Rethinking the Significance of Reovirus in Water and Wastewater

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Received: 19 April 2016 / Accepted: 13 June 2016 / Published online: 18 June 2016
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Abstract The genus *Orthoreovirus* contains nonenveloped viruses with double-stranded gene segments encased in a double-layered icosahedral capsid shell. These features constitute major determinants of virion stability in the environment and virion resistance against physical and chemical agents. Reovirus (ReoV) is the general term most commonly used for all virus strains that infect humans and nonhuman animals. Several studies have demonstrated the frequent occurrence of ReoV in wastewaters and natural waters, including surface and ground waters from different geographical areas. Most of these studies have reported higher concentrations of ReoV than any other enteric virus analyzed. They are more commonly isolated in chlorine-disinfected wastewaters than other enteric viruses, and appear to survive longer in water. The ability of ReoV to form large aggregates, even with different types of enteric viruses (e.g., poliovirus) and their ability to undergo mechanisms of gene segment reassortment among different serotypes may also explain their greater stability. Different approaches have been applied for concentration of ReoV from water; however, the recovery efficiency of the filtration methods has not been fully evaluated. Recently, molecular methods for identification of ReoV strains and quantification of virus genome have been developed. Studies have shown that the overall detection sensitivity of ReoV RNA is enhanced through initial replication of infectious virions in cell culture. More studies are needed to specifically address unresolved issues about the fate and

distribution of ReoV in the environment since this virus is not commonly included in virological investigations.

Keywords Reovirus · Transmission · Water · Wastewater

Introduction

Reovirus (acronym for *respiratory enteric orphan virus*) belongs to a highly diverse group of nonenveloped and double-stranded RNA viruses (dsRNA) classified within the *Reoviridae* family. *Orthoreovirus* is the genus name (the true “reovirus”) for one of the 15 genera of the *Reoviridae* viruses that infect mammals, birds, and reptiles. This designation distinguishes the orthoreoviruses from other members of the family that infect plants, fish, mollusks, fungi, and insects (Clarke and Tyler 2010; Day 2009; Dermody et al. 2013). The *Mammalian orthoreovirus* (MRV, prototypical member of the *Reoviridae*) is the type species of the *Orthoreovirus* genus that was initially associated with a subgroup of respiratory and enteric viruses present in healthy humans with minimal or no symptoms of respiratory or enteric disease; therefore, the virus was considered an “orphan” (Sabin 1959; Jackson and Muldoon 1973; Norman and Lee 2000; Day 2009; Dermody et al. 2013). Reovirus (ReoV) is a general term that refers to the MRV and is most frequently used throughout this review. Current studies indicate that ReoV may be the most common enteric virus found in raw and treated wastewater; however, the concentration efficiency of ReoV in these matrices including natural waters has not been thoroughly evaluated. This review summarizes previous and recent findings about the occurrence of ReoV in water. Recent progress in concentration and molecular-based detection methods combined with traditional cell

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culture techniques will undoubtedly lead to more effective approaches to uncover the significance of ReoV in water and wastewater.

Taxonomy and Biological Properties of Reoviruses

The *Orthoreovirus* genus is divided into two distinct phylogenetic subgroups: the fusogenic orthoreoviruses (i.e., induce fusion of infected cells through formation of large, multinucleated syncytia) which infect mammals, birds, and reptiles; and the nonfusogenic mammalian orthoreoviruses which infect virtually all mammals, including humans (Day 2009; Dermody et al. 2013).

The *Reoviridae* are further divided into two subfamilies based upon virion morphology: the *Sedoreovirinae* subfamily, which includes genera containing “smooth” viruses almost spherical in appearance; and the *Spinareovirinae* subfamily which contains viruses with large “spikes” or “turrets” at the 12 icosahedral vertices of the core particle. The *Orthoreovirus* genus is included within the *Spinareovirinae* subfamily (Day 2009; Dermody et al. 2013).

Three morphologically similar groups of ReoV have been described based on genetic divergence and antigenic properties among serotypes as described later in this review. Based on available sequence data, the current taxonomic organization of the orthoreoviruses comprises five species groupings (I–V): (I) the prototypical mammalian ReoV, plus Ndelle virus which correspond to non-fusogenic viruses; (II) avian reoviruses; (III) Nelson Bay virus and related bat-origin reoviruses such as Paulu virus (PuIV) and Melaka virus (MeIV); (IV) baboon reovirus; and (V) the reptilian reoviruses. Species group II, III, IV, and V belong to the fusogenic viruses (Clarke and Tyler 2010; Day 2009; Dermody et al. 2013).

ReoVs are ubiquitous in their geographical distribution, and virtually all mammals, including humans, serve as host for infection (Scott 1971; Mochizuki et al. 1992; Decaro et al. 2005; Dermody et al. 2013). ReoVs infect their hosts by the fecal–oral and respiratory routes. Infectious virions enter the body by ingestion of contaminated food or inhalation of virus-containing aerosols. At both portals of entry, ReoVs infect epithelial cells and disseminate to peripheral sites where they cause disease (Boehme et al. 2013). Studies indicate that ReoVs are neurotropic viruses that use both bloodborne and neural pathways to spread systemically within their hosts to cause disease (Boehme et al. 2013). Most ReoV infections in humans occur in infancy or early childhood; therefore, full seroconversion against all three major serotypes is attained by late childhood, (around 5 years of age) (Norman and Lee 2000; Day 2009; Dermody et al. 2013). Human ReoVs are rarely

associated with disease, except in the very young. They are known to cause either asymptomatic or mild symptoms of upper respiratory and intestinal infections, in some cases febrile exanthema with fever (Jackson and Muldoon 1973; Clarke and Tyler 2010; Dermody et al. 2013). ReoVs have been occasionally found in fecal specimens associated with childhood diarrhea (Giordano et al. 2005). More recently novel ReoVs from nonhuman animals have been identified with some infecting humans and thus raising concerns about their zoonotic potential (Chua et al. 2007; Zhang et al. 2011; Kohl et al. 2012; Dermody et al. 2013; Yamanaka et al. 2014; Kawagishi et al. 2016).

Organization of the Reov Particle and Genome

Fine structural analysis by cryoelectron microscopy (cryo-EM) and three-dimensional image reconstruction techniques has revealed that the ReoV virions are spherical, nonenveloped particles approximately 85 nm in diameter (Day 2009; Dermody et al. 2013). The virions have a particle mass of approximately 1.3×10^8 Daltons of which about 15 % is RNA, while the remainder is protein. In cesium chloride gradients, virion particles band at a buoyant density of 1.36–1.38 g/cm³ (Silverstein et al. 1976). Table 1 provides general features of reovirus particles.

All members of the *Orthoreovirus* genus contain 10 linear segments of double-stranded RNA with collinear and complementary [+] and [–] strands encased in a double-layered icosahedral capsid shell (Dermody et al. 2013). These features constitute major determinants of virion stability in the environment and virion resistance against physical and chemical agents (Gomatos and Tamm 1963; Harris et al. 1987; Drayna and Fields 1982a, b; Norman and Lee 2000). The inner-capsid shell or core particle has an internal diameter of approximately 50–60 nm and encloses the genome. In addition, the inner capsid possesses 12 prominent projections or spikes situated as though on vertices of a regular icosahedron. The outer-capsid shell is 38 nm in diameter and surrounds the core particle (Dermody et al. 2013). The 10 dsRNA genome segments are divided into three size classes based upon their migration in polyacrylamide gels: three large (L: L1, L2, L3), three medium (M: M1, M2, M3), and four small (S: S1, S2, S3, S4) segments. These segments encode mRNAs for synthesis of 12 viral proteins that execute the ReoV replication program in a variety of cell types within diverse animal hosts. Eight of these segments encode a single protein, while two encode two proteins each. Eight of the 12 proteins are structural components of virions (λ 1, λ 2, λ 3, μ 1, μ 2, σ 1, σ 2, and σ 3) and four are nonstructural (μ NS, μ NSC, σ 1S, and σ NS). The function of these virus-

Table 1 General features of Reovirus

Taxonomy

Family *Reoviridae*Subfamily *Spinareovirinae*Genus *Orthoreovirus*Species *Mammalian orthoreovirus* (Reovirus)

Physical characteristics of the virion

Nonenveloped virus

Spherical with icosahedral symmetry

70–80 nm diameter

Buoyant density in CsCl: 1.36–1.38 g/cm³

Isoelectric point measured for serotype 3 (T3D) varies between 3.8 (electrophoretic mobility using light microscopy for detection) and 3.9 (isoelectric focusing using dense aqueous solution)

Reovirus genome

Genome consists of 10 linear double-stranded RNA (dsRNA) gene segments that enables reassortment and viral evolution

Gene segments of three size classes:

Three large segment (L1, L2, L3), three medium size segments (M1, M2, M3), and four small segments (S1, S2, S3, and S4)

Genome encased in a double concentric icosahedral capsid shell (inner shell or core and outer shell)

Gene segments are transcribed into full-length mRNAs

Gene segments encode one or two proteins: λ , μ , and σ size class proteins

encoded proteins has been described in detail elsewhere (Schiff 1998; Dermody et al. 2013). Cell attachment $\sigma 1$ protein is the most type-specific reovirus protein. It is also the reovirus-hemagglutinin and is a major determinant of host-range/tissue specificity and of the nature of interaction of reovirus with cells of the immune system (Schiff 1998; Dermody et al. 2013). Interestingly, studies have shown that proteins $\sigma 3$ and $\mu 1$ interact extensively within the outer capsid and the extensive contacts between $\sigma 3$ and $\mu 1$ molecules have been suggested to stabilize outer-capsid structure and thereby facilitate survival of virions during transmission of ReoV between hosts (Nibert et al. 1995; Schiff 1998). Furthermore, outer-capsid proteins $\sigma 3$, $\mu 1$, and $\sigma 1$ have each been associated with the stability of ReoV particles against particular physicochemical agents (Drayna and Fields 1982a, b).

Three morphologically similar groups of ReoV have been described based on genetic divergence and antigenic properties among serotypes. These occur mainly in the gene coding for cell attachment $\sigma 1$ protein that gives rise to antibodies with strong, completely type-specific hemagglutination inhibition (HI) activity; strong, almost completely type-specific neutralization activity; and ability to precipitate ReoV particles (Gaillard and Joklik 1982; Cashdollar et al. 1985; Douville et al. 2008; Dermody et al. 2013). The three classically recognized serotypes represent the prototype strains: type 1 Lang (T1L), type 2 Jones (T2J), type 3 Dearing (T3D), and type 3 Abney (T3A). A putative fourth serotype strain isolated from a mouse in the

Cameroon has been identified and named Ndelle (T4N) (Clarke and Tyler 2010; Day 2009; Kohl et al. 2012; Dermody et al. 2013). These three serotypes currently circulate in humans and other mammals (Boehme et al. 2013). They are closely related in sequence and can exchange their ten genomic dsRNA segments by genetic recombination which occurs primarily through reassortment. This process generates some progeny (reassortants) that stably behaves as the wild type that can be grown under nonpermissive temperature conditions (Shiff 1998). It is known that T1L and T3D share approximately 25 % identity in their $\sigma 1$ protein, while other outer capsid and viral core proteins are highly conserved with 90–98 % identity (Douville et al. 2008). Serotypes 1, 2, and 3 differ in their response to a variety of chemical and physical inactivating agents as discussed below.

ReoV replication and assembly occurs in the cytoplasm of host cells within cytoplasmic structures called viral factories (VFs) or viral inclusion bodies (VIBs). Following virus replication, different morphotypes of reovirus particles can be found in infected cells, including genome-containing virions, infectious or intermediate subvirion particles (ISVPs), core particles, and genome-lacking particles (Dermody et al. 2013).

The size of the ReoV genome is approximately 23,500 base pairs (Day 2009; Dermody et al. 2013). Genomes of the prototype strains and several fusogenic *orthoreovirus* isolates have been completely sequenced (National Center for Biotechnology Information) including novel reassortant

MRV (Anbalagan et al. 2014; Lelli et al. 2015). Studies have indicated that 75 % of the RNA (15×10^6 Daltons) in the virion is contained in these 10 dsRNA segments, while about 25 % of the RNA in the virions ($\sim 4.5 \times 10^6$ Daltons) is composed of short single-stranded adenylic-rich oligonucleotides. Around 70 % of these oligonucleotides are 2–9-residue products of abortive transcription and terminate with 5'-GC(U)(A). The remainder (~ 30 %) are oligoadenylates that range in length from 2 to 20 residues (Silverstein et al. 1976; Dermody et al. 2013).

Occurrence of Reov in Natural Waters and Waste Waters

The frequent occurrence of ReoV in natural waters including surface and ground waters from different geographical areas has been documented in previous studies either as presence/absence—qualitative results (i.e., typing with conventional neutralization tests, distinctive cytopathogenic effects, immunofluorescence with specific antibodies, or molecular methods)—or as virus titers using different quantitative assays of infectivity in cultured cells. Most of the published work on ReoVs in ambient waters and wastewaters, with some exceptions (Dahling et al. 1989; Symonds et al. 2009) reported higher concentrations of these viruses than any other enteric virus analyzed. Table 2 summarizes selected studies on the occurrence of ReoVs in natural waters including streams, source waters (groundwater and surface waters) used for drinking water supply, and seawater.

Between the late 1960s and the mid-1980s, several studies demonstrated the frequent occurrence of ReoVs in wastewater (Adams et al. 1982; England et al. 1967; England 1972; Berg 1972; Sattar and Westwood 1978; Sellwood et al. 1981; Ridinger et al. 1982; Payment et al. 1986). Adams et al. (1982) using antibody immunofluorescence in cell culture was able to detect up to 210,000 ReoV per liter of untreated wastewater. Overall these studies documented higher levels of ReoV than any other enteric virus co-occurring in raw sewage and treated wastewater (Table 3). Surprisingly, ReoV occurrence in wastewaters was less frequently reported in subsequent years. This may be due to the types of cell lines used and the incubation time. The most commonly used cell line for testing for viruses in water is the BGM cell line. While ReoV will grow in this cell line, it usually requires 14 days or longer incubation for the observation of cytopathogenic effects.

More recent investigations confirmed the frequent occurrence of ReoV in raw sewage and treated effluent in the United States (Sedmak et al. 2005), the Netherlands (Lodder and de Roda Husman 2005), and Taiwan (Lim et al. 2015). The comparative reductions of ReoV and other human

enteric viruses after wastewater treatment have been documented in previous studies and also summarized in Table 3.

Sedmak et al. (2005) conducted the longest term study of the relative occurrence of ReoV. They used different cell lines for detection of infectious virus in raw sewage, activated sludge-treated disinfected effluent, and the intake to a drinking water treatment plant. ReoVs were the most commonly isolated viruses followed by enteroviruses and adenoviruses. All of their isolates were from BGM cells which had been maintained for 14 days. The occurrence of ReoV relative to the other enteric viruses after disinfection increased suggesting greater resistance of the ReoV to removal by activated sludge treatment and/or chlorine disinfection.

Recently Qiu et al. (2015) conducted a study on the removal of enteric viruses by activated sludge treatment, disinfection, and ultrafiltration at a full-scale wastewater treatment facility in Canada. Virus removal was assessed by both qPCR and integrated cell culture PCR (ICC-PCR). Interestingly ReoV, in contrast to the other enteric viruses, was rarely detected in the primary effluent and after other steps of the treatment process. However, it was the most commonly isolated virus by the ICC-PCR infectivity assay after secondary treatment, UV light disinfection, chlorination, and ultrafiltration. It was the only infective virus isolated after disinfection and ultrafiltration.

Sewage Sludge

ReoV appears to be common in sewage sludge and have been detected in raw, anaerobically, aerobically digested, and lagoon sewage sludge by infectivity assays (Wellings et al. 1976; Sattar and Westwood 1978; Gallagher and Margolin 2007). The relative concentration to other enteric viruses is difficult to judge because of different methods for recovery from the sludge and cell lines used for assay in the different studies. Also, quantitative assessment of removal of naturally occurring ReoV is difficult because extracting data on the operation of the processes is often not provided in enough detail. Using a primary cell line from a primate Cliver (1975) isolated ReoV in anaerobically digested sludge more commonly and in greater numbers than enteroviruses. Also, using a primary cell line Subrahmanyam (1983) reported the almost exclusive isolation of ReoV. It was also the most common isolate before sludge digestion. Laboratory studies suggest that ReoV may be more resistant to thermal inactivation than poliovirus. Brewster et al. (2005) found that ReoV was more resistant than poliovirus 1 at thermophilic (50–55 °C) and mesophilic (30–35 °C) temperatures used in anaerobic sludge treatment. However, Coxsackie and polio virus were shown to have similar inactivation rates during drying of sewage

Table 2 Occurrence of reovirus in natural waters

Water type and location	Methods	Detection rates/levels/remarks	Reference
Groundwater sites vulnerable to fecal pollution (multiple sampling locations across North America)	Filtration (1-MDS Virosorb filter). Multiplex reverse transcription PCR used for detection of enteric viruses.	62 % of the sites positive for viruses ReoV most frequently detected virus 10 % samples positive for ReoV 5 % samples positive for EV 3 % samples positive for Norwalk virus 1 % samples positive for HAV	Fout et al. (2003)
Aquifer and drinking water supply wells (Florida, USA)	Filtration (1-MDS Virosorb filter). BGM cells used for detection of enteric viruses. Typical CPE in BGM cells used for identification of ReoV.	ReoVs found in 3 of 4 active water wells (0.0002–0.0015 MPN/L)	Betancourt and Rose (2005)
Source waters for drinking water supply (Lake Michigan, USA)	Filtration (1-MDS Virosorb filter). Cell culture using multiple cell lines (BGM, Caco-2, RD, and HEP-2) for detection of total culturable viruses (MPN/L). Typical CPE in BGM cells used for identification of ReoV. Quantification of ReoV by DFA test using an antireovirus polyclonal fluorescent antiserum of caprine origin.	Culturable viruses detected in 18 of 204 (8.8 %) samples ReoV was the only virus detected. Virus titer ranged between 15.8 and 59.0 MPN/L.	Sedmak et al. (2005)
Surface waters used for potable water (12 sampling locations in different states across North America)	Filtration (1-MDS Virosorb filter). Combined cell culture (BGM cells) and RT-PCR assay using primers targeting the L3 gene that encodes the $\lambda 3$ major core protein for detecting and genotyping ReoV	ReoV detected in 5 of 26 (19.2 %) virus CPE-positive samples. Sequence analysis of L3 gene products revealed significant sequence diversity among ReoV isolates. ReoV proposed as valuable targets for monitoring viral water contamination	Spinner and DiGiovanni (2001)
Surface waters, drinking water, karst springs, treated raw water	Concentration by aluminum hydroxide flocculation and filtration (1-MDS Virosorb filter). Detection by cell culture and SDS-PAGE to determine dsRNA electrophoretic patterns.	ReoV was detected in every type of water. Constant electrophoretic patterns were associated with reovirus type 1 and 3.	Milde et al. (1995)
Coastal waters (estuary and seawater) of the middle northern Adriatic Sea, Italy	Prefiltration of water samples through polypropylene membranes followed by tangential flow filtration using polysulfone membrane with a 10,000 molecular weight exclusion size, elution in 3 % beef extract, and a final concentration step using a polysulfone membrane. BGM and Hep-2cells used for isolation of viruses. Cell culture homogenous lysates tested for EVs by RT-PCR and ReoV dsRNA using nondenaturing polyacrylamide gel electrophoresis.	ReoV found with higher frequency than EV in all samples. 27 out of 40 samples (67.5 %) positive for viruses based on CPE. EV was detected in 8 of 25 (32 %) of samples while both EV and ReoV were detected in 18 of 25 (72 %).	Muscillo et al. (1997)
Seawater samples from two areas of the Adriatic Sea	Prefiltration of water samples through polypropylene membranes followed by tangential flow filtration using polysulfone membrane with a 10,000 molecular weight exclusion size, elution in 3 % beef extract, and a final concentration step using a polysulfone membrane. BGM cells and Hep-2 cells used for isolation of viruses. Cell culture homogenous lysates tested for ReoV by RT-PCR and PAGE and EV by RT-PCR	ReoV detected after ICC-PCR in 22/72 samples. No EV was detected. Multiple blind passages (up to four) on BGM cells the presence of viral particles Increased enough to be detectable by RT-PCR or both RT-PCR and PAGE	Muscillo et al. (2001)

Table 2 continued

Water type and location	Methods	Detection rates/levels/remarks	Reference
River water samples from Safo River, Nara Prefecture, Japan.	Centrifugation followed by hydroextraction with polyethylene glycol plus an additional centrifugation step and final suspension of the pellet in 3 % beef extract. Plaque assay technique used for virus enumeration. Identification of reoviruses based on the hemagglutination inhibition test	ReoV most frequently isolated throughout the 5-year study. Types 1 and 2 most frequently isolated. Levels of ReoV ranged from 0 to 325 PFU/L (average 56.2 PFU/L). Peak of ReoV found in winter (Nov to Mar). Levels of EV ranged from 0 to 190 PFU/L (average 40.6 PFU/L). Peak found in summer (May–Sept).	Tani et al. (1995)
Stream sites from multiple locations in major water systems of the United States	Sample processing included prefiltration with a 10- μ m polypropylene prefilter cartridge and filtration with a 1MDS cartridge filter following U.S. EPA ICR method. Cell culture and multiplex RT-PCR used for detection of viruses (EV, ReoV, RV, HAV, NoV). Results confirmed by dot-blot hybridization.	Culturable EV detected in 13 of 15 (87 %) of the samples ReoV detected in 9 of 30 (30 %) RV detected in 6 of 30 (20 %) HAV detected in 5 of 30 (17 %) NoV was not detected.	Denis-Mize et al. (2004)
Source, finished and tap water	Virus concentration via filtration through 1 MDS filters. TCVA and ICC-PCR used for detection of multiple enteric viruses.	ReoV detected at a higher frequency (89 %) than AdV and EV (between 15 and 21 %) after testing CPE-positive cell culture using reovirus-specific primers	Lee and Jeong (2004)
Source water for drinking water production at three locations in the Netherlands	Adsorption–elution with electronegative cartridges used for primary concentration of viruses in the samples. Secondary concentration accomplished by ultrafiltration and the two-phase separation method BGM cells used for detection of ReoV and EV. Other viruses by PCR.	Detection rates for ReoV ranged from 83 to 100 %, while detection of EV ranged from 30 to 75 %. Mean concentrations of ReoV varied between 0.003 and 5.9 PFU/L. Mean concentrations of EV ranged from 0.0052 to 2.4 PFU/L. NoV and RV RNA detected in 45 and 48 % of the samples, respectively.	Lodder et al. (2010)
Surface water samples from two large rivers in the Maas and Waal Rivers, The Netherlands	Adsorption–elution with electronegative cartridges (Nominal 1.2 μ m pore-sized filter) used for primary concentration of viruses. Secondary concentration accomplished by ultrafiltration and the two-phase separation BGM cells used for detection of ReoV and EV. Other viruses studied included NoV, RV by PCR.	ReoV: 2–10 PFU/L EV: 0.3–2 PFU/L NoV: 29–4,900 PDU/L RV: 57–5,386 PDU/L	Lodder and de Roda Husman (2005)
Riverbank filtration system in a managed aquifer recharge site. Aurora, Colorado, United States	Adsorption–elution using NanoCeram filters. Secondary concentration accomplished by organic flocculation. BGM cells used for detection of cultivable viruses. Confirmation of ReoV RNA by PCR	ReoV RNA was the only infectious virus detected in a well with a subsurface residence time of 5 days	Betancourt et al. (2014)

CPE cytopathic effect; *Hep*: human laryngeal carcinoma, *DFA*: Direct Fluorescent Assay, *RD*: Human Rhabdomyosarcoma cells, *MPN/L*: most probable number per liter, *SDS-PAGE*: sodium dodecyl sulfate polyacrylamide gel electrophoresis, *TCVA*: Total culturable virus assay, *ICC-PCR*: integrated cell culture—polymerase chain reaction; *RT-PCR*: reverse transcription—PCR; *ReoV*: Reoviruses; *EV*: Enteroviruses, *HAV*: Hepatitis A virus, *RV*: Rotaviruses; *NoV*: Noroviruses; *AdV*: Adenovirus; *dsRNA*: double-stranded ribonucleic acid; *U.S-EPA-ICR*: United States Environmental Protection Agency-Information Collection Rule, *PFU*: Plaque forming units

sludge (Ward and Ashley 1977). The mechanisms of ReoV inactivation in sewage sludge are complex depending on temperature, pH, ammonia, and the presence of detergents. Inactivation of ReoV results from the breakdown of the viral particles (Ward 1983). ReoV appears to show similar resistance to enteroviruses and MS2 phage to lime

treatment of sludges (Ward 1983; Brewster et al. 2005; Katz and Margolin 2007). In lime-treated fecal sludge, from latrines, the reduction of ReoV type 3 was found to be similar to adenovirus type 1 and related to ammonia concentrations above pH 9 (Magri et al. 2015). The existing data suggest that ReoV may be more abundant than

Table 3 Reovirus occurrence in wastewaters and reduction through conventional and advanced wastewater treatment processes

Sample location and type	Methods	Detection rates/levels/remarks	Reference
Conventional activated sludge	Sampling sites included raw sewage, settled water, effluent after activated sludge treatment, and sludge. Viruses concentrated by the Viradel method using electronegative filters (Duo-Fine filter) and organic flocculation. Plaque assay (BGM cells) and immunoassay used for detection of multiple enteric viruses.	ReoV and coxsackieviruses appeared to be more resistant to the activated sludge treatment than polio 3	Payment et al. (1986)
Activated sludge	Influent and effluent Virus concentrated by organic flocculation. Multiple cell lines (BGM, Caco-2, RD, and HEp-2) used for detection of multiple enteric viruses. Reoviruses identified by typical CPE in BGM cells and Direct Fluorescent Antibody (DFA) test using an antireovirus polyclonal fluorescent antiserum of caprine origin.	Frequent detection (98 %) of culturable viruses in wastewater Influent—wastewater: ReoV detected in 90.6 % of the samples followed by EV (85 %) and AdV (63.6 %). Virus titer highest for ReoV (12,027 MPN/L) compared to EV (3,347 MPN/L) Treated effluent: culturable viruses detected in 29 % of samples. Virus titer highest for ReoV (221 MPN/L), followed by AdV (8 MPN/L) and echovirus (4 MPN/L)	Sedmak et al. (2005)
Activated sludge	Raw and effluent samples. Virus concentration included membrane filtration (AP20 prefilter and 0.45 µm mixed cellulose membrane filter). Cell culture (monkey kidney, BGM, HeLa-R, HEp-2, human embryonic lung cells) and neutralization tests used for virus detection.	ReoV found at higher concentrations (2,150 IU/L) and frequency than EV (1400 IU/L) and AdV (1,950 IU/L) in raw sewage. ReoV reduced less efficiently (28 %) than AdV (85 %) and EV (93 %).	Irving and Smith (1981)
Trickling filter and activated sludge. Fifteen sewage treatment plants in selected Puerto Rican communities	Filtration (1-MDS Virosorb filter). Combined cell culture (BGM by the suspended-cell procedure for EVs and Madin Darby bovine kidney (MDBK) cell line by the cell monolayer procedure for reoviruses) EV identification accomplished with the Lim Benyesh-Melnick antiserum pools (LBM pool analysis). Viruses isolated from MDBK were assayed by the same method using ReoV antiserum.	ReoV detected in raw sewage from nondetected to 1,247 PFU/L Effluent samples: from nondetects to 21 PFU/L EV detected from 100 to 242,500 PFU/L in influents and 1–224,000 PFU/L in effluents	Dahling et al. (1989)
Activated sludge City of Apeldoorn, The Netherlands	Adsorption–elution with electronegative filter cartridges for primary concentration. Secondary concentration accomplished by the two-phase separation method BGM cells used for detection of ReoV and EV. Other viruses studied included NoV, rotaviruses detected by RT-PCR. Quantification of viruses by plaque assay (PFU/L) or by RT-PCR diluted RNA) expressed as PCR-detectable units per liter (PDU/L)	ReoV: Raw: 111–2,143 PFU/L Effluent: 8–92 Removal: 1.1–1.4 log ₁₀ EV Raw: 140–833 PFU/L Effluent: 5–39 Removal: 0.7–1.8 log ₁₀ NoV: Raw: 4.6x10 ⁴ –8.5x10 ⁵ PDU/L Effluent: 896–7499 PDU/L Removal: 0.2–1.9 log ₁₀ Rotavirus Raw: 339–2.9 × 10 ⁴ PDU/L Effluent: 598–2.9 × 10 ⁴ PDU/L Removal: 0–1.1 log ₁₀	Lodder and de Roda Husman (2005)

Table 3 Reovirus occurrence in wastewaters and reduction through conventional and advanced wastewater treatment processes

Sample location and type	Methods	Detection rates/levels/remarks	Reference
Activated sludge. Rome, Italy	Virus concentration via filtration through electropositive membranes (1 MDS membrane filters) Cytopathogenic Units (MPNCU) through inoculation onto BGM (Buffalo Green Monkey) cell monolayers and MPN quantification. Identification of viruses: Enteroviruses were typed by serum neutralization tests using pools of antisera. ReoV was confirmed polyacrylamide gel electrophoresis (PAGE)	Raw: 100 % ReoV, 83 % EV Primary sewage 100 % ReoV, 75 % EV Secondary sewage 100 % ReoV, 27 % EV Effluent: 100 % ReoV, 54 % EV	Aulicino et al. (1996)
Activated sludge with nutrient removal followed by advanced treatment: ultraviolet (UV) disinfection, ultrafiltration (UF) and chlorine disinfection Edmonton, Canada	Virus concentration by NanoCeram 90-mm laminated disc filter and organic flocculation. Detection by ICC-qPCR using BGM cells	Infectious ReoV was the only virus detected by ICC-qPCR after chlorine disinfection (12 %, 3 of 25) and ultrafiltration (8 %, 1 of 13) of the final effluent	Qiu et al. (2015)

enteroviruses in sewage sludge and may be more resistant to digestion than enteroviruses. ReoV has been suggested as a better model for sludge treatment than enteroviruses (Brewster et al. 2005).

Methods for Concentration and Detection of ReoV in Water

Different approaches have been applied for concentration of ReoV from sewage including cotton gauze swab, precipitation by protamine sulfate treatment (salmine) and isoelectric coagulation (i.e., flocculation). Early studies demonstrated that protamine sulfate treatment provided an efficient method for concentrating ReoV and adenoviruses which tended to be overgrown by more rapid replicating enteroviruses in cell culture (England 1972). The efficiency of the protamine sulfate method with some modifications for the recovery of ReoV from waste waters and sewage was later confirmed by another study in the U.S. (Adams et al. 1982). In this study, infectious ReoV and potentially infectious ReoV particles (particles with complete outer coat) were concentrated from water and sewage by protamine sulfate precipitation followed by addition of 0.25 % fetal bovine serum and 0.005 % protamine sulfate for the first precipitation of the sample plus 0.0025 % for the second. The study reported virus recoveries between 80 and 100 % with levels of infectious ReoV greater than 10^5 per liter of untreated sewage, which suggested that the modified protamine precipitation method when combined with fluorescent-antibody staining was useful in determining the potential roles of the infectious virus and ReoV particles in water quality testing.

Another study (Bottiger 1973) investigated the viral content of sewage collected with gauze swabs at the inlet of three major sewage treatment plants of the Stockholm area. Echovirus type 1 and ReoV were found throughout the entire period of the study using primary monkey kidney cells. Later, a comprehensive study from the United Kingdom (Sellwood et al. 1981) conducted over a 3-year period at a local sewage works (biological oxidation beds) found that multiple cell lines were necessary for efficient evaluation of the virus content of raw sewage and treated effluent. In this study, Vero and primary monkey kidney cells yielded the most Cocksackievirus and ReoV isolates, which were frequently isolated along with echovirus from the plant inlet (raw sewage) and outlet swabs. In a similar study in the United States, the sensitivities to ReoV (serotypes 1, 2, and 3) of 12 continuous cell lines was compared with sewage isolates precipitated by protamine sulfate (Ridinger et al. 1982). Among the cell lines evaluated, Madin–Darby bovine kidney (MDBK) was the most sensitive for isolation of ReoV, and when combined with an immunofluorescent cell count assay, the detection rate gave a ReoV titer in excess of 10^4 /liter of raw sewage.

Adsorption/elution methods (e.g., membrane, cartridge, and glass wool filtration) and entrapment or size exclusion techniques which involve ultrafiltration systems (e.g., tangential flow filtration) have been applied for processing large volumes of water including treated sewage and surface waters (Milde et al. 1995; Muscillo et al. 1997; Fout et al. 2003; Lodder and de Roda Husman 2005; Sedmak et al. 2005). Following primary concentration, different secondary concentration procedures have been used to further isolate ReoV and other enteric viruses. Two-phase separation with dextran and polyethylene glycol (PEG)—or PEG and NaCl—beef extract elution, and PEG

precipitation have been most commonly used for the secondary concentration step (Wyn-Jones 2007). Very few studies have evaluated the recovery efficiency of ReoV from water. It is very important since the sorption behavior of virus particles is governed by electrostatic interactions which are given by the virus isoelectric point (IEP); this is a characteristic parameter of the virion in equilibrium with its environmental water chemistry (Michen and Graule 2010). In this situation, the sorption behavior of a particular virus may substantially differ from the most common human viruses (i.e., poliovirus) or viral surrogates (i.e., bacteriophages) used in recovery efficiency experiments. For instance, reported isoelectric points for poliovirus strains range from 4.0 to 7.4, MS2 coliphage 2.2–4.0, and ReoV type 3 from 3.8 to 3.9 (Michen and Graule 2010).

The detection of ReoV in water concentrates has been accomplished through different assay methods. As previously mentioned, virus replication in continuous cell lines of monkey kidneys was initially applied for isolating ReoV from water; however, they have the ability to infect many continuous mammalian cell lines maintained in clinical laboratories (Dermoddy et al. 2013). The identification of ReoV in water based on observation of typical cytopathic effect (CPE) has been applied simultaneously with quantitative assays such as hemagglutination or immunological methods which quantify the presence of infectious and noninfectious virus particles (Condit 2013). ReoV produces a very characteristic cytopathogenic effect (CPE) in monkey kidney cell lines. The cells become granular and do not slough off as readily as with enteroviruses. Often they remain fasten to the glass by a single process and flutter in the medium as the flask/tube is agitated during microscopic examination. The addition of the proteolytic enzyme can be used to enhance infectivity in cell culture (Spendlove and Schaffer 1965). Cell culture assay methods have been concomitantly applied for quantification of ReoV infectivity such as most probable number assays (MPN) which derive MPN titers (i.e., virus titer) in replicates of cell monolayers inoculated with different equivalent volumes of a water concentrate (Lee and Jeong 2004; Sedmak et al. 2005). Studies have also shown that multiple blind passages on BGM cells may increase the presence of virus particles for subsequent detection of ReoV RNA by RT-PCR or polyacrylamide gel electrophoresis (PAGE) (Muscillo et al. 2001). Interestingly, these studies have also demonstrated that the simultaneous presence of ReoV and enteroviruses can have a masking effect by hindering enterovirus amplification and detection in environmental samples highly contaminated with ReoV (Carducci et al. 2002).

ReoV RNA obtained from cell culture homogenous lysates has been used to study dsRNA segment patterns separated on a nondenaturing polyacrylamide gel (Milde

et al. 1995; Muscillo et al. 1997). Further combination of cell culture with molecular amplification of viral RNA sequences (ICC-PCR) added another feasible and relatively more sensitive approach applicable to ReoV detection in water (Spinner and DiGiovanni 2001; Lee and Jeong 2004).

Genome-based molecular techniques offer rapid detection and identification of ReoV. These assays involve reverse transcriptase polymerase chain reaction (RT-PCR) with primers designed to amplify specific viral RNA sequences. Genomic sequencing and phylogenetic analysis are subsequently used to identify specific genotypes and/or to confirm the isolate serotype as well as to reveal virus diversity. Most studies included initial replication of the virus in cell culture to increase viral genomes available for molecular identification and typing assays (Spinner and DiGiovanni 2001; Muscillo et al. 2001).

Primers that target conserved regions of the ReoV L1, L3, and S2 genes have been developed and applied for molecular detection of ReoV RNA in water (Muscillo et al. 2001; Spinner and DiGiovanni 2001; Leary et al. 2002). The sensitivity of amplification of ReoV RNA with these primers was evaluated differently. The detection sensitivity with primers against the L1 gene was evaluated with serial dilutions of the purified PCR-positive control. The lowest concentration in which a band could be visualized defined the sensitivity of the assay which corresponded to 100 targets per reaction (Symonds et al. 2009). Spinner and DiGiovanni (2001) used serially diluted purified nucleic acids from ReoV types 1, 2, and 3 to determine the sensitivity of the ReoV primers against the L3 gene. The detection sensitivities corresponded to approximately 3, 30, and 0.3 PFU per reaction mixture, respectively. The sensitivity of the RT-PCR reactions with primers against the S2 region was tested on the RNA extracted from serial dilutions of virus stock ranging from 10^5 tissue culture infectious dose (TCID₅₀) to 0.1-fold. In this case, the S2 reactions were positive on the reference strains with a sensitivity of less than 10^2 – 10 TCID₅₀ viral particles (Muscillo et al. 2001).

Additionally, reverse transcription quantitative real-time PCR (RT-qPCR) methods have been developed for sensitive, rapid, and specific detection of ReoV RNA in wastewater samples (Gallagher and Margolin 2007; Qiu et al. 2015). These assays have not been tested for detection of ReoV in other water matrices.

ReoV Stability in the Environment

Studies have shown that ReoV is very environmentally stable; its high stability outside the host enables this virus to remain viable for up to a year at 4 °C (Berard and

Coombs 2009; Nibert et al. 1995). Additional studies conducted under controlled laboratory conditions using distilled water and wastewater demonstrated that among different enteric viruses evaluated ReoV and rotavirus survived more than 30 days at 26 °C and longer than 1 year at 8 °C (McDaniels et al. 1983). Similarly, studies which involved the incubation of ReoV and poliovirus in a buffered solution (2× Saline Sodium Citrate: 300 mM NaCl, 30 mM sodium citrate, pH 7.0) at different temperatures (22, 37, 39.5, 42, 45, and 50 °C) indicated that survival of ReoV as determined by plaque assay was greater than for poliovirus. This study provided good evidence to support ReoV being more resistant to temperature-induced inactivation than PV (Brewster et al. 2005). ReoVs have been shown to be resistant to high temperature (55 °C) for short periods of time (McVey et al. 2013).

ReoV is very stable in aerosols in relative humidity above 75 % or below 40 % and may be expected to survive spray irrigation with wastewater (Spendlove and Fannin 1982; Sattar and Springthorpe 1999).

Resistance to Disinfection

Several studies have demonstrated that ReoV are more resistant to Ultraviolet (UV) light inactivation than single-stranded human RNA viruses (Chevrefils et al. 2006; Lytle and Saripanti 2005; Harris et al. 1987; Hill et al. 1970). Doses of UV light for ReoV type 1 (Lang) have been observed to be almost double of poliovirus type 1 for a 99.9 % reduction in titer (24 vs. 46 mW/sec/cm²) (Harris et al. 1987). McClain and Spendlove (1966) observed differences in UV light sensitivity of ReoV 1–3 at higher doses of UV, with ReoV type 3 being the most resistant. They also observed a nonlinear rate of inactivation and attributed to it experimentally to multiplicity of reactivation, i.e., two or more viruses infecting the same cell with different regions of damaged genome. They also observed that multiplicity of reactivation occurred between the different ReoV types, i.e., ReoV 1 could serve to complement ReoV 2 infecting the same cell. It has also been suggested that the greater resistance of ReoV to UV light is due to its double-stranded segmented genome, a thicker protein coat than the single-stranded enteroviruses, and greater ability to aggregate (Harris et al. 1987). In a study of resistance of naturally occurring coliphages and ReoV to UV light in secondary effluent, the rate of ReoV inactivation was found to be almost identical to F-specific coliphages (Nieuwstand et al. 1991).

Liu et al. (1971) reported that highly purified ReoV types 1–3 were more sensitive to chlorine in buffered water and Potomac River water than various types of adenovirus, poliovirus, echovirus, and Coxsackievirus. The most

detailed studies on ReoV inactivation by disinfectants was conducted by Sharp et al. (1975). They found that inactivation of ReoV and enteroviruses was highly dependent on the size and number of viral aggregates. The viruses could be made to aggregate and deaggregate by changing the pH, salt concentration, and the types of salts in solution (Floyd and Sharp 1977; 1978a; 1978b). Their studies also suggested that the observed tailing effect during bromine disinfection was due to aggregates larger than 1,000 virions. These “super sized” aggregates were too rare to be seen under the electron microscope, but by sonication they could break some of these resulting in an increase in titer. They suggested that these “super” aggregates were formed in cell culture and in addition to aggregated virus might also contain cell debris. Another phenomenon observed was the ability of ReoV and poliovirus type 1 to form aggregates together (Floyd 1979). Thus, dissimilar viruses could form aggregates. Since they found that 10⁵–10⁶ virions were needed to form aggregates, they concluded this was not a significant factor in viral resistance in wastewater. However, with modern molecular methods such as qPCR for quantifying genome copies of viruses, concentrations of 10⁵–10⁶ per mL of adenoviruses, Aichi viruses, and pepper mild mottle virus (PMMoV) are known to occur in wastewater (Kitajima et al. 2014). In addition, the formation of disinfectant-resistant viral aggregates may be possible, especially considering that the total concentration of all human, plant, and bacterial viruses likely exceeds 10⁷ per mL in domestic wastewater (Kitajima et al. 2014).

Reovirus exhibits greater resistance to inactivation by preformed chloramines and ozone than poliovirus, but is more sensitive to inactivation by chlorine and chlorine dioxide, 95 % ethanol and quaternary ammonium disinfectants, such as Roccal ROCCAL[®]-D Plus used in veterinary practice based on laboratory studies of purified virus (Drulak et al. 1984; Liu et al. 1971; McVey et al. 2013).

Concluding Remarks

ReoV has not been studied as much in the environment, as enteroviruses and adenoviruses, especially using qPCR. Existing data suggest that they may be among the most common enteric viruses in wastewater, if not the most common. Studies on disinfected wastewater suggest that they may be more sensitive to chlorine than enteroviruses. However, they are more resistant to ultraviolet light disinfection than enteroviruses because of their dsRNA genome. ReoV also appears to be more environmentally stable than enteroviruses. Fout et al. (1996) found ReoV to be the most common viruses in groundwater in the United

States. Studies at managed aquifer sites also suggest that they may travel further than other enteric viruses in the subsurface (Betancourt et al. 2014). However, their role in human disease is unclear, but given the role of bats in emerging zoonotic diseases such as SARS, Ebola, and MERS and zoonotic potential (including bats) of ReoV, additional studies on routes of human exposure are needed.

Moreover, the ubiquitous presence of ReoV in mammals, frequent occurrence in sewage and relative resistance to ambient conditions suggest that these viruses may be used to determine the vulnerability of watersheds, coastal areas, and groundwater sites to fecal pollution and consequently as an indication of enteric virus contamination (Muscillo et al. 1997; Spinner and DiGiovanni 2001; Fout et al. 2003; Shoeib et al. 2009). This review suggests that environmental transmission of ReoV clearly needs more study both to better assess its potential for transmission and its usefulness of an indicator of enteric viruses in the environment.

Acknowledgments This work was supported by the United States Department of Agriculture-National Institute of Food and Agriculture, Grant number 20166800725064, that established CONSERVE: A Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health.

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