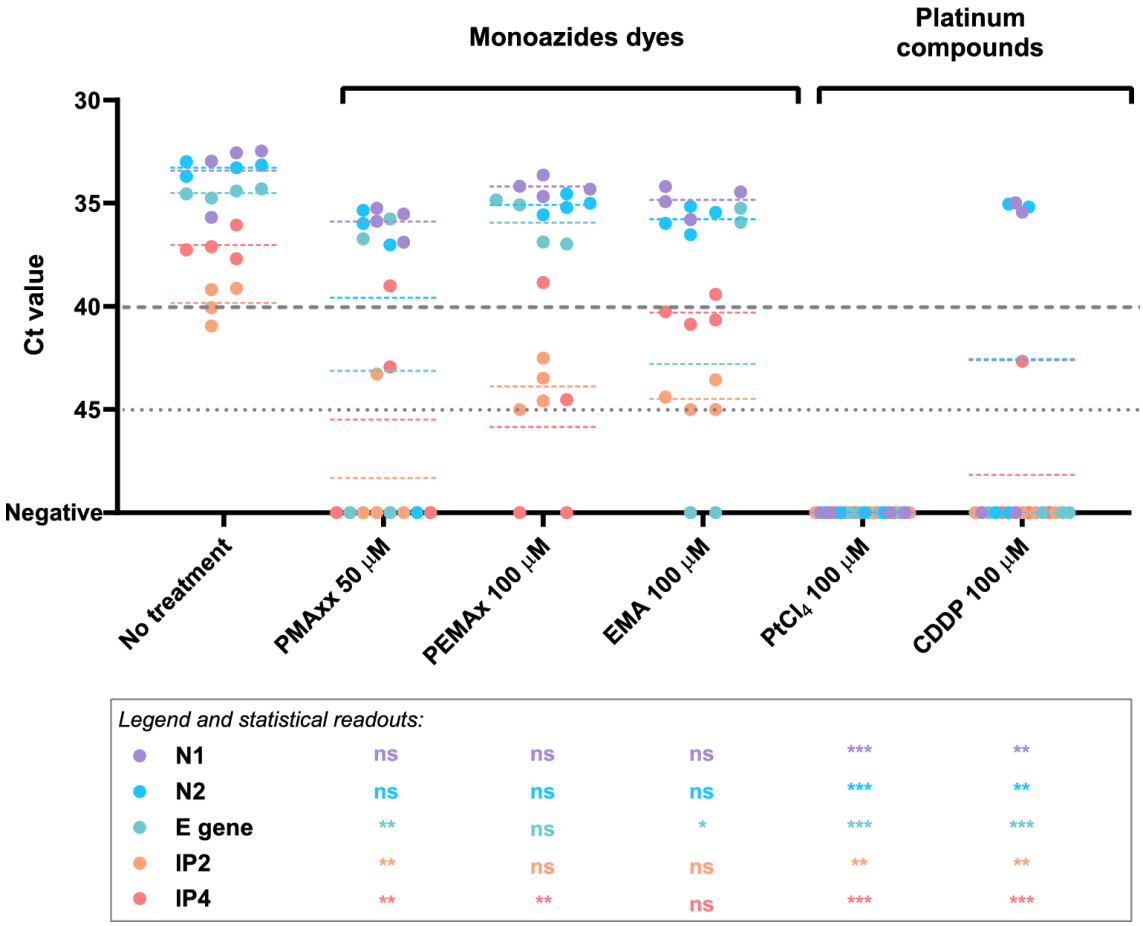
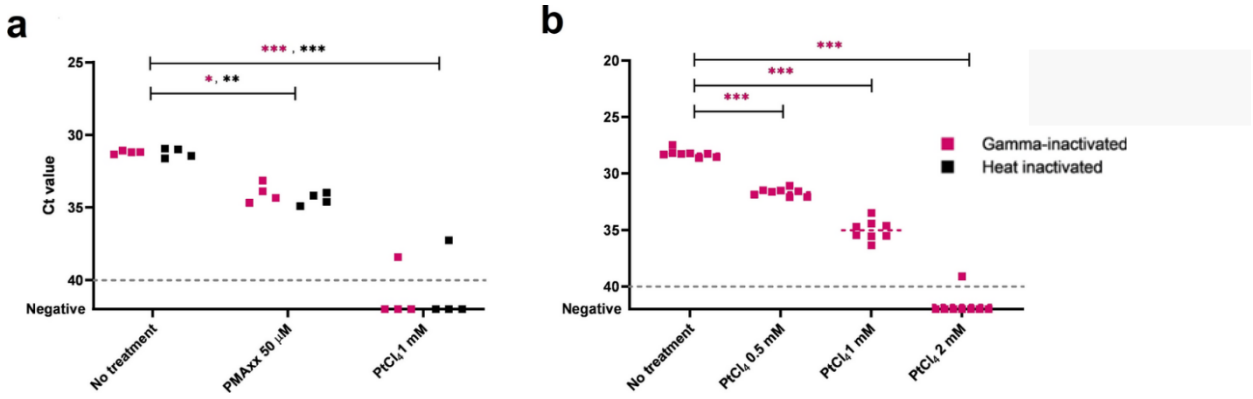


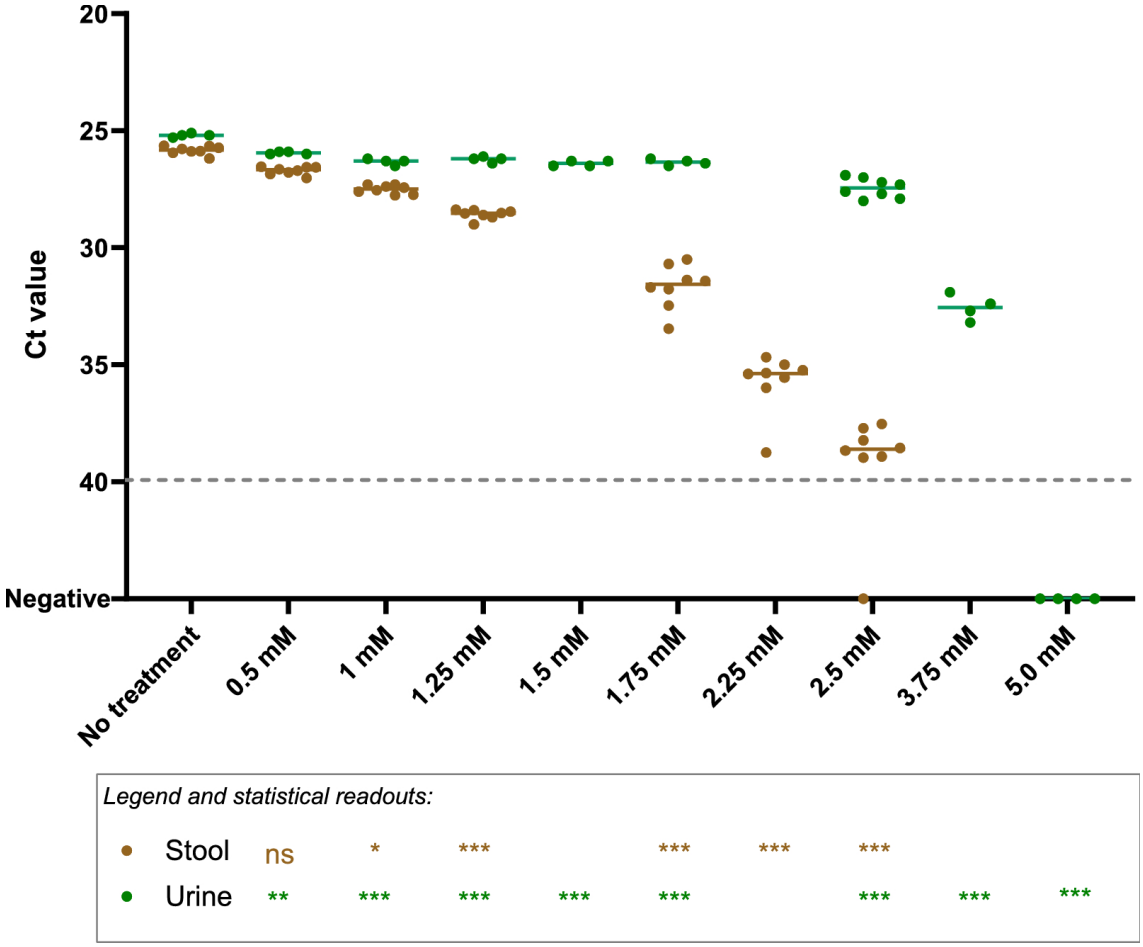
Figure 2. Assessment of viability markers on inactivated SARS-CoV-2 viral particles suspended in PBS buffer at different concentrations. RT-qPCR assays targeted N1 region. (a) Comparison of PMAxx and platinum chloride ($PtCl_4$) viability RT-qPCRs on low (ca. 103 gc/mL) gamma- and heat inactivated SARS-CoV-2 viral particles. (b) Viability RT-qPCR optimization using increasing concentration of $PtCl_4$ on high concentrations of gamma-inactivated SARS-CoV-2 viral particles (ca. 105 gc/mL). Dashed grey lines represent RT-qPCR theoretical limit of detection. Asterisks indicate significant difference from untreated control: *p < 0.01; **p < 0.001; ***p < 0.0001.



Effect of sample complexity on viability RT-qPCR

To determine the effect of sample matrix on viability RT-qPCR, we spiked tenfold diluted stool suspensions (1% w/v final dilution) and urine specimen (10% v/v final dilution) with approximately 105 gc/mL gamma-inactivated SARS-CoV-2, and applied up to 5.0 mM $PtCl_4$ as viability marker. Compared to the untreated control, significant differences were observed for 1.0 mM $PtCl_4$ in urine samples or 1.25 mM PtCl4 in stool suspensions (Fig. 3). However, a concentration of 5.0 mM was needed to completely remove the PCR signals in urine, while 2.5 mM PtCl4 prevented the amplification of 1 out of 8 replicates in stool. Although the complete inhibition of amplification signals was achieved to a limited extent, a sharp difference above one logarithm of genomic copies (ΔCts≈3.3) was observed in stool and urine samples processed with 1.25 and 3.75 mM $PtCl_4$, respectively.

Figure 3. Platinum chloride ($PtCl_4$) viability RT-qPCR on ten-fold diluted faecal suspensions (1% w/v final dilution) (brown dots) and urine specimens (10% v/v final dilution) (green dots) spiked with approximately 105 gc/mL gamma-inactivated SARS-CoV-2. Dashed grey line represents RT-qPCR theoretical limit of detection. Asterisks indicate significant difference from untreated control: *p < 0.01; **p < 0.001; ***p < 0.0001; ns, not significant.



Viability RT-qPCR validation on positive clinical samples and naturally contaminated wastewater

Additional experiments were set up to validate viability $PtCl_4$ RT-qPCR on nasopharyngeal swabs from COVID-19 positive patients and on naturally contaminated wastewater samples. Initial experiments using undiluted samples achieved unsuccessful results (data not showed), thus both clinical

and wastewater samples were ten-fold diluted in PBS buffer. Nine tenfold diluted nasopharyngeal swabs and the corresponding heat-inactivated (95 °C for 10 min) subsamples were processed by RT-qPCR alone and viability RT-qPCR with either 1.0 or 2.5 mM $PtCl_4$ (Fig. 4). Applying 1.0 mM $PtCl_4$ viability RT-qPCR, consistent amplification signals were observed in both naïve and heat-treated samples with minimal Ct differences compared to RT-qPCR alone. Increased concentration to 2.5 mM led to a sharper discrimination of PCR signals (ΔCt = 9.24 ± 3.59). Similarly, the complete prevention of RT-qPCR signals occurred in one out of four samples at 1.0 mM $PtCl_4$, and in three out of five samples at 2.5 mM (Fig. 4). Regardless of the viability marker concentration applied, the complete prevention of amplification was observed in samples with initial low viral titer (Ct values ≥ 30). Moreover, the 2.5 mM PtCl4 viability RT-qPCR was further validated on six wastewater samples naturally contaminated with SARS-CoV-2. The results showed that 2.5 mM $PtCl_4$ completely prevented the amplification in all samples (Fig. 5).

Figure 4. Validation of viability RT-qPCR with either 1 mM or 2.5 mM $PtCl_4$ on ten-fold diluted nasopharyngeal swabs from COVID-19 positive patients. Plotted dots represents the median cycle threshold value (Ct) of naïve and heat-inactivated (95 °C for 10 min) subsamples assayed by RT-qPCR alone and viability RT-qPCR both targeting N1. Dashed grey line represents RT-qPCR theoretical limit of detection.

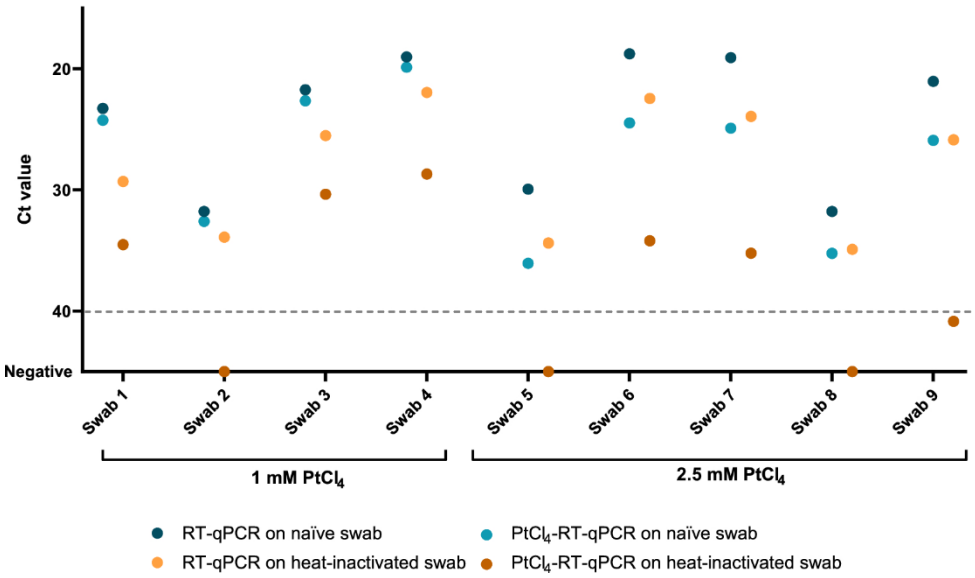
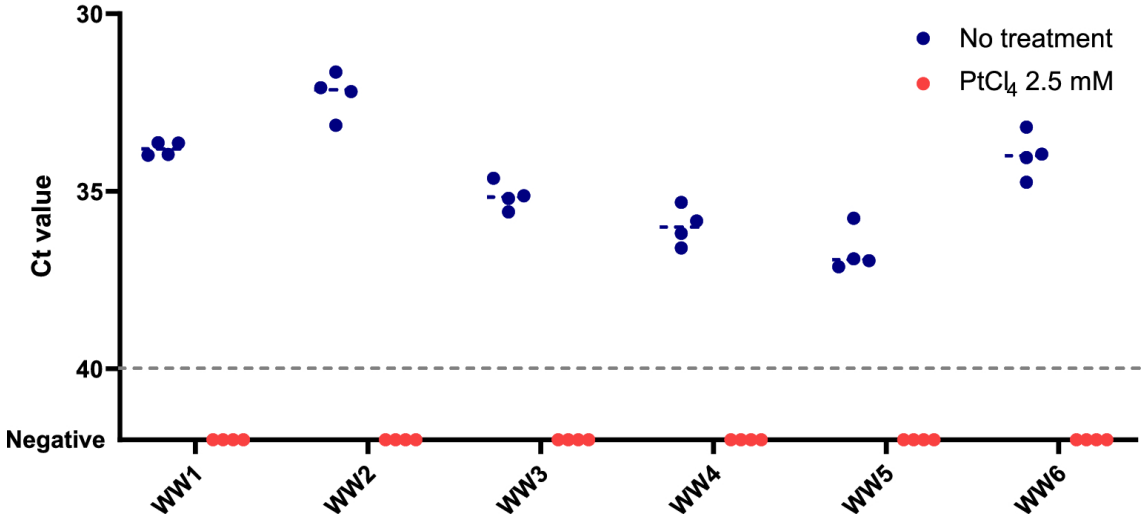


Figure 5. Validation of 2.5 mM $PtCl_4$ viability RT-qPCR on ten-fold diluted naturally contaminated wastewater samples. Dashed grey line represents RT-qPCR theoretical limit of detection.



4. Discussion

Currently, research projects aiming to assess the risk of transmission and exposure to infectious virus either in clinical and environmental settings have been limited by the biosafety level-3 (BSL-3) conditions needed to handle infectious SARS-CoV-2. The research effort of this investigation intended to provide a rapid and sensitive analytical method that selectively detects potentially infectious SARS-CoV-2 in a significantly shorter time than the traditional cell-culture based method and that can be used in a wide range of applications, including clinical and environmental COVID-19 monitoring programs as epidemiological response to the pandemic that is causing such a public health emergency.

This study evaluated photoactivatable monoazide dyes and metal compounds as viability markers applied prior to nucleic acid extraction to prevent amplification of RNA from non-viable viral particles, thus enabling amplification only of viable/infectious viruses in downstream RT-qPCR assay. Selecting platinum chloride as the best performing viability marker, we demonstrated that viability RT-qPCR efficiently discriminated free RNAs and inactivated SARS-CoV-2 inoculated in buffer, stool and urine suspensions. Then, we further proved that the method inferred SARS-CoV-2 infectivity better than RT-qPCR alone in both nasopharyngeal swabs from positive COVID-19 patients and in naturally contaminated wastewater samples. In the case of complex matrices, increased $PtCl_4$ concentration of 2.5 mM and ten-fold sample dilution are recommended because of the high presence of organic matter, suspended solids, and inhibitors that hinder the efficacy of the viability treatment.

Our investigation initially included five well-established molecular assays since the length of the amplicon and/or the richness of secondary structures of targeted RNA may affect the efficiency of viability treatments (Contreras et al., 2011; Soejima et al., 2011). We show that metal compounds performed better than monoazide dyes, irrespective of RT-qPCR assays. However, RT-qPCR targeting N1 region is recommended because of its superior sensitiveness. This aspect is of importance because complex matrices needed to be diluted to achieve a more efficient inference of viral infectivity, as demonstrated in spiked stool and urine, in positive swabs and in contaminated wastewater. Similarly, sample dilution was needed to implement a viability RT-qPCR targeting norovirus in sewage (Randazzo et al., 2016). Moreover, N1 assay better fits the testing on samples with expected low viral concentrations (e.g.,

environmental samples) and/or PCR inhibitors (e.g., concentrated wastewater, stool). As N1 assay has been validated in many laboratories worldwide, this viability method could also be easily and widely implemented.

To the best of our knowledge, this is the first report comparing different conditions to optimize a rapid molecular assay independent from viral replication in cell culture developed to test SARS-CoV-2 infectivity in clinical and environmental samples. A recent investigation by our group demonstrated the suitability of viability RT-qPCRs to infer the infectivity of porcine epidemic diarrhea coronavirus, member of the genus Alphacoronavirus within Coronaviridae family (Puente et al., 2020). Interestingly, we were able to demonstrate that PMAxx viability RT-qPCR matched the thermal inactivation pattern obtained by cell culture better than other viability markers, including $PtCl_4$, and RT-qPCR alone. Alternative rapid methods to assess the viability of enveloped viruses have been explored with inconclusive results. These included propidium monoazide and immunomagnetic separation tested on laryngotracheitis virus and ethidium monoazide on avian influenza virus (Bindari et al., 2020; Graiver et al., 2010). It is worth to report that during the reviewing process of this study few studies have been reporting the use of PMA or PMAXX to discriminate live and dead SARS-CoV-2 in environmental samples.

we also tested on inactivated SARS-CoV-2 suspensions a porcine gastric mucine in situ capture RT-qPCR, a method that was originally implemented in our laboratory for human enteric viruses (Falcó et al., 2019). Although proper SARS-CoV-2 infectious controls could not be included, those experiments resulted in inconclusive outcomes (data not shown).

Our viability RT-qPCR results of nasopharyngeal swabs from positive COVID-19 patients indicate the potential infectivity of the samples, while naturally contaminated wastewater are unlikely to contain infectious viral particles. These later findings reflect the viral replication in cell culture from RNA positive stool and respiratory samples as well as the unsuccessful attempts to isolate and cultivate SARS-CoV-2 from wastewater samples (Rimoldi et al., 2020; Singanayagam et al., 2020; Wölfel et al., 2020).

Our findings are clinically relevant as RT-qPCR has become the primary method to diagnose COVID-19. However, as it detects RNA, its ability to determine the infectivity of patients is limited (Atkinson and Petersen, 2020; Krupp et al., 2020; Romero-Gómez et al., 2021; Widders et al., 2020). In addition, the immune system can neutralise SARS-CoV-2 preventing subsequent infection but not eliminating nucleic acid, which degrades slowly

over time. This has been confirmed in cohort studies that concluded that seroconversion does not necessarily lead to the elimination of viral RNA, with cases being RT-PCR positive up to > 63 days after symptom onset despite having neutralizing antibodies (Liu et al., 2020; Molina et al., 2020; Vibholm et al., 2021). Furthermore, some reports correlated the infectiousness of upper respiratory tract samples with RT-qPCR Ct values in COVID-19 cases. Analysing a large dataset (n=324), Singanayagam and colleagues demonstrated that the probability of viral recovery from samples with 27.5 < Ct < 30 was ≈66%, decreasing to ≈ 28% for 30 < Ct < 35, and to 8.3% for Ct > 35 (Singanayagam et al., 2020). Similarly, Bullard observed SARS-CoV-2 cell infectivity only for respiratory specimens sampled < 8 days symptom onset with Ct < 24 (Bullard et al., 2020). However, viral replication was also obtained from samples with elevated Ct values of 36-39 ("Clinical and virologic characteristics of the first 12 patients with coronavirus disease 2019 (COVID-19) in the United States.," 2020; Romero-Gómez et al., 2021). Notwithstanding, the correlation between RNA and virus isolation remains unclear.

Unfortunately, we had no access to clinical samples with higher Ct values which are likely to contain non-infectious particles to contrast such hypothesis by the proposed viability RT-qPCR. In addition, the ratio between viral shedding and infectivity has been reported to vary along the course of the infection (Owusu et al., 2021; Widders et al., 2020; Wölfel et al., 2020). This information regarding epidemiological characteristics, symptom history and relevant sampling details included in medical records could have explained at least in part the different performances of viability RT-qPCR among clinical samples, however it could not be retrieved as de-identified specimens were analysed in this study.

Despite the viability treatment, we detected residual signals in heat inactivated nasopharyngeal swabs. This could be attributed to the viral envelop and nucleoproteins that limit the access and/or the binding of viability markers to SARS-CoV-2 RNA, as hypothesised for avian influenza virus and bacteriophage T4 (Fittipaldi et al., 2010; Graiver et al., 2010). The enveloped structure of coronaviruses may also explain the increased concentration of viability markers needed for SARS-CoV-2 and PEDV compared to human enteric viruses (Puente et al., 2020; Randazzo et al., 2018, 2016). This cumbersome finding obtained by the proposed viability procedure suggests that the overestimation of the infectivity of a given sample may occur which, although warranting a careful interpretation, represents a conservative prediction. Despite the fact that this pretreatment is a step forward to better interpret quantification of SARS-CoV-2, improvements in the procedure needs

to be undertaken. For instance, it has recently suggested to combine viability dyes with surfactans. Moreover, by collecting clinical samples in PBS buffer has the disadvantage in terms on biohazard samples management.

With regards to wastewater samples that tested positive for SARS-CoV-2 RNA, they most probably contained detergents and chemicals that are detrimental to viral infectivity further supporting the efficacy of the viability RT-qPCR in discriminating potentially infectious and inactivated viral particles (Khokhar et al., 2020). The ultimate confirmation on the infectivity of the samples by cell culture, although recommendable, could not be provided. Nonetheless, recent laboratory scale investigations on SARS-CoV-2 persistence in wastewater estimated that the infectious titer of spiked virus decreases by one logarithm (T90) in 1.5-1.9 days at room temperature, and in few minutes at higher temperatures (Aaron Bivins et al., 2020; de Oliveira et al., 2021). This evidence suggests that naturally contaminated wastewater samples are unlikely to contain infectious particles. Detection of SARS-CoV-2 by either culture and viability RT-qPCR is valuable as a proxy for infectiousness; however, as the human infectious dose remains unknown, the significance of low titres of infectious virus for human-to-human transmission remains uncertain. Above all, as some individuals reportedly remain PCR positive weeks after SARS-CoV-2 infection recovery, knowing whether viral RNA in these persistent carriers is contagious provides key insights for quarantine policy, to safely discontinue self-isolation and contact tracing as essential public health measures to definitively prevent transmission (Esteve et al., 2020; Vibholm et al., 2021; Widders et al., 2020). Besides some limitations, the proposed viability RT-qPCR effectively reduced the amplification signals of non-infectious and free RNA of SARS-CoV-2 in complex matrices finally providing a better estimation of the infectiousness of samples. Thus, mathematical models derived from laboratory scale experiments comparing viability RT-qPCR and viral replication could correlate viral load and infectivity, finally providing relevant tools of interest based on rapid molecular assay for prevention strategies and risk assessment.

In conclusion, the use of pre-treatments to prevent RT-qPCR amplification of RNA from non-infectious SARS-CoV-2 using platinum chloride as a viability marker of infectivity was implemented in stool and urine samples and successfully validated in naturally contaminated wastewater samples, supporting the idea that SARS-CoV-2 present in sewage is not infectious. Residual amplification signals in nasopharyngeal swabs exposed to heat-inactivation overestimated the amount of viable virus, still providing a conservative interpretation of the infectiousness of the sample. The authors

believe that the described platinum-based viability RT-qPCR method would be of great use in the research field, especially when involving environmental samples such as wastewater. As a limitation, the high viral loads that characterize nasopharyngeal samples from infected individuals resulted in less reliable outcomes thus making the method not optimal for clinical monitoring routines. Even so, as for future work, it would be interesting to apply this protocol in samples from infected patients who have already been vaccinated or are in the final stage of the disease and therefore present higher Cq values.

Overall, our study proposes a rapid analytical tool based on viability RT-qPCR to infer SARS-CoV-2 infectivity with potential application in risk assessment, and prevention and control in public health programmes.

5. Methods

Viral materials, viability markers and optimization of viability treatment

SARS-CoV-2 genomic RNA (VR-1986D™, ATCC, VA, US), gamma-irradiated (5 × 10^6 RADs) (NRC-52287, BEI Resources, VA, US) and heat inactivated (65°C for 30 min) (NR-52286, BEI Resources, VA, US) viral particles preparations all obtained from isolate USA-WA1/2020 were used for initial screening of viability markers. Specifically, monoazide photoactivatable dyes and platinum compounds were initially screened as viability marker candidates using SARS-CoV-2 genomic RNA, gamma-inactivated, and heat inactivated SARS-CoV-2 suspensions. Viability marker stock solutions were prepared as follows and stored at −20 °C for later use: ethidium monoazide (EMA™, Geniul, Spain) was diluted in dimethylsulfoxide (DMSO) to 2.0 mM, PEMAX™ (Geniul, Spain) and propidium monoazide (PMAxx™, Biotium, CA, US) were diluted in nuclease-free water to 4.0 mM, platinum (IV) chloride ($PtCl_4$; Acros Organics, NJ, US) and cis-diamineplatinum (II) dichloride (CDDP; Sigma-Aldrich, MO, US) salts were dissolved in DMSO to 1.0 M and further diluted in nuclease-free water to 50 mM. Viability assays were carried out by treating 300 µL of either genomic SARS-CoV-2 RNA (approx. 10^3 gc/mL), gamma-inactivated (approx. 10^5 gc/mL), and heat inactivated SARS-CoV-2 (approx. 10^5 gc/mL) suspensions with final concentrations of 50-100 µM photoactivatable dyes (PMAxx™, PEMAX™, or EMA™) or 0.1-2.0 mM platinum compounds (CDDP or $PtCl_4$) in DNA LoBind tubes (Eppendorf, Germany). Photoactivation of monoazide dyes was achieved by 10 min of dark-incubation in an orbital shaker (150 rpm) at room temperature (RT) followed by 15 min blue LED light exposure in a photo-activation system (Led-Active Blue, GenIUL). Alternatively, 30 min incubation

at RT in an orbital shaker (150 rpm) were used for viability treatments with platinum compounds. A control consisting of genomic RNA or virus suspension without viability marker was included in each assay. Following the viability treatment, the viral RNA was immediately purified as described hereafter.

Assessment of $PtCl_4$ viability RT-qPCR in artificially inoculated and validation in naturally contaminated samples

Platinum (IV) chloride was selected as the most reliable viability marker and tested at final concentrations of 0.5 to 5.0 mM for viability RT-qPCR optimization in stool, urine, nasopharyngeal swabs and wastewater samples.

For the initial optimization, stool and urine specimens that had tested negative for SARS-CoV-2 were retrieved from IATA biobank. Faecal material was resuspended 1% w/v in phosphate-buffered saline (PBS), and supernatant recovered by centrifugation at 2000 × *g* for 5 min. Direct and ten-fold diluted urine, and ten-fold diluted faecal suspension (final 1% *w*/*v* faecal dilution) were spiked with either gamma- and/or heat inactivated SARS-CoV-2 to approximately 10^5 gc/L final concentration.

Then, nasopharyngeal swabs from positive COVID-19 patients and naturally contaminated wastewater samples were used to validate the viability $PtCl_4$ RT-qPCR. Nasopharyngeal swabs (n=9) from COVID-19 positive patients were originally collected at Hospital Clínico Universitario de Valencia (Valencia, Spain) and included in this study once de-identified. To test whether the detection of viral RNA was exclusive for infectious particles, nasopharyngeal swab subsamples were inactivated at 95 °C for 10 min, included in the experiments along with naïve specimen and both assayed by RT-qPCR and viability $PtCl_4$ RT-qPCR.

SARS-CoV-2 positive wastewater grabbed samples (n=6) were collected in June-October, 2020 from different wastewater treatments plants involved in a WBE monitoring programme. The samples were originally concentrated by an aluminium precipitation procedure and tested positive for at least two RT-qPCR targets (N1, IP4 or E gene) (Randazzo et al. 2020). To exclude additional viral inactivation due to the concentration procedure, wastewater were freshly concentrated by Centricon-Plus 70 centrifugal ultrafilters units with a cut-off of 100 kDa (Merk-Millipore, MA, US) (Medema et al. 2020). Samples were all diluted in PBS as specified. Viability treatment, RNA extraction and SARS-CoV-2 detection were carried out as hereafter detailed.

Viral RNA purification and SARS-CoV-2 detection

Viral RNA was extracted using Maxwell® RSC 16 instrument and Maxwell RSC Pure Food GMO and authentication kit (Promega, Spain) and detected by RT-qPCR targeting N1, N2, E gene, IP2 and IP4 regions (Pérez-Cataluña et al. 2021). Viral RNA from nasopharingeal samples was extracted using a KingFisher™ Flex (Thermo Fisher Scientific) instead. Given the superior sensitivity of N1 RT-qPCR resulting from the initial screening, this target was used for subsequent determinations. Each RT-qPCR assay was performed in duplicate and included nuclease-free water as negative control, and SARS-CoV-2 complete genomic RNA (VR-1986D™, ATCC, VA, US), E gene plasmid (10006896, 2019-nCoV_E Positive Control from Charité/Berlin, IDT, Belgium) or N1/N2 plasmid (10006625, 2019-nCoV_N_Positive Control from CDC, IDT, Belgium) as positive controls. Ten-fold RNA dilutions were consistently tested to check RT-qPCR inhibition due to viability marker residues or inhibitory substances in the sample.

Statistical analysis

All data were compiled from three independent experiments with at least two technical replicates for each variable. Data are presented as median ± SD. Significant differences in median cycle threshold (Ct) were determined by using either one- or two way(s) ANOVA followed by Dunnett's multiple comparisons test on GraphPad Prism version 8.02 (GraphPad Software, US). Differences in means were considered significant when the p was <0.05.

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GENERAL DISCUSSION

IV. GENERAL DISCUSSION

Development of procedures to concentrate and detect Hepatitis E virus in water samples

At the time of starting this thesis, when it came to the detection of enteric viruses, validated protocols for the concentration of HuNoVs, HAVs, and RVs in water samples had already been published (Bosch et al. 2011; Haramoto et al. 2018a; Rusiñol et al. 2014), but there was a noticeable gap in the literature regarding the concentration of hepatitis E virus (HEV) in these types of samples (Fenaux et al. 2019). Furthermore, HEV had recently been acknowledged as an emerging pathogen in industrialized countries, especially in Europe, and the need for standard methods for its concentration and detection had been highlighted by the EFSA (Ricci et al. 2017). Therefore, establishing concentration methodologies and validating RT-qPCR procedures for the detection and quantification of HEV in different types of water samples was one of the specific objectives of this thesis, as indicated in the objectives section. Thus, in study 1.1 an ultracentrifugation-based technique (UC) and an aluminium adsorption-precipitation approach (Al) were evaluated by using artificially contaminated influent wastewater samples to provide information on the efficacy of the HEV detection methods in environmental waters. The analysed concentration methods displayed average HEV recoveries of 15.2%, 19.9%, and 16.9% in influent, effluent, and drinking water samples, respectively, and detection limits ranged from 10^3 to 10^4 international units (IU)/L. Obtained results were comparable to, or slightly lower than, the values previously reported for other enteric viruses in influent wastewaters (about 10^4–10^5 genome copies/L) (Nordgren et al. 2009; Randazzo et al. 2019). Results related to viral recovery were in line with the literature (Miura et al. 2016). In addition to the concentration protocol, it is important to evaluate the subsequent extraction and amplification processes, as the limit of detection ($LOD_{95\%}$) may vary depending on the extraction method or RT-qPCR kit used. Thus, the performances of column-based and magnetic beads-based extraction methods were compared in this study along with three different RT-qPCR assays for HEV RNA detection.

In addition to wastewater samples, in study 1.1 a procedure for concentrating HEV from drinking water samples based on a Dead End Hollow Ultrafiltration (DEUF) protocol followed by a PEG secondary concentration step was

assessed. Results showed that DEUF protocol paired with the column-based extraction kit efficiently recovered HEV from 20L of artificially contaminated drinking water samples.

The results of this study validated the appropriateness of the selected methods on HEV concentration and were used both in study 1.1 itself and in subsequent published works where the presence of HEV and other enteric viruses as well as SARS-CoV-2 was monitored in wastewater, drinking water and surface water samples.

Impact of library preparation when characterising the virome of influent and effluent samples from wastewater treatment plants.

In recent years, there has been a surge in the use of metagenomics for the characterisation of viromes and its application to wastewater research, particularly in wastewater-based epidemiology. However, the lack of reference procedures can produce differing outcomes depending on the selected analysis parameters. Study 2.1 compared two distinct library preparation kits to examine the virome profiles in influent and effluent wastewater samples obtained from four wastewater treatment plants: the ScriptSeq v2 RNA-Seq (LS) and the NEBNext Ultra II RNA (NB) library preparation kits.

Results from this study revealed significant disparities in the ability to identify viral faecal markers, as well as viruses themselves, when using high-throughput sequencing. Resulting data demonstrated the heterogeneity of the virome investigations as well as the impact of the library employed. Also, the sensitivity of each library was evaluated utilising mengovirus (MgV) as a process control for both metagenomic and RT-qPCR studies; sensitivity was greater when using the NEBNext Ultra II RNA (NB) library. In contrast with Adriaenssens et al. (2018), where no MgV reads were retrieved from samples of spiked water and sediment, between 6.0-95.1% and 98.4-100% of the MgV full genome was recovered from the ScriptSeq v2 RNA-Seq (LS) collection and the NB library, respectively. The authors in Adriaenssens et al. 2018 claim that this was most likely caused by the addition of a DNase inactivation phase at 75°C, which may have worsened the effects of the RNase step. Therefore, when comparing sequencing libraries, using models of a virus of interest may be a very useful technique for library selection as it adds extra information of whole process efficiency.

Furthermore, results from both libraries were combined to analyse the virome of influent and effluent wastewaters. In high concentrations, influent wastewaters contained phages such crAssphage, Aeromonas phages, Escherichia phages, or viruses from the *Microviridae* family. Even though further research, including a larger sampling design, must be done, the lack of these viruses in effluent samples may have been attributable to the sanitation practices used in WWTPs. These findings were consistent with other research demonstrating a significant prevalence of bacteriophage families in influent sewage samples (Aw, Howe, and Rose 2014; Cantalupo et al. 2011; Fernandez-Cassi et al. 2018; Rusiñol et al. 2020; Wang et al. 2018). However, other investigations indicated that *Virgaviridae* was the most prevalent virus family (Furtak et al. 2016). Virome profiling may have differed from other studies due to the impact of library sequencing as well as the inherent properties of the virome that are specific to the sample and the area of study. On the other hand, a decrease in viruses that were highly present in influent waters after the WWTP treatment may have been the cause of the increased relative presence of other viruses or even their detection only in effluent samples.

It has been reported that treated wastewater contains human enteric viruses (Adriaenssens et al. 2018a). However, metagenomics investigations don't always detect all these pathogenic viruses (Adriaenssens et al. 2018a). This shows that when viral diseases are in low abundance among a vast background of bacteriophages, then metagenomics has limited sensitivity in identifying them. While Adriaenssens et al. (2018) reported between 10 and 110 million, the amount of produced paired-reads per sample in study 2.1 was 3.2 million and 11.5 million for LS and NB, respectively. This difference greatly increased the likelihood of retrieving whole or partial viral genomes. The selection of target RNA before library preparation with a capture utilising VirCapSeq-VERT target enrichment, as reported for norovirus, is an alternative way to characterise and identify individual viruses (Strubbia et al. 2019). These findings underlined the need for further studies to elucidate the influence of sequencing procedures in virome profiles in wastewater matrices, in order to improve knowledge of the virome in the water environment.

Prevalence of enteric viruses and viral indicators in influent and effluent water samples from wastewater treatment plants

Given the efficient performance results of the aluminium chloride adsorption-precipitation virus concentration method in study 1.1, the method was subsequently used to monitor the prevalence of HEV and other enteric viruses, as well as crAssphage novel viral faecal contamination indicators in influent and effluent water samples. Samples were collected from four different WWTPs located in different areas of the Valencian region (Spain) over a one-year period.

Although the incidence rates across WWTPs varied greatly, study 1.1 found that HEV was extensively distributed (30.6%) in Valencian influent wastewater samples. Similar prevalence (from 13.5 to 43.5% in influent waters, with absence or low detection of HEV in effluent waters) had been seen in studies performed in Barcelona (Spain) (Clemente-Casares et al. 2003; Rodriguez-Manzano et al. 2010; Rusiñol et al. 2015). Study 1.1 revealed HEV contamination in influent wastewaters ranging from around $1.3x10^3$–$3.5x10^4$ IU/L. These results were similar to values previously reported (Fenaux et al. 2019; Randazzo et al. 2018). In effluent waters, HEV genomes were not found. These findings agreed with the majority of studies conducted in Europe, even those conducted after an outbreak was formally verified (Fenaux et al. 2019; Miura et al. 2016). This implies that, even though a decrease of 1–2 $\log_{10}$ GC/L would result in concentrations below $LoD_{95\%}$, treatments used at WWTPs were effective in eliminating HEV. Therefore, more progress is required to boost the methodologies used to detect virus content in effluent waters. Besides this, 56 samples were collected upstream and downstream of two drinking water treatment plants (DWTPs), and 20L water samples were concentrated by DEUF protocol in conjunction with PEG precipitation, a column-based extraction method, and RT-qPCR analysis. Although all samples showed the required minimum recovery rate of MgV (1%), none of the influent and effluent drinking water samples tested positive for HEV, which suggests that the sanitation process used by the DWTP was efficient.

In study 3.1, influent and effluent wastewater samples from the same four WWTPs in the Valencian region (as in studies 1.1 and 2.1) were processed in parallel using a capsid-integrity RT-qPCR protocol (PMAxx-RT-qPCR) to find the presence of potentially infectious HuNoV GI, HuNoV GII, HAV, RV, and HAstV, as well as crAssphage indicators. Overall, HuNoV GI, HuNoV GII, and RV were

detected in practically all the processed influent water samples and in half of the tested effluent samples, thus implying a potential hazard for wastewater discharge or reuse. This represents a real risk for public health as more than 80% of wastewater around the globe (up to 95% in some of the least developed nations) is said by United Nations reports to flow back into the environment untreated (UN WWDR, 2017). Results showed average reductions in upstream and downstream wastewater of 2–3 $\log_{10}$ GC/L, in keeping with the recently published literature (Sabar, Honda, and Haramoto 2022), however these do not comply with the recent European regulation regarding reclaimed water. To validate monitoring programmes of reclaimed water used for agricultural irrigation, it is necessary to see a decrease in rotavirus, total coliphages, or at least one of them (F-specific or somatic coliphages), by 6 $\log_{10}$ GC/L (Regulation (EU) 2020/741, 2020). However, as noted by the scientific community and water managers, explicit rules need to be developed internationally (Gerba, Betancourt, and Kitajima 2017; Sano et al. 2016).

Due to its specificity to human faecal contamination, its high concentration in sewage, and its global distribution in recent years, crAssphage has become a viral water quality indicator (Bivins et al. 2020; Farkas et al. 2019; Honap et al. 2020). In influent and effluent wastewater samples from study 3.1, crAssphage concentrations ranged from 7.41 to 9.99 $\log_{10}$ GC/L and 4.56 to 6.96 $\log_{10}$ GC/L, respectively. These values were up to 3–4 $\log_{10}$ GC/L greater than those of the targeted human enteric viruses. All samples tested positive for crAssphage, and effluent samples had a mean drop of 2.73±0.68 $\log_{10}$ GC/L compared to influent samples, which is in concordance with the literature (Sabar et al. 2022).

Recent research analysing wastewaters (Crank et al. 2020; Farkas et al. 2019; Malla et al. 2019; Tandukar, Sherchan, and Haramoto 2020), sludge (Zu et al. 2020), and other faecal contaminated fluids has documented the association between crAssphage and human viral infections (Jennings et al. 2020). Overall, the consistently high levels of crAssphage found in all influent and effluent samples support the use of the phage as a marker for faecal contamination of wastewater. Although this was the main hypothesis addressed in study 3.1, correlation readouts did not conclusively support the use of crAssphage as an indicator for the presence of potentially infectious enteric viruses in wastewater. Therefore, for both research and monitoring, it makes sense to use a method that specifically targets each viral target rather than one that focuses merely on phage detection. In concordance with earlier reports, the

findings of study 3.1 also showed that *E. coli*, which is used in the existing regulation as a faecal biomarker, and physicochemical criteria are not suitable as markers for the viral contamination of wastewater (Ahmed, Kitajima, et al. 2020; Stachler et al. 2018).

Implementing a SARS-CoV-2 monitoring system in wastewater: Wastewater Based Epidemiology (WBE) as an early-warning tool for pandemic response management.

Given the previous work at the start of this thesis on the monitoring of enteric viruses (studies 1.1 and 3.1), implementing a SARS-CoV-2 monitoring system in wastewater was added as a specific objective shortly after the COVID-19 pandemic emerged. As a result, in response to the ongoing pandemic scenario, the intention was to establish a wastewater monitoring system to detect the variation of COVID-19 cases in certain geographic locations that would serve as an early-warning tool for COVID-19 tracking. Obtained SARS-CoV-2 titers in influent wastewater samples from Murcia (Spain) were similar to the ones that had been reported in Massachusetts and France by that time, where peaks of 4 and 5 to more than 6 $\log_{10}$ GC/L had been recorded (Wu et al. 2020; Wurtzer et al. 2020). Digital RT-qPCR (dRT-qPCR) research could have been done to determine a more accurate way to estimate SARS-CoV-2 levels in wastewater. Although it might not have been the most practical and financially viable option for environmental surveillance (Abachin et al. 2018), dRT-qPCR could have been used to quantify samples with low viral loads, as published for HuNoVs in wastewater (Monteiro and Santos 2017) and SARS-CoV-2 in clinical samples (Dong et al. 2021; Suo et al. 2020).

According with study 4.1., when cases were diagnosed within the municipality, RT-qPCR amplification signals were typically seen in wastewaters. In low-frequency localities positive wastewater samples were found. Interestingly, positive wastewater samples were found in some of the sampled towns even 12–16 days before COVID-19 cases were reported.

Similar research carried out in Paris (France) showed that SARS-CoV-2 genome could be found before the epidemic's exponential phase (Wurtzer et al. 2020). Nevertheless, findings in study 4.1 showed that SARS-CoV-2 could be found weeks before the first verified clinical case. Early discovery of SARS-CoV-2 RNA in wastewater might have warned about the imminent risk, allowing management enough time to plan and put measures in place to stop the

disease's spread. These findings thus suggested that WBE might be employed as a community-wide early warning system for COVID-19 infection. On the other hand, this environmental monitoring could also be used as a tool to lessen the chance that restriction measures would be lifted too soon. How to lessen the possibility of a "second wave" and/or recurrent local outbreaks, for instance, was a crucial concern. Although large-scale population testing is generally the preferred option, wastewater monitoring of SARS-CoV-2 RNA can provide a trustworthy picture of the present situation in its absence. The prevalence of COVID-19 verified cases did not quantitatively match the resulting wastewater data. For a better understanding of the wastewater monitoring data, a quantitative model that considers and corrects all factors should be used. For example, SARS-CoV-2 RNA is not always excreted in faeces by COVID-19 positive patients, and, when it is, the concentration and the length of shedding differ across people and over time (He et al. 2020; Pan et al. 2020; Wölfel et al. 2020; Xu et al. 2020). However, because of the significant proportion of moderate or asymptomatic carriers that were not being counted in epidemiological data, the actual number of positive cases in each location was still unclear.

Remarkably, data generated in this thesis was necessary to elucidate the most suitable methodologies for SARS-CoV-2 detection in wastewater for the use of WBE as an early warning system to help public authorities in Spain to face the pandemic. This study represents a crucial contribution to the national and global implementation of the WBE in monitoring the spread of COVID-19. The method developed in this work has been implemented nationally within the framework of the Spanish National SARS-CoV-2 Wastewater Surveillance System (VATar COVID-19) from the Spanish Ministry for the Ecological Transition and the Demographic Challenge (MITECO) (https://www.miteco.gob.es/es/agua/temas/concesiones-y-autorizaciones/protocolo-deteccion-sars-cov-2-en-aguas-residuales_tcm30-528265.pdf) and is being used by ENAC as a reference method for the official accreditation of laboratories. The results obtained by analysing SARS-CoV-2 in wastewater using these methods are transmitted weekly to MITECO and are currently used by the Spanish Ministry of Health in decision-making to contain the pandemic.

Spatial and temporal distribution of SARS-CoV-2 diversity circulating in wastewater

During the course of this thesis, a large number of wastewater samples were analysed in the Environmental Virology and Food Safety lab as a result of

collaboration with MITECO and several companies for monitoring the spread of COVID-19 using RT-qPCR molecular techniques. Protocols for metagenomic approaches from wastewater samples had been previously assessed in the lab for the analysis of the virome of influent and effluent wastewater samples (study 2.1), so study 4.2 focused on the combination of RTq-PCR based WBE together with high-throughput sequencing protocols to evaluate SARS-CoV-2 variants that were circulating in wastewater samples from 14 WWTPs in different regions of Spain. This is how study 4.2 sequencing outcomes efficiently detected the presence of the SARS-CoV-2 Alpha variant in Spain in wastewater samples collected prior to its clinical detection. Study 4.2 was the first research conducted in Spain to examine the variety of SARS-CoV-2 strains found in wastewater during the three epidemic waves that took place between 2020 and 2021. Amino acid substitutions in the spike protein that hadn't been previously described in Spain were found in the sequenced samples, some of them months before their first detection in a clinical context. These findings demonstrated the ability of sewage sequencing to identify new SARS-CoV-2 mutations and lineages, which is crucial for efforts to track down SARS-CoV-2 mutants that could escape the immunisation process after vaccination.

This research has proven the usefulness of sewage sequencing in efficiently tracking Variants of Concern, and this, together with clinical testing, can play an important role in aiding public health responses to the pandemic.

Evaluation of different procedures for coronaviruses' nucleic acid detection in water matrices.

The aforementioned study 4.1 suggested that tracking the spread of COVID-19 within certain locations by SARS-CoV-2 RNA monitoring in wastewater samples is extraordinarily efficient and accurate. Subsequently, two additional pieces of work included in this thesis were published with the aim of selecting optimal approaches in detecting coronaviruses from distinct water matrices.

In research 5.1, the suitability of SARS-CoV-2 detection in wastewaters of two concentration (Aluminium chloride adsorption-precipitation vs PEG precipitation) and extraction (a semi-automated and a column-based) techniques was examined to optimise and simplify the processes for SARS-CoV-2 monitoring. The studied concentration and extraction procedures did not significantly differ in the SARS-CoV-2 and MgV mean recoveries. Based on

those findings, no concentration and extraction procedure evaluated in this investigation would appreciably alter recoveries of SARS-CoV-2 and MgV. These findings demonstrated how well-suited the selected approaches were for analysing enveloped viruses in wastewater. Ahmed, Bertsch, et al. (2020) reported comparable mean recoveries utilising murine hepatitis virus as a surrogate for SARS-CoV-2 concentrated from wastewater by ultracentrifugation, filtering, and flocculation techniques. Gonzalez et al. (2020) found that when utilising InnovaPrep and electronegative filtration procedures for viral concentration, bovine coronavirus recovery percentages were 5.5% and 4.8%, respectively. These recovery numbers were more consistent with the non-enveloped MgV results from studies 1.1 and 3.1. In that research, the aluminium chloride adsorption-precipitation approach (total duration less than 2h) was chosen for additional comparisons over the PEG protocol, which includes an overnight incubation stage and thus takes longer to be performed.

Also, through the study of serially diluted spiking samples, detection limits of five SARS-CoV-2 genome targets in wastewater were assessed in research 5.1. RT-qPCR techniques for SARS-CoV-2 detection in wastewater samples were also tested for bias. To assess the sensitivity of each RT-qPCR test, naturally polluted wastewater samples were subjected to analysis using five distinct SARS-CoV-2 genomic segments (N1, N2, E, IP2, and IP4). Depending on the selected target, there were some variations in the detection of SARS-CoV-2. These outcomes demonstrated the variation that might be seen in positive samples based on the applied primer set. Additionally, SARS-CoV-2 detection reproducibility differed within each genetic target. In concordance with previously published data, N1 genomic region had the greatest percentage of positive duplicates (77%) of any target (Muenchhoff et al. 2020). Targeting SARS-CoV-2 gene E may have lesser sensitivity when identifying gamma-irradiated SARS-CoV-2 because the primer binding site may have mutations that could prevent the amplification and, hence, its detection (Artesi et al. 2020). This highlights the importance of checking the generic set of primers used for SARS-CoV-2 amplification to confirm that new emerging variants of the virus do not contain any mutation affecting the primer-probe region.

Furthermore, standard curves were created utilising synthetic plasmids containing genes N and E and the whole genomic RNA of SARS-CoV-2 (ATCC VR-1986D) for the viral quantification of genes N1, N2, and E gene. Remarkably, synthetic RNA has transpired to be an optimal tool to rapidly obtain reference material for new SARS-CoV-2 emerging variants. When

comparing quantification results from various research, it is important to note that quantification bias was seen depending on the reference material employed. Furthermore, this bias must be appropriately evaluated prior to the integration of quantification values into predictive epidemiological models given the final WBE purpose, which is the prediction of the number of infected individuals in a particular community. The findings of study 5.1 demonstrate the existing variability provoked by the selected extraction technique, the molecular target, and the surrogate utilised as a process control to validate the analysis.

During the pandemic, institutions and researchers became interested in detecting SARS-CoV-2 in different types of water that could be contaminated by the virus (e.g., surface water, seawater, drinking water). With this in mind, study 5.2 characterised the analytical performances of numerous versions of a DEUF approach (validated in study 1.1 to concentrate HEV from drinking water) to concentrate enveloped (PEDV) and non-enveloped (MgV) viruses from large amounts of tap, ocean, and surface waters of interest in order to understand the possible contamination of water resources by SARS-CoV-2. Studies to evaluate the effectiveness of concentration techniques in water matrices have, up until recently, primarily focused on non-enveloped viruses, such as human enteric viruses (reviewed by Bofill-Mas and Rusiñol 2020; Haramoto et al. 2018; Ikner, Gerba, and Bright 2012; Matrajt et al. 2018). However, the need to look at enveloped viruses along the water cycle was already raised following SARS-CoV-1 and MERS epidemics (Wigginton et al. 2015). This request for verified analytical tools had foresight and was rooted in the structural and biochemical distinctions between non-enveloped and enveloped viruses, raising the question whether techniques created for the former would still be suitable to concentrate the latter.

Additionally, two quantitative detection kits were contrasted to see which was less sensitive to inhibitors: one was advertised as being optimised for low RNA levels (SENS-kit), and the other as being extremely resistant to a range of inhibitory chemicals (NoInh-kit). Study 5.2 results showed a distinct sensitivity of RT-qPCR tests to co-concentrated inhibitory compounds, with the SENS-kit being less susceptible to such limiting factors, despite using a contaminant/inhibitor removing reagent prior to RNA extraction (Plant RNA Isolation Aid). A well-known method to assess the presence of inhibitors in complex matrices is nucleic acid dilution (ISO 15216-1:2017; McKee, Spear, and Pierson 2015). However, the sensitivity of the test declines with the amount of dilution used. Recent research on SARS-CoV-2 detection in river

water found little to no inhibitor carryover in samples concentrated between 1 and 5L (Guerrero-Latorre et al. 2020; Haramoto et al. 2020; Rimoldi et al. 2020).

When testing a secondary concentration technique for tap water, results indicated that centrifugal filtering was more effective at recovering MgV whereas PEG-precipitation was the best approach for concentrating PEDV, an enveloped virus proposed as a SARS-CoV-2 surrogate. On the other hand, the recovery of both spiking viruses from seawater did not substantially differ between high and low centrifugation rates. Up to now, monitoring the potential SARS-CoV-2 contamination of reclaimed waters used for irrigation or leisure, as well as drinking water resources in environments with limited access to water, sanitation, and hygiene, calls for more investigation (Street et al. 2020). In the meantime, the evaluated approaches offer practical ways to detect the presence of SARS-CoV-2 in various water resources, enable on-site sampling of huge volumes of water, and have been used for subsequent COVID-19 health risk assessment projects and analyses.

Implementing rapid molecular methods to infer SARS-CoV-2 infectivity.

In parallel with the SARS-CoV-2 monitoring efforts (studies 4.1 and 4.2), a new specific objective was set consisting of the development of a rapid molecular approach to indirectly assess the potential infectiousness of the SARS-CoV-2 present in positive wastewater samples based on the already existing capsid-integrity RT-qPCR concept. Thus, study 6.1 evaluated the performance of several viability markers applied prior to nucleic acid extraction to prevent amplification of RNA from non-infectious viral particles, thus enabling amplification only of infectious viruses in downstream RT-qPCR assay. The selection of platinum chloride as the best performing viability marker demonstrated that capsid-integrity RT-qPCR efficiently discriminated free RNAs and inactivated SARS-CoV-2 inoculated in buffer, stool, and urine suspensions.

When published, this was the first report comparing different conditions to optimise a rapid molecular assay independent from viral replication in cell culture developed to test SARS-CoV-2 infectivity in clinical and environmental samples. A prior investigation demonstrated the suitability of capsid integrity

RT-qPCRs to infer the infectivity of porcine epidemic diarrhoea coronavirus, a member of the genus *Alphacoronavirus* within *Coronaviridae* family (Puente et al. 2020). Interestingly, it was possible to demonstrate that PMAxx viability RT-qPCR matched the thermal inactivation pattern obtained by cell culture better than other viability markers, including $PtCl_4$, and RT-qPCR alone. Canh et al. (2021) identified CDDP at a concentration of 100 µM as the most efficient reagent for the selective detection of infectious murine hepatitis virus (MHV, used as SARS-CoV-2 surrogate) by RT-qPCR. Alternative rapid methods to assess the viability of enveloped viruses have been explored with inconclusive results. These include propidium monoazide and immunomagnetic separation tested on laryngotracheitis virus and ethidium monoazide on avian influenza virus (Bindari, Walkden-Brown, and Gerber 2020; Hamza et al. 2011). It is worth reporting that during the review process for this study, few others reported the use of PMA or PMAxx to discriminate between live and dead SARS-CoV-2 in environmental samples (Polo et al. 2021; Hong et al. 2021; Wurtzer et al. 2021; Monteiro et al. 2022).

With regards to wastewater samples that tested positive for SARS-CoV-2 RNA, they most probably contained detergents and chemicals that are detrimental to viral infectivity further supporting the efficacy of the viability RT-qPCR in discriminating potentially infectious and inactivated viral particles (Khokhar et al. 2020). The ultimate confirmation on the infectivity of the samples by cell culture, although recommendable, could not be provided. Nonetheless, recent laboratory-scale investigations on SARS-CoV-2 persistence in wastewater estimated that the infectious titer of spiked virus decreases by one logarithm in 1.5–1.9 days at room temperature, and in a few minutes at higher temperatures (Aaron Bivins et al. 2020; de Oliveira et al. 2021).

Besides some limitations, the proposed viability RT-qPCR effectively reduced the amplification signals of non-infectious and free RNA of SARS-CoV-2 in complex matrices, finally providing a better estimation of the infectivity of samples. Thus, mathematical models derived from laboratory-scale experiments comparing viability RT-qPCR and viral replication, could correlate viral load and infectivity, finally providing relevant tools of interest based on a rapid molecular assay for prevention strategies and risk assessment.

Lastly, even though the SARS-CoV-2 RNA detection in wastewater is functional for WBE purposes, the risk for human health associated with the water cycle is still under debate as infectivity of viral particles in sewage and faeces remains to be confirmed together with their potential faecal-oral transmission. Rimoldi et al. (2020) suggests that the risk of infection from

wastewater and rivers is negligible given the failure in cell culturing SARS-CoV-2 from water samples despite the high number of RNA copies.

CONCLUSIONS

V. CONCLUSIONS

- Regarding the development of procedures for emerging enteric viruses' concentration in wastewater samples, the aluminium hydroxide adsorption-precipitation protocol efficiently concentrated HEV enabling its sensitive detection in influent and effluent wastewater samples. Moreover, the Dead End Ultrafiltration procedure effectively recovered HEV from artificially contaminated drinking water samples.

- In concordance with monitoring the prevalence of enteric viruses and viral indicators in influent and effluent water samples from wastewater treatment plants (WWTPs), this thesis provided insights on the quantitative occurrence of crAssphage, and intact capsid HAV, HuNoVs, RVs, and HAstVs in influent and effluent wastewaters from different WWTPs located in the Valencian region of Spain. Besides, correlation outcomes indicated that crAssphage might not be an optimal indicator for enteric virus infectivity in reclaimed wastewater.

- Regarding the characterisation of the virome of water samples from WWTPs, the present thesis described a benchmark procedure that allows the detection and characterisation of viral populations in influent and effluent wastewater samples. This work also showed the bias on virome profiles resulting from different sequencing libraries. Overall, this investigation shed light on the diversity of the viral communities in influent and effluent wastewaters by providing valuable information also in terms of viral faecal indicators.

- As for the specific objective of implementing a SARS-CoV-2 monitoring system in wastewater, the results of this thesis demonstrated wastewater-based epidemiology (WBE) is an efficient approach to estimate the presence and even the prevalence of COVID-19 in communities and to serve as an early-warning tool for public health responses to pandemic situations. Also, this thesis includes the first study carried out in Spain that made a metagenomic analysis of the diversity of SARS-CoV-2 present in wastewater in the three first epidemiological waves which occurred between 2020 and 2021. These results further confirmed the potential of sewage sequencing to detect new mutations and lineages of SARS-CoV-2. Finally, this thesis also compared and optimised coronaviruses' nucleic acid concentration, extraction, and detection protocols from wastewater

samples and from large volume water samples. Thus, this work expanded the knowledge of analytical procedures and their efficiency in SARS-CoV-2 detection in wastewater constituting a step forward for the global implementation of COVID-19 WBE.

- Related to the development and optimisation of rapid molecular methods to infer SARS-CoV-2 viral infectivity, this thesis implemented a capsid-integrity RT-qPCR protocol based on platinum chloride acting as a viability marker to prevent RT-qPCR amplification of RNA from non-infectious SARS-CoV-2 and successfully validated it in naturally contaminated wastewater samples. Thus, the results of this thesis support the idea that SARS-CoV-2 present in sewage is not infectious. Overall, this work proposed a rapid analytical tool based on viability RT-qPCR to infer SARS-CoV-2 infectivity with potential application in risk assessment, prevention, and control in public health programmes.

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VII. REFERENCES

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ANNEXES

VIII. ANNEXES

Annex A. List of publications included in this thesis

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ORIGINAL RESEARCH
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HEV Occurrence in Waste and Drinking Water Treatment Plants

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Hepatitis E virus (HEV), particularly zoonotic genotype 3, is present in environmental waters worldwide, especially in industrialized countries. Thus, monitoring the presence of HEV in wastewater treatment plants (WWTPs) is an emerging topic due to the importance of reusing water on a global level. Given the limited data, this study aimed to monitor the occurrence of HEV in influent and effluent water in waste- and drinking-water treatment plants (WWTPs and DWTPs). To this end, different procedures to concentrate HEV in influent and effluent water from WWTPs and DWTPs were initially evaluated. The evaluated procedures resulted in average HEV recoveries of 15.2, 19.9, and 16.9% in influent, effluent, and drinking water samples, respectively, with detection limits ranging from 10^3 to 10^4 international units (IU)/L. Then, a one-year pilot study was performed to evaluate the performance of the selected concentration method coupled with three RT-qPCR assays in influent and effluent water samples from four different WWTPs. HEV prevalence in influent water varied based on both the RT-qPCR assay and WWTP, while HEV was not detected in effluent water samples. In addition, HEV prevalence using only RT-qPCR3 was evaluated in influent (n = 62) and effluent samples (n = 52) from four WWTPs as well as influent (n = 28) and effluent (n = 28) waters from two DWTPs. The present study demonstrated that HEV circulated in the Valencian region at around 30.65% with average concentrations of 6.3×10^3 IU/L. HEV was only detected in influent wastewater samples, effluent samples from WWTPs and influent and effluent samples from DWTPs were negative. However, given that the infective dose in waterborne epidemics settings is not yet known and the low sensibility of the assay, unfortunately, no direct conclusion could be achieved on the risk assessment of environmental contamination.

Keywords: Hepatitis E virus, wastewater, drinking water, water quality, RT-qPCR, occurrence

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Bias of library preparation for virome characterization in untreated and treated wastewaters

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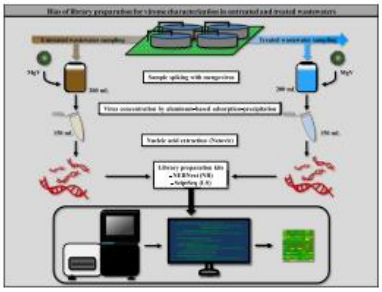
HIGHLIGHTS

- Work-flow procedure for the characterization of virome in wastewaters
- Sequencing libraries lead to different virome profiles.
- Not all proposed viral indicators correlate with the presence of enteric viruses.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of metagenomics for virome characterization and its implementation for wastewater analyses, including wastewater-based epidemiology, has increased in the last years. However, the lack of standardized methods can led to highly different results. The aim of this work was to analyze virome profiles in upstream and downstream wastewater samples collected from four wastewater treatment plants (WWTPs) using two different library preparation kits. Viral particles were enriched from wastewater concentrates using a filtration and nuclease digestion procedure prior to total nucleic acid (NA) extraction. Sequencing was performed using the ScriptSeq v2 RNA-Seq (LS) and the NEBNext Ultra II RNA (NB) library preparation kits. Cleaned reads and contigs were annotated using a curated *in-house* database composed by reads assigned to viruses at NCBI. Significant differences in viral families and in the ratio of detection were shown between the two library kits used. The use of LS library showed *Virgaviridae*, *Microviridae* and *Siphoviridae* as the most abundant families; while *Ackermannviridae* and *Helleviridae* were highly represented within the NB library. Additionally, the two sequencing libraries produced outcomes that differed in the detection of viral indicators. These results highlighted the importance of library selection for studying viruses in untreated and treated wastewater. Our results underline the need for further studies to elucidate the influence of sequencing procedures in virome profiles in wastewater matrices in order to improve the knowledge of the virome in the water environment.

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Monitoring Human Viral Pathogens Reveals Potential Hazard for Treated Wastewater Discharge or Reuse

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Wastewater discharge to the environment or its reuse after sanitization poses a concern for public health given the risk of transmission of human viral diseases. However, estimating the viral infectivity along the wastewater cycle presents technical challenges and still remains underexplored. Recently, human-associated crAssphage has been investigated to serve as viral pathogen indicator to monitor fecal impacted water bodies, even though its assessment as biomarker for infectious enteric viruses has not been explored yet. To this end, the occurrence of potentially infectious norovirus genogroup I (GI), norovirus GII, hepatitis A virus (HAV), rotavirus A (RV), and human astrovirus (HAstV) along with crAssphage was investigated in influent and effluent water sampled in four wastewater treatment plants (WWTPs) over 1 year by a PMAxx-based capsid integrity RT-qPCR assay. Moreover, influent and effluent samples of a selected WWTP were additionally assayed by an *in situ* capture RT-qPCR assay (ISC-RT-qPCR) as estimate for viral infectivity in alternative to PMAxx-RT-qPCR. Overall, our results showed lower viral occurrence and concentration assessed by ISC-RT-qPCR than PMAxx-RT-qPCR. Occurrence of potentially infectious enteric virus was estimated by PMAxx-RT-qPCR as 88–94% in influent and 46–67% in effluent wastewaters with mean titers ranging from 4.77 to 5.89, and from 3.86 to 4.97 $\log_{10}$ GC/L, with the exception of HAV that was sporadically detected. All samples tested positive for crAssphage at concentration ranging from 7.41 to 9.99 $\log_{10}$ GC/L in influent and from 4.56 to 6.96 $\log_{10}$ GC/L in effluent wastewater, showing higher mean concentration than targeted enteric viruses. Data obtained by PMAxx-RT-qPCR showed that crAssphage strongly correlated with norovirus GII ($\rho = 0.67$, $p < 0.05$) and weakly with HAstV and RV ($\rho = 0.25$–0.30, $p < 0.05$) in influent samples. In effluent wastewater, weak ($\rho = 0.27$–0.38, $p < 0.05$) to moderate ($\rho = 0.47$–0.48, $p < 0.05$) correlations between crAssphage and targeted viruses were observed. Overall, these results corroborate crAssphage as an indicator for fecal contamination in wastewater but a poor marker for either viral occurrence and viral integrity/infectivity. Despite the viral load reductions detected in effluent compared to influent wastewaters, the estimates of viral infectivity based on viability molecular methods might pose a concern for (re)-using of treated water.

Keywords: enteric viruses, capsid integrity RT-qPCR, wastewater, crAssphage, fecal contamination indicator

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SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 200,000 reported COVID-19 cases in Spain resulting in more than 20,800 deaths as of April 21, 2020. Faecal shedding of SARS-CoV-2 RNA from COVID-19 patients has extensively been reported. Therefore, we investigated the occurrence of SARS-CoV-2 RNA in six wastewater treatments plants (WWTPs) serving the major municipalities within the Region of Murcia (Spain), the area with the lowest COVID-19 prevalence within Iberian Peninsula. Firstly, an aluminum hydroxide adsorption-precipitation concentration method was validated using a porcine coronavirus (Porcine Epidemic Diarrhea Virus, PEDV) and mengovirus (MgV). The procedure resulted in average recoveries of 10 ± 3.5% and 10 ± 2.1% in influent water (n = 2) and 3.3 ± 1.6% and 6.2 ± 1.0% in effluent water (n = 2) samples for PEDV and MgV, respectively. Then, the method was used to monitor the occurrence of SARS-CoV-2 from March 12 to April 14, 2020 in influent, secondary and tertiary effluent water samples. By using the real-time RT-PCR (RT-qPCR) Diagnostic Panel validated by US CDC that targets three regions of the virus nucleocapsid (N) gene, we estimated quantification of SARS-CoV-2 RNA titers in untreated wastewater samples of 5.4 ± 0.2 $\log_{10}$ genomic copies/L on average. Two secondary water samples resulted positive (2 out of 18) and all tertiary water samples tested as negative (0 out 12). This environmental surveillance data were compared to declared COVID-19 cases at municipality level, revealing that members of the community were shedding SARS-CoV-2 RNA in their stool even before the first cases were reported by local or national authorities in many of the cities where wastewaters have been sampled. The detection of SARS-CoV-2 in wastewater in early stages of the spread of COVID-19 highlights the relevance of this strategy as an early indicator of the infection within a specific population. At this point, this environmental surveillance could be implemented by municipalities right away as a tool, designed to help authorities to coordinate the exit strategy to gradually lift its coronavirus lockdown.

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Spatial and temporal distribution of SARS-CoV-2 diversity circulating in wastewater

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SARS-CoV-2
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Variants of interest

ABSTRACT

Wastewater-based epidemiology (WBE) has proven to be an effective tool for epidemiological surveillance of SARS-CoV-2 during the current COVID-19 pandemic. Furthermore, combining WBE together with high-throughput sequencing techniques can be useful for the analysis of SARS-CoV-2 viral diversity present in a given sample. The present study focuses on the genomic analysis of SARS-CoV-2 in 76 sewage samples collected during the three epidemiological waves that occurred in Spain from 14 wastewater treatment plants distributed throughout the country. The results obtained demonstrate that the metagenomic analysis of SARS-CoV-2 in wastewater allows the detection of mutations that define the B.1.1.7 lineage and the ability of the technique to anticipate the detection of certain mutations before they are detected in clinical samples. The study proves the usefulness of sewage sequencing to track Variants of Concern that can complement clinical testing to help in decision-making and in the analysis of the evolution of the pandemic.

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Comparing analytical methods to detect SARS-CoV-2 in wastewater

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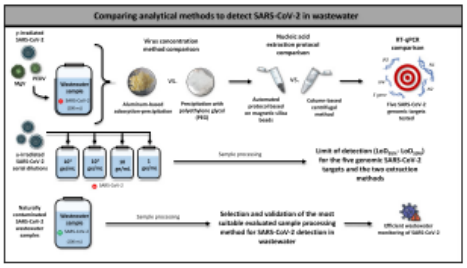
HIGHLIGHTS

- Gamma-irradiated SARS-CoV-2 was used to assess method performance.
- Different methods were compared for SARS-CoV-2 WBE surveillance.
- Tested methods showed no significant differences for SARS-CoV-2 recovery from wastewater.
- Automated nucleic acid extraction showed lower $LoD_{95\%}$ than column based method.
- Different sensitivity of RT-qPCR assays was observed.

GRAPHICAL ABSTRACT



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Aluminum-based adsorption-precipitation
Wastewater based epidemiology
RT-qPCR

ABSTRACT

Wastewater based epidemiology (WBE) has emerged as a reliable strategy to assess the coronavirus disease 2019 (COVID-19) pandemic. Recent publications suggest that SARS-CoV-2 detection in wastewater is technically feasible; however, many different protocols are available and most of the methods applied have not been properly validated. To this end, different procedures to concentrate and extract inactivated SARS-CoV-2 and surrogates were initially evaluated. Urban wastewater seeded with gamma-irradiated SARS-CoV-2, porcine epidemic diarrhea virus (PEDV), and mengovirus (MgV) was used to test the concentration efficiency of an aluminum-based adsorption-precipitation method and a polyethylene glycol (PEG) precipitation protocol. Moreover, two different RNA extraction methods were compared in this study: a commercial manual spin column centrifugation kit and an automated protocol based on magnetic silica beads. Overall, the evaluated concentration methods did not impact the recovery of gamma-irradiated SARS-CoV-2 nor MgV, while extraction methods showed significant differences for PEDV. Mean recovery rates of 42.9 ± 9.5%, 27.5 ± 14.3% and 9.0 ± 2.2% were obtained for gamma-irradiated SARS-CoV-2, PEDV and MgV, respectively. Limits of detection ($LoD_{95\%}$) for five genomic SARS-CoV-2 targets (N1, N2, gene E, IP2 and IP4) ranged from 1.56 log genome equivalents (ge)/mL (N1) to 2.22 log ge/mL (IP4) when automated system was used; while values ranging between 2.08 (N1) and 2.34 (E) log ge/mL were observed when using column-based extraction method. Different targets were also evaluated in naturally contaminated wastewater samples with 91.2%, 85.3%, 70.6%, 79.4% and 73.5% positivity, for N1, N2, E, IP2 and IP4, respectively. Our benchmarked comparison study suggests that the aluminum precipitation method coupled with the automated nucleic extraction represents a method of acceptable sensitivity to provide readily results of interest for SARS-CoV-2 WBE surveillance.

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Short Communication

Recovering coronavirus from large volumes of water

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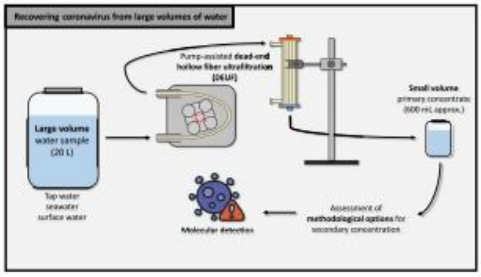
HIGHLIGHTS

- Suitable options to investigate coronavirus in tap water, seawater and surface water were assessed.
- DEUF coupled with PEG-precipitation and SENS-kit better recovered PEDV in tap water.
- High and low centrifugation speeds do not differ in recovering PEDV and mengovirus from seawater.
- Co-concentration of inhibitory substances may occur in seawater and surface water.

GRAPHICAL ABSTRACT



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ABSTRACT

The need for monitoring tools to better control the ongoing coronavirus disease (COVID-19) pandemic is extremely urgent and the contamination of water resources by excreted viral particles poses alarming questions to be answered. As a first step to overcome technical limitations in monitoring SARS-CoV-2 along the water cycle, we assessed the analytical performance of a dead end hollow fiber ultrafiltration coupled to different options for secondary concentrations to concentrate viral particles from large volume of spiked tap water, seawater and surface water together with two quantitative RT-qPCR detection kits. Spiking the porcine epidemic diarrhea virus (PEDV), an enveloped virus surrogate for SARS-CoV-2, together with the mengovirus, we demonstrated that PEG-precipitation and SENS-kit better recovered PEDV (13.10 ± 0.66%) from tap water, while centrifugal filtration resulted the best option to recover mengovirus regardless of the detection kit used. No statistical significant differences were found when comparing high (10,000 ×g) and low (3500 ×g) centrifugation speeds for the secondary PEG- based concentration of spiked seawater, while considerable inhibition was observed for both viruses detected by NoInh-kit assay. Similarly, the co-concentration of PCR inhibitors and viral particles was observed in surface waters detected with either SENS-kit or NoInh-kit and RNA dilution was needed to achieve acceptable recoveries at the expenses of the overall sensitivity of the method. These methodologies represent suitable options to investigate SARS-CoV-2 occurrence in different water resources and allow to conduct on site sampling of large volume of water.

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Platinum chloride-based viability RT-qPCR for SARS-CoV-2 detection in complex samples

Enric Cuevas-Ferrando[1]✉, Walter Randazzo[1], Alba Pérez-Cataluña[1], Irene Falcó[1], David Navarro[2,3], Sandra Martin-Latil[4], Azahara Díaz-Reolid[1], Inés Girón-Guzmán[1], Ana Allende[5] & Gloria Sánchez[1]

Isolation, contact tracing and restrictions on social movement are being globally implemented to prevent and control onward spread of SARS-CoV-2, even though the infection risk modelled on RNA detection by RT-qPCR remains biased as viral shedding and infectivity are not discerned. Thus, we aimed to develop a rapid viability RT-qPCR procedure to infer SARS-CoV-2 infectivity in clinical specimens and environmental samples. We screened monoazide dyes and platinum compounds as viability molecular markers on five SARS-CoV-2 RNA targets. A platinum chloride-based viability RT-qPCR was then optimized using genomic RNA, and inactivated SARS-CoV-2 particles inoculated in buffer, stool, and urine. Our results were finally validated in nasopharyngeal swabs from persons who tested positive for COVID-19 and in wastewater samples positive for SARS-CoV-2 RNA. We established a rapid viability RT-qPCR that selectively detects potentially infectious SARS-CoV-2 particles in complex matrices. In particular, the confirmed positivity of nasopharyngeal swabs following the viability procedure suggests their potential infectivity, while the complete prevention of amplification in wastewater indicated either non-infectious particles or free RNA. The viability RT-qPCR approach provides a more accurate ascertainment of the infectious viruses detection and it may complement analyses to foster risk-based investigations for the prevention and control of new or re-occurring outbreaks with a broad application spectrum.

Annex B. List of publications not included in this thesis

International Journal of Hygiene and Environmental Health 230 (2020) 113621

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

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Short communication

Metropolitan wastewater analysis for COVID-19 epidemiological surveillance

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ARTICLE INFO

Keywords:
Epidemiological surveillance
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COVID-19
Early warning

ABSTRACT

The COVID-19 disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a rapidly emerging pandemic which has enforced extreme containment measures worldwide. In the absence of a vaccine or efficient treatment, cost-effective epidemiological surveillance strategies are urgently needed. Here, we have used RT-qPCR for SARS-CoV-2 detection in a series of longitudinal metropolitan wastewaters samples collected from February to April 2020, during the earliest stages of the epidemic in the Region of Valencia, Spain. We were able to consistently detect SARS-CoV-2 RNA in samples taken in late February, when communicated cases in that region were only incipient. We also find that the wastewater viral RNA context increased rapidly and anticipated the subsequent ascent in the number of declared cases. Our results strongly suggest that the virus was undergoing community transmission earlier than previously believed, and suggest that wastewater analysis could be sensitive and cost-effective strategy for COVID-19 epidemiological surveillance. Routine implementation of this surveillance tool would significantly improve our preparedness against new or re-occurring viral outbreaks.

microorganisms

MDPI

Article

Assessment of ISO Method 15216 to Quantify Hepatitis E Virus in Bottled Water

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Abstract: Hepatitis E virus (HEV) is one of the causative agents of water-borne human viral hepatitis and considered in Europe an emerging zoonotic pathogen. Analysis of bottled water through a standard method validated for HEV can contribute towards the risk management of this hazard. Putting some recent reports by the European Food Safety Authority in place, this study aimed to assess the performance of the concentration and extraction procedures described in ISO 15216-1:2017 for norovirus and hepatitis A virus on HEV detection. Following the ISO recommendation, the bottled water samples were spiked using serially diluted HEV fecal suspensions together with mengovirus as process control and concentrated by filtration via positively charged nylon membranes. In order to extract viral RNA from the resulting concentrates, two different methods were compared in this study: The one recommended in the ISO norm, NucliSens® MiniMag® system (NS), and an alternative commercially available kit NucleoSpin®RNA virus kit (MN). Finally, three reverse transcription quantitative PCR (RT-qPCR) assays were used to quantify HEV titers. The evaluated procedures resulted in average HEV recoveries of 14.08 ± 4.90% and 3.58 ± 0.30% for the MN and NS methods, respectively. The limit of detection ($LoD_{95\%}$) was 1.25×10^4 IU/L for both extraction methods combined with the three RT-qPCR assays tested, with the exception of NS extraction coupled with RT-qPCR1 that showed a $LoD_{95\%}$ of 4.26×10^3 IU/L. The method characteristics generated in this study support the limited suitability of the ISO 15216-1:2017 concentration procedure coupled with the evaluated RT-qPCR assays for detecting HEV in bottled water.

Keywords: Hepatitis E Virus (HEV); bottled water; concentration method; RT-qPCR

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Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Discrimination of non-infectious SARS-CoV-2 particles from fomites by viability RT-qPCR

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ARTICLE INFO

Keywords:
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SARS-CoV-2
Fomites
Viability RT-qPCR
Transmission risk

ABSTRACT

The ongoing coronavirus 2019 (COVID-19) pandemic constitutes a concerning global threat to public health and economy. In the midst of this pandemic scenario, the role of environment-to-human COVID-19 spread is still a matter of debate because mixed results have been reported concerning SARS-CoV-2 stability on high-touch surfaces in real-life scenarios. Up to now, no alternative and accessible procedures for cell culture have been applied to evaluate SARS-CoV-2 infectivity on fomites. Several strategies based on viral capsid integrity have latterly been developed using viability markers to selectively remove false-positive qPCR signals resulting from free nucleic acids and damaged viruses. These have finally allowed an estimation of viral infectivity. The present study aims to provide a rapid molecular-based protocol for detection and quantification of viable SARS-CoV-2 from fomites based on the discrimination of non-infectious SARS-CoV-2 particles by platinum chloride (IV) ($PtCl_4$) viability RT-qPCR. An initial assessment compared two different swabbing procedures to recover inactivated SARS-CoV-2 particles from fomites coupled with two RNA extraction methods. Procedures were validated with human (E229) and porcine (PEDV) coronavirus surrogates, and compared with inactivated SARS-CoV-2 suspensions on glass, steel and plastic surfaces. The viability RT-qPCR efficiently removed the PCR amplification signals from heat and gamma-irradiated inactivated SARS-CoV-2 suspensions that had been collected from specified surfaces. This study proposes a rapid viability RT-qPCR that discriminates non-infectious SARS-CoV-2 particles on surfaces thus helping researchers to better understand the risk of contracting COVID-19 through contact with fomites and to develop more efficient epidemiological measures.

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Combined kinetic analysis of SARS-CoV-2 RNAemia, N-antigenemia and virus-specific antibodies in critically ill adult COVID-19 patients

Rosa Costa[1,5], Juan Alberola[2,5], Beatriz Olea[1], Roberto Gozalbo-Rovira[2], Estela Giménez[1], Enric Cuevas-Ferrando[3], Ignacio Torres[1], Eliseo Albert[1], Nieves Carbonell[4], José Ferreres[4], Gloria Sánchez[3], Jesús Rodríguez-Díaz[2], María Luisa Blasco[4] & David Navarro[1,2]✉

Combined kinetic analysis of plasma SARS-CoV-2 RNAemia, Nucleocapsid (N)-antigenemia and virus-specific antibodies may help ascertain the role of antibodies in preventing virus dissemination in COVID-19 patients. We performed this analysis in a cohort of 71 consecutive critically ill COVID-19 patients (49 male; median age, 65 years) using RT-PCR assay, lateral flow immunochromatography method and receptor binding domain (RBD) and N-based immunoassays. A total of 338 plasma specimens collected at a median of 12 days after symptoms onset were available for analyses. SARS-CoV-2 RNAemia and N-antigenemia were detected in 37 and 43 specimens from 26 (36.5%) and 30 (42.2%) patients, respectively. Free RNA was the main biological form of SARS-CoV-2 found in plasma. The detection rate for both viral components was associated with viral load at the upper respiratory tract. Median time to SARS-CoV-2-RBD antibody detection was 14 days (range, 4–38) from onset of symptoms. Decreasing antibody levels were observed in parallel to increasing levels of both RNAemia and N-antigenemia, yet overall a fairly modest inverse correlation (Rho = −0.35; $P < 0.001$) was seen between virus RNAemia and SARS-CoV-2-RBD antibody levels. The data cast doubts on a major involvement of antibodies in virus clearance from the bloodstream within the timeframe examined.

foods

MDPI

Article

Occurrence and Accumulation of Human Enteric Viruses and Phages in Process Water from the Fresh Produce Industry

Enric Cuevas-Ferrando [1], Ana Allende [2], Alba Pérez-Cataluña [1], Pilar Truchado [2], Natalia Hernández [2], Maria Isabel Gil [2] and Gloria Sánchez [1,*]

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Abstract: The virological quality of process water (PW) used by the produce industry has received limited attention. As a first step to overcoming technical limitations in monitoring viruses in PW, the analytical performance of ultrafiltration was assessed to concentrate viral particles from 20 L of spiked PW. The selected method used for sample concentration of PW was carefully validated, thus enabling the accurate quantification and estimation of viral titers of human enteric viruses and phages. PW from the produce industry was collected periodically from the washing tanks of commercial facilities. The analysis of coliphages was performed by plaque assay, while the occurrence of enteric viruses and crAssphage was determined by molecular techniques. Significant differences in the physicochemical composition of PW, mostly due to the different nature of fresh produce types and differences in the sanitizer used in commercial operation, were observed. Accumulation of crAssphage and coliphages was observed in PW, but correlation with human enteric viruses was not possible due to the low prevalence of these pathogens in the PW analyzed. The obtained results showed that depending on the type of product washed, the product/water ratio and the residual concentrations of the sanitizers, the prevalence and concentration of bacteriophages changed significantly.

Keywords: human enteric viruses; viral indicator; bacteriophages; molecular methods; infectivity; produce; wash water; food safety

Citation: Cuevas-Ferrando, E.; Allende, A.; Pérez-Cataluña, A.; Truchado, P.; Hernández, N.; Gil, M.I.; Sánchez, G. Occurrence and Accumulation of Human Enteric Viruses and Phages in Process Water from the Fresh Produce Industry. *Foods* **2021**, *10*, 1853. https://doi.org/10.3390/foods10081853

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Research note

RNA loads of severe acute respiratory syndrome coronavirus 2 in patients with breakthrough coronavirus disease 2019 caused by the Delta and Omicron variants

Paula de Michelena [1], Ignacio Torres [1], Enric-Cuevas Ferrando [2], Beatriz Olea [1], Fernando González-Candelas [3], Gloria Sánchez [2], David Navarro [1, 4]

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