Detection and Characterization of Orthoreovirus in Alberta's Environmental Waters

by

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Abstract

Reoviruses have been shown to infect a very broad range of mammalian sources and infections in humans have been associated with gastrointestinal and respiratory illnesses. While the presence of reovirus in several different water matrices and clinical samples has been reported, the clinical implications of reovirus in Alberta's water and clinical samples have not been studied because of the lack of an appropriate screening method. To improve reovirus detection, a novel reverse-transcription real time PCR (qRT-PCR) assay was designed, developed, and implemented. Two sets of novel qPCR primers and probes (targeting the M1 and L3 genetic fragments) were designed and found to have sensitivities of 5 genetic copies per qPCR for M1 and 50 genetic copies per qPCR for L1, respectively. Using the newly developed qRT-PCR assay, reoviruses were detected in wastewater (15/16) and environmental surface water (20/216) samples obtained from various sites in Alberta, while no reoviruses were observed in clinical fecal samples obtained from patients suffering from gastroenteritis. Phylogenetic trees produced from S4 gene fragment sequences indicate that a variety of S4 alleles are present in the environment in Alberta, which is consistent with data previously published regarding reoviruses in the environment.

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1.0 Literature Review

1.1 History of Reoviruses

The first reovirus was isolated and cultured in the 1950s, along with a group of other enteric viruses, from both healthy children as well as from children with documented aseptic meningitis (20, 51, 94). Reoviruses were initially described as an unusual group of viruses, as they were capable of rapid growth in monkey-kidney tissue cultures, yet lacked any noticeable pathogenicity when introduced to laboratory mice (94, 95).

Upon its discovery, reovirus was initially referred to as an Enteric Cytopathogenic Human Orphan (ECHO) virus; specifically, ECHO Type 10 Lang, due to its presence in the gastrointestinal system. Soon after the initial discovery of the new group of viruses, screening of fecal and oral swabs from nursery children with observable enteric problems, such as diarrhea and abdominal discomfort, in Washington in the late 1950s revealed an outbreak of the newly discovered virus (92, 93). It was noted at the time that the virus' presence in rectal swabs could be detected for up to five weeks after the speculated time of infection (89, 90, 91). The name "reovirus" was introduced to describe a growing group of cytopathogenic viruses that were believed to cause both respiratory and enteric symptoms in clinical patients, but were not directly attributed to any particular known disease condition. Hence, Respiratory Enteric Orphan viruses became known as the reoviruses (95). The recently described ECHO Type 10 Lang, discovered in healthy children as well as children with aseptic meningitis or gastrointestinal symptoms, was renamed reovirus serotype 1 Lang (92, 93).

Although initially discovered in human fecal and oral swabs, reoviruses infect a very broad range of different species including mammals, birds, fish, and reptiles (71). Due to increasing membership in the family *Reoviridae*, specific genera were described, with reoviruses that preferentially use mammals, birds, and reptiles as their host being grouped together as the genus *Orthoreovirus*. Members of the *Orthoreovirus* genus have several common traits, including a genome that is composed of 10 genetic fragments, infection of a vertebrate host, and a capsid that is composed of an inner and outer layer (106). When investigating potential candidates for the *Orthoreovirus* genus, one approach sought to determine if the new candidate was capable of reassorting its genetic fragments with those of a known orthoreovirus (23). To further distinguish members of the *Orthoreovirus* genus, members are described based upon which species (or groups of species) they infect, with *Orthoreovirus* members who infect mammals (including humans) being described as the mammalian orthoreoviruses (or MRVs) (71).

1.2 Structure and Taxonomy of Reovirus

Reoviruses are large, non-enveloped particles of approximately 70-80 nm and feature a double-layered capsid (71). The inner capsid layer and the viral genome combined are collectively referred to as the viral core (71).

The family *Reoviridae* can be divided into two subfamilies based on the presence or absence of an internal turret-like protein structure that is attached to the inner capsid layer. This turret-like protein is encoded by the L2 gene fragment and is known as λ2. The *Spinareovirinae*, which contains nine distinct genera including *Orthoreovirus* have this turret-like λ2 protein present. The six genera of the subfamily *Sedoreovirinae* do not have the λ2 turret-like protein present on their inner capsid layer (8).

The *Orthoreovirus* genus can be further divided into two subgroups: fusogenic and non-fusogenic orthoreoviruses. Fusogenic orthoreoviruses are capable of causing infected cells to fuse together, creating a multinucleated cell known as a syncytium. Inversely, non-fusogenic orthoreoviruses are not capable of causing cell-to-cell fusion (8, 26, 27, 28, 67). While there are fusogenic orthoreoviruses that infect mammalian species (the Nelson Bay virus and baboon reovirus), the three prototypic mammalian orthoreovirus serotypes are known to be non-fusogenic (25, 26).

There are three serotypes of mammalian orthoreoviruses: serotype 1 Lang (T1L), serotype 2 Jones (T2J), and serotype 3 Dearing (T3D) (71, 95). T1L was initially isolated from an anal swab of a healthy child; T2J from a child with diarrhea; and T3D from children with diarrhea and/or respiratory illness (83 - 88). The three serotypes of mammalian orthoreoviruses were differentiated based upon hemagglutination inhibition studies (89). All three mammalian orthoreovirus subtypes have been recovered from patients who were voluntarily inoculated, suggesting that the virus is well suited to surviving within the human respiratory and enteric tracts (90).

Reoviruses form a secondary particle apart from the traditional virion. Reoviruses may degrade their outer protein capsid layer and form a secondary particle known as an infectious subviral particle (ISVP) during their life cycle. The ISVP has been shown to have increased infectivity relative to typical mammalian orthoreovirus virions, but have been shown to attach to host cells in the same manner (9, 10).

During infection, the reovirus σ1 protein (encoded by the S1 genomic fragment) is responsible for the attachment of reoviruses to junctional adhesion molecule-A (JAM-A) in host cells in the presence of sialic acid (8, 21, 40, 57, 70, 110, 122). In humans and

other mammals, JAM-A is responsible for formation and resealing of tight junctions between cells (9, 62). After attachment, the orthoreovirus virion or ISVP enters the host cell by endocytosis (57). The particle then digests the endosome by proteolysis, and mammalian orthoreovirus begins the first round of RNA synthesis: (+)-strand transcription (8), followed by protein synthesis, and (-)-strand replication (3). After infection and replication, mammalian orthoreoviruses cause apoptosis to a variety of host cells (18, 19).

Members of the family *Reoviridae* are known to have 10 to 12 fragments of double-stranded RNA (71). Mammalian orthoreoviruses contain a segmented genome of approximately 23,500 nucleotides featuring 10 genomic fragments with varied sizes (99). The genome is composed of three large (L) segments, three medium (M) segments, and four small (S) fragments and encodes eight structural and three non-structural proteins (71).

It has been demonstrated that reoviruses, being double-stranded RNA viruses, have both a high mutation rate and are prone to frequent gene reassortment between strains (17, 79, 117). This presents an issue when molecular detection is considered, as increased diversity and mutations may reduce the amount of potential targets for molecular detection. Previous efforts have paid attention to the similarities and differences found between the genomic fragments of the three mammalian orthoreovirus serotypes (13, 37, 114, 115, 116). The lengths of the genomic fragments range in size from 1196 nucleotides for S4 to 3916 nucleotides for L2 (71). In terms of homology between serotypes, it has been demonstrated that serotypes 1 and 3 are more closely related genetically to one another than they are to serotype 2, as demonstrated by

hybridization of single-stranded RNA between serotypes (37). It has been shown that nine of the ten mammalian orthoreovirus gene fragments show increased homology between serotypes 1 and 3. The only gene that strays from this paradigm is the S1 gene fragment, which encodes the σ1-protein (14). The three *L* show the highest rate of homology between the three serotypes, followed by the three *M* genes, and finally, the four *S* genes (37).

The lengths of the genetic fragments are fairly well conserved across the three serotypes, with nine of the ten fragments being either identical in length or differing by less than ten base pairs (as illustrated in Table 1.1). The S1 fragment is the only fragment that differs in size between the three serotypes by more than ten base pairs. This is an interesting feature of reovirus' genome as this fragment encodes the σ1 protein, which is responsible for viral attachment to host cells (40). The conserved size of fragments across reovirus' serotypes becomes important during PCR primer design for sequencing of reoviruses, as conserved fragments across the three serotypes provides potential targets for amplification across all serotypes with a single primer set.

Table 1.1 Comparison of the actual lengths in base pairs of large (L), medium (M), and short (S) genomic fragments of the three mammalian orthoreovirus subtypes (75).

Genetic Fragment	Seroype 1 Lang	Serotype 2 Jones	Serotype 3 Dearing
L1	3860	3854	3854
L2	3916	3915	3916
L3	3901	3901	3901
M1	2304	2304	2304
M2	2203	2203	2203
M3	2241	2241	2241
S1	1462	1440	1416
S2	1331	1331	1331
S3	1198	1198	1198
S4	1196	1196	1196

1.3 Human and Animal Reovirus

Reoviruses have been demonstrated as having a wide array of potential hosts (71, 106). It has been shown that reoviruses can infect a variety of animals including, but not limited to, mammals, birds, fish, and reptiles (22). Although the impulse for many in the health sciences may be to focus on humans, there exist several types of reoviruses that pose a significant risk to their animal hosts. Grass carp are an economically important fish species in China. Grass carp reovirus (GCRV) poses a significant threat to the Chinese economy and aquaculture as it causes grass carp hemorrhage, a disease that proves fatal to fingerling grass carp (121, 123). Similarly, avian reoviruses have caused a substantial threat to chicken populations: it has been demonstrated that strains of reovirus are responsible for viral arthritis and pale bird syndrome in chicken populations (98). The potential risk to chickens has lead to increasing efforts to develop a reovirus vaccination for poultry (118).

Mammalian orthoreoviruses have been implicated in biliary atresia in humans. Biliary atresia is most frequently detected in newborns and is characterized as the complete lack of or malformation of the extrahepatic bile duct (32, 41, 64, 85, 107). Reovirus has been detected by PCR in 21 of 64 liver biopsies taken from patients with biliary atresia who were undergoing Kosai portoenterostomy (85). In addition, reoviruses have been isolated from patients suffering from acute enteric and respiratory symptoms as well as necrotizing encephalopathy (90 - 93, 95).

A series of antibody detection studies have demonstrated that reovirus-specific antibodies can be detected in up to 75% of newborns, with a gradual loss of antibody around one year of age. Furthermore, it was shown that 50% of children aged 5 to 6

possess anti-reovirus antibodies, suggesting that exposure to reovirus in common in young children (104). Another study exploring the presence of reoviruses in patients suffering from gastroenteritis investigated stool samples obtained over a period of twenty years. These stool samples were screened using polyacrylamide gel electrophoresis (PAGE) of the entire RNA genome with additional electron microscopy of potential positives and found reoviruses to be present in only 3 of 2854 (0.10%) stool samples, indicating that reovirus is not a causative agent of gastroenteritis (38).

Contradictory studies in the 1950s showed that six of eight prisoners that were initially seronegative were converted to seropositive after they were voluntarily infected with reovirus (90). Three of these volunteers were eventually hospitalized with diarrhea, fatigue, and headache, which lasted up to one week (84). Another report by the same group indicated that T1L was the cause of a diarrheal outbreak in children and that reovirus was detected at 5 weeks after the presentation of symptoms (92). These studies indicated that reovirus could be detected from anal and throat swabs, stool samples and urine.

Finally, sequence homology detected between reoviruses isolated from various source organisms may indicate that reoviruses are transmitted between species (80, 91, 103). A study of a novel strain of reovirus isolated from stool from a 17-month old male from Slovenia indicated a high degree of similarity with a strain of serotype 2 reovirus isolated from a bat in Germany: both nucleotide and amino acid sequences were found to be most closely related to a reovirus obtained from a bat (103). However, no bat stool samples were obtained from Slovenia so no direct assumptions about the zoonotic transmission of the virus can be made (103). Additionally, viral inactivation studies

suggest that it is possible that reoviruses can be transmitted indirectly through exposure to fomites (49). As such, no study until this point had investigated any correlations between strains of reovirus observed in humans and those found in the surrounding environment in Alberta.

1.4 Reoviruses in the Environment

In the United States, approximately one-half of all waterborne disease outbreaks were caused by improperly treated groundwater (34). In Canada, viruses and bacterial pathogens pose a significant threat to the quality of drinking and recreational waters (44). In a recent study of groundwater samples in the United States commissioned by the United States Environmental Protection Agency (US EPA) which sought to detect the presence of a variety of viruses, it was illustrated that reoviruses were more frequently detected than hepatitis A virus, norovirus, and rotavirus (34). Furthermore, in another study reoviruses were determined to be more abundant than enteroviruses, echoviruses, and adenoviruses in surface water (69). The presence of reovirus in environmental waters has been viewed as potentially troubling because, although the virus has been observed in a variety of environments, the relationship between the presence of reovirus and human disease is not currently well understood (69, 101).

Apart from their detection in humans and other mammals, reoviruses have been detected in a wide variety of aqueous environments: surface water (58, 69, 101), seawater (73, 74), and groundwater (2), as well as in activated sludge collected from sewage treatment plants (49). Reoviruses are frequently detected in wastewater (49, 69, 97, 105). Reoviruses show clear seasonality when observed over time: a recent study performed over nine years showed that reoviruses were detected in larger quantities from August to

December (97, 105). The seasonal incidence of reovirus was corroborated by another study published in Japan (105).

Due to reovirus' occasional presence in human fecal samples and municipal wastewater, it has been suggested that reovirus may have potential use as an indicator for viral and/or fecal contamination in treated wastewater (33, 58).

1.5 Detection of Reoviruses

A variety of techniques ranging from viral cell culture to molecular biological methods have been used to isolate and detect reoviruses in a variety of settings.

1.5.1 Cell Culture

In terms of reovirus specifically, it has been noted that reovirus grows exceptionally well in laboratory cell lines (90, 95, 101). While culture-based methods have been previously shown to be effective for detecting reoviruses, the method itself has a number of drawbacks with the main issue being specificity. The culturing method requires a single sample to be placed into a cell line and monitored for cell lysis (101). While reoviruses have been shown to grow readily in cell lines, reovirus is not the only virus that is capable of growth in a cell line, and therefore, it is possible that a given sample may contain other viruses that could contribute a positive CPE result, causing a false positive (20, 101). As such, it would be beneficial to combine this culturing technique with an additional technique that would specifically identify reovirus, such as real-time PCR, which would allow for rapid molecular-based detection of the virus.

1.5.2 Immunological Testing

Hemagglutination inhibition testing was used in an effort to categorize the different reoviruses into subtypes. A hemagglutination assay entails a serial dilution of

prepared virus/bacterial solution being applied to mammalian blood cells. After incubation for a period of time the blood cells will aggregate together. In a hemagglutination inhibition assay, blood cells that have been challenged with virus aggregate together and are exposed to increasing levels of viral antibody until a level is reached that inhibits hemagglutination (89).

1.5.3 Molecular Assays

Advancements in the field of molecular biology have allowed for the used of a variety of techniques to be applied to the detection of reoviruses. The polymerase chain reaction (PCR) technique for the amplification of specific DNA sequences has been applied to reovirus in a variety of matrices (23, 36, 58). In the case of reovirus, the double stranded RNA must first be reverse transcribed to complementary DNA (cDNA) before PCR can be used to amplify the fragment of interest (58, 73, 74, 101). A variety of genetic fragments have been explored as molecular targets for detection of reovirus using conventional PCR (Table 1.2).

Table 1.2 Various gene fragment targets used in PCR detection assays for reoviruses.

Authors	Genetic Fragment Targeted for Amplification
Decaro et al., 2005 (23)	S1
Gallagher and Margolin, 2007 (36)	M2
Leary et al., 2002 (58)	L1
Muscillo et al., 2001 (74)	S2
Spinner and Di Giovanni, 2001 (101)	L3
Uchiyama and Besselsen, 2003 (108)	M3

One major issue that has been identified with the use of only conventional PCR to screen for the presence of viruses is the fact that the assay can only detect the presence of the virus' nucleic acid. As such, the assay is capable of detecting both live viral nucleic acid as well as free nucleic acid. The detection of both types of nucleic acid presents an

issue in that the assay is not capable of differentiating between free nucleic acid and infectious, replicating viruses. The second issue with conventional PCR is contamination: the protocol amplifies anything that contains the same primer sequences, leading to potential false positives (72). Finally, conventional PCR requires that agarose gels be run to verify the size of the PCR products, adding an additional amount of time for each group of samples that are run.

1.5.4 Integrated Cell Culture and PCR

In order to address the issue of detecting only viable, infective, replicating reoviruses, a combination of cell-culturing methods with molecular detection is often used (1, 2, 86, 101). The combination of cell culture and PCR is known as integrated cell culture and PCR (ICC-PCR) and is one of the currently used methods for the enhanced detection of infectious reovirus (1, 2, 36, 39, 56, 86, 87). First, samples are subjected to culturing on mammalian cell lines to promote growth of the viruses. Several cell lines have been previously utilized for amplifying reovirus in culture including, but not limited to, Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), buffalo green monkey (BGM), and MA-104 (36, 39, 56). The cells are then lysed; the nucleic acid is extracted and amplified by conventional PCR (86, 101). ICC-PCR is a more reliable method than PCR alone as culturing the virus before molecular screening allows for growth and replication of the virus in specific cells which are susceptible to the virus. Growth of viral particles in culture improves detection of viruses (36, 39).

1.5.5 Integrated Cell Culture and Real-Time qPCR

In order to address some of the issues that exist with conventional PCR, real time quantitative PCR (qPCR) has been adapted to detect reovirus. qPCR relies on the same

principle as conventional PCR in that it amplifies a specific segment of DNA (or cDNA) based on forward and reverse PCR primers, with the further addition of an oligonucleotide probe. The oligonucleotide is bound to a reporter and a quencher dye that during amplification fluoresces to indicate that replication is occurring. A major advantage of this technique is that the replication and fluorescence can in turn be quantified directly (7, 65, 68).

Using a specific probe provides increased specificity over conventional PCR. The probe-annealing site must have a high degree of homology in order to bind, leading to enhanced specificity over the traditional two primer reaction (7, 68).

A further advantage of qPCR over PCR is that qPCR takes place within a closed-tube system under strict conditions. The qPCR samples follow a standardized master mixture, similar conditions, and are analyzed directly by computerized software in the instrument during and after the run, avoiding the potential risk of contamination.

qPCR can be further combined with a culturing method (ICC-qPCR) as described in Section 1.5.4. This allows for combination of a sensitive culturing method with an ultra-sensitive molecular diagnostic technique, providing the user with a means of enhanced cultural and molecular detection of reovirus with the advantages of real-time detection, the ability to quantify the level of viruses, and increased specificity (36, 65, 68).

A potential drawback to the use of qPCR and qRT-PCR is that qPCR amplicons are much shorter in length than conventional PCR amplicons, typically ranging from 50 to 150 base pairs in length (7). This presents an issue in regards to downstream processing of positive samples: since the qPCR amplicon is so short, it does not provide

any substantial sequencing data, facilitating the need for an additional PCR to produce a longer amplicon more suitable for sequencing.

1.5.6 Other Reovirus Detection Methods

Other detection methods, such as immunofluorescence in conjunction with plaque assays, high performance liquid chromatography (HPLC), and particle-associated nucleic acid PCR (PAN-PCR) have been used as attempts for detection of reoviruses (16, 55, 88). Another (but costly) assay is the use of complete genome pyrosequencing, followed by sequence analysis (80). The methods listed in this section have been met with varying degrees of success and generally have a higher cost than molecular detection.

1.6 Study Hypothesis and Objectives

While reoviruses can be detected frequently in environmental water samples (58, 82, 101), the link between the presence of reoviruses and human disease is not well documented. As such, many other viral targets often take precedence when water quality is taken into consideration.

Recent research has focused upon the development of a multiplex quantitative PCR (qPCR) panel that would be capable of detecting norovirus, sapovirus, coxsackievirus, echovirus, rotavirus, astrovirus, and adenovirus in clinical specimens and in environmental samples as well (82). This study used qPCR in conjunction with cell culturing to detect the presence of infectious viruses both by molecular means (qPCR) and by cell culture cytopathogenic effect (82). Interesting results were observed from this study: several surface, wastewater, and drinking water samples that did not provide a positive qPCR result for any of the viruses in the panel still yielded a positive culture result, while other samples positive for qPCR resulted in negative cell culture outcomes.

These discordant samples had RNA extracted, reverse transcribed for sequencing and NCBI BLAST searches from the positive culture results was shown to be associated with the presence of reovirus (81, 82). The appearance of reovirus in surface and wastewater was an interesting observation, as the presence of reovirus had not been previously studied in Alberta waters.

While reoviruses have been frequently detected in a variety of water sources, as well as in humans, a link between the presence of reovirus in the environment and the risk of human disease is still largely unknown. Thus, the presence of reoviruses and the origin of the source of contamination in both Albertan and Canadian waters need to be investigated.

1.6.1 Hypothesis

Infectious and non-infectious strains of mammalian orthoreovirus are present in untreated surface water and wastewater samples in Alberta; and this presence is likely linked to human fecal contamination. Furthermore, reoviruses detected in environmental surface water and wastewater will show a high degree of homology to strains of reovirus isolated from human fecal samples as the source of the water contamination is likely human.

1.6.2 Objectives

1. Development of real-time qPCR assay for detection and quantification of reoviruses
2. Detection and characterization of reoviruses in environmental surface water, wastewater, and clinical stool samples

2.0 Development of Real-Time qPCR Assay for Detection and Quantification of Reoviruses

2.1 Introduction

Reoviruses have been isolated from a wide variety of biological sources, as well as a variety of aqueous environments. In terms of mammalian sources, the virus has been found in a very wide range of mammalian species including humans, wherein it has been suggested that mammalian orthoreoviruses may be responsible for upper respiratory tract illness, mild gastrointestinal symptoms, and potentially meningitis (23, 55, 85, 89 - 95). In addition to their observed presence in mammalian sources, reoviruses have also been observed in a variety of different water matrices, including groundwater, wastewater, surface water, and seawater (2, 34, 49, 58, 69, 73, 74, 97, 101, 105). As reoviruses have such a seemingly universal presence, several different techniques have been investigated as a means of detecting the virus.

Initial efforts for detecting reoviruses in the environment focused entirely on the use of viral culturing techniques. In terms of culturing, reovirus, like other viruses, was cultured on a mammalian cell line in an effort to observe the presence of CPE (90 - 93, 101). A major drawback of the use of culture-based techniques for detecting viruses is that the cell line may be infected by multiple types of virus at once and only the fastest growing will be observed (101). With the advent of molecular-based diagnostic tools that relied on the presence of genetic material to infer the presence of a biological entity, there was a shift to the use of molecular diagnostic tools. Reverse transcription and conventional PCR subsequently became the primary molecular method used in the detection of reoviruses (23, 36, 58). PCR was directed at a variety of reovirus' ten genetic

fragments in an effort to detect the virus in a variety of media (78, 101, 108). However, a major downfall is that the use of reverse transcription PCR (RT-PCR) does not provide any information regarding the infectivity of the virus present in a given sample. Thus, RT-PCR was combined with cell culturing techniques to create an integrated cell culture PCR method (ICC-PCR)(1, 2, 86). This type of assay provides additional enhanced quantitative detection of infective viruses present in the sample, as culturing aids reproduction, thereby enhancing the total amount of viruses present in a sample (36, 39, 56, 87). One of the first attempts at using ICC-RT-PCR for detecting reoviruses in literature was the use of primers directed towards the L3 genetic fragment and screened for the presence of reoviruses in surface water (101). These PCR primers produced a 320-bp amplicon from all three reovirus serotypes, albeit with different internal sequences for the respective serotypes (101). These PCR primers were used again for screening laboratory mice for reovirus infections (47). An Italian study of reoviruses observed in bats utilized five different primer sets: two nested primer sets that were capable of broad detection based on the L1 genetic fragment were used to screen for the virus and three serotype-specific PCRs based on the S1 genetic fragment to be used for serotyping (23). Further studies have also focused their attempts at broad detection of reovirus on the L1 genetic fragment, typically using similar primers to those found in the initial 2001 study (56, 58).

Additional attempts at reovirus detection by PCR have focused on the M3 fragment for the specific detection of serotype 3 Dearing in neonatal laboratory mice (108).

Real-time qPCR has a number of advantages over conventional PCR including increased specificity, decreased assay times, and the entire reaction takes place in a closed system. The closed reaction system of qPCR is a major improvement over conventional PCR: once samples are prepared and placed inside the hardware they are amplified and analyzed directly within the hardware, removing the need to transfer amplified samples elsewhere to perform gel electrophoresis. This reduces the amount of time required to analyze samples and also reduces the potential for contamination (5, 7).

Due to the lack of long stretches of conserved genetic material in the reovirus genome, a common approach to detecting MRVs using qPCR relies on using three different primers and probes (one for each reovirus serotype). In the case of one study, untreated and treated sludge from wastewater treatment facilities in Texas, Pennsylvania, and New Hampshire (36). The three primer/probe sets were designed to be specific to each viral serotype's M2 genetic fragment. Ultimately, 24 raw and treated sludge samples (15 treated and 9 untreated) were screened by the three sets of primers and probes resulting in 13 positive ICC-qRT-PCR results (12 untreated and 1 treated). While using three primer and probe sets allows for immediate serotyping, a major issue with using multiple probes for each serotype is that it requires three times the amount of primers and probes, resulting in three times the cost as opposed to using only a single primer/probe set.

An additional use of real-time PCR in regards to reoviruses has been to investigate the bio-distribution of the oncolytic drug ReoLysin® in clinical trials. In the case of ReoLysin®, only serotype 3 Dearing is present, so all efforts to create a qRT-PCR assay have been focused on only this viral serotype, with serotypes 1 and 2 being

neglected (15, 60). While both the L1 and L2 genetic fragments were explored for use in these assays, ultimately the assays were disregarded because of their specificity to only serotype 3 (15, 60).

While a small number of studies have previously investigated the use of real-time qRT-PCR primers and probes for detecting MRVs, it would be beneficial to decrease the number of probes required, as this would allow for a reduction in the overall cost required to screen each sample. In the case of the M2-primed qRT-PCR that utilized three different primer and probe sets, each sample was screened by three individual qRT-PCRs, increasing the overall cost and time associated with running the 24 samples (36). Therefore, a new qPCR assay was designed in the hopes of reducing the amount of primers and probes required. Furthermore, all previously existing conventional PCR primers that were discussed previously were investigated for use in real-time qRT-PCR, as either primer or probe sequences. However, none of the sequences were found suitable for use, as they were located in regions that exhibited genetic variation between serotypes. Additionally, pairs of conventional PCR primers were not selected for use as the amplicons produced would be too large for the acceptable range of TaqMan® qPCR (50 to 150 bp) (7).

A published study regarding the conservation of the different genes indicated that the *L* and *M* groups of genes show the highest degree of homology between the three serotypes (37). Furthermore, published studies focused on the L1 gene fragment, which encodes the virus' RNA-dependent RNA polymerase, have shown success in developing an RT-PCR test for detection of reoviruses from water (58, 101). As such, the *L* and *M*

gene fragments were investigated as potential targets as these fragments are likely to have a higher degree of homology than the *S* fragments (13).

2.2 Materials and Methods

2.2.1 Study Design

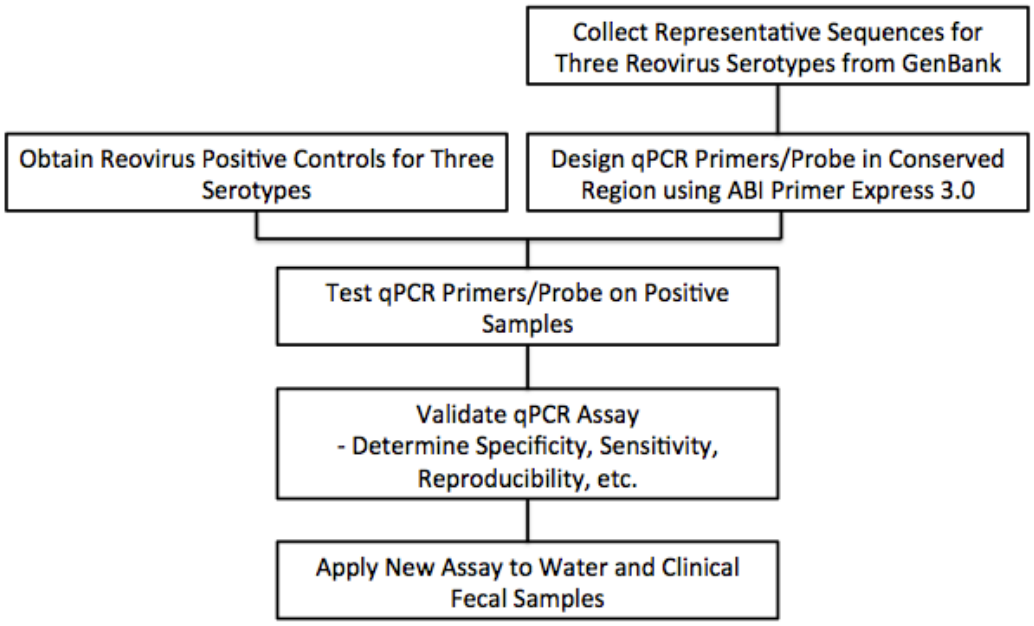

Figure 2.1 Study design flow chart for the development of qPCR primers and probes for screening environmental water and clinical stool samples.

2.2.2 Positive Control Samples

Positive reovirus control samples were obtained from three different sources. Conventional PCR-positive and sequence-positive reovirus samples for the three serotypes (six total positive samples) were previously isolated from wastewater and a surface water in Alberta. Additionally, a single clinical isolate provided by Dr. Julie Fox (Calgary Provincial Laboratory for Public Health) was also included during qPCR assay development.

2.2.3 qPCR Primer and Probe Design

In the current study, novel qPCR primers and probes were designed using reovirus strains obtained from the National Center for Biotechnology Information's (NCBI) GenBank and Nucleotide databases (NCBI, USA). DNA sequences for all ten genetic fragments were aligned using ClustalX Multiple Sequence Alignment Tool (SFI, Ireland) and MAFFT (Multiple Alignment using Fast Fourier Transform) from EMBL-EBI (European Molecular Biology Laboratory, UK) to identify genetic fragments that showed a high degree of conservation across the three serotypes. The strains used in this study can be found below in Appendix A.

Once fragments of interest were identified, regions of high conservation from the fragments were used by ABI's Primer Express 3.0 software to create potential qPCR primers and probes. Sequences used in the alignments can be found in Table 2.1.

Table 2.1 Serotype reference strains of mammalian orthoreovirus sequences used in the design of qPCR primers and probes from NCBI's Nucleotide database.

Genetic Fragment	Serotype	Accession Numbers
L1	1	M31058.1
	2	M24734.1
	3	NC_004282.1
L2	1	AF378003.1
	2	AF378007.1
	3	NC_004275.1
L3	1	AF129820.1
	2	AF129821.1
	3	NC_004274.1
M1	1	X59945.1
	2	GU196309.1
	3	NC_004280.1
M2	1	M19407.1
	2	GU196310.1
	3	NC_004278.1
M3	1	AF174382.1
	2	AF174383.1
	3	NC_004281.1
S1	1	M14779.1
	2	EU049607.1
	3	NC_004277.1
S2	1	L19774.1
	2	L19775.1
	3	NC_004279.1
S3	1	M14325.1
	2	M18390.1
	3	NC_004283.1
S4	1	X61586.1
	2	X60066.1
	3	NC_004276.1

Once a region of high conservation within a genetic fragment was identified, the sequence of interest was analyzed using Primer Express 3.0® under default qPCR conditions and settings to identify a suitable probe and pair of primers that can be used to detect the three reovirus serotypes. Designed primers and probes were then used as individual queries against NCBI's BLAST database in an effort to deduce their specificity to the intended target gene fragment and to identify any potential mismatches.

The qPCR primers and probes designed by Primer Express 3.0 are listed below in Table 2.2.

Table 2.2 List of qPCR primers and probes designed and used throughout the study for gross detection of mammalian orthoreoviruses.

Name	Type	Sequence (5'→3')	Gene	Location	Serotype Coverage
REOM1F1	Forward Primer	AGT TGC TGA ACG CAA ATT ATT TTG	M1	1611 – 1634	T1L, T2J, T3D
REOM1R1	Reverse Primer	TGC GAA TCA GAT TAA CCT GTG T		1667 – 1692	
REOM1P1	Probe	FAM-TAT TGC GAC TAA AAA TAC C-MGB		1641 - 1659	
REOC1F1	Forward Primer	GTC GTG ATT GCC GCA TCT C	L3	3246 – 3264	Clinical Strain
REOC1R1	Reverse Primer	GCA CCA TCA TAC CCG TCT CAT		3329 – 3349	
REOC1P1	Probe	FAM-ATG AAC GGA GCG GCC-MGB		3272 - 3286	

When designing the REOM1 qPCR set, all complete records for the reovirus M1 gene were initially aligned to visualize potential conserved regions and to identify representative sequences for the three main serotypes. The REOM1 qPCR set was designed specifically from representative sequences for reovirus' M1 gene fragment listed below in Table 2.3. In the case of the M1 fragment, complete records for the three serotypes on GenBank show a maximum difference of 4.5% in terms of sequence identity across the entire fragment.

Table 2.3 Record of the sequences used from NCBI's GenBank as representative sequences when designing the M1 qPCR primers and probes.

Gene Fragment (Protein)	MRV Serotype	GenBank Accession Number
M1 (μ2)	1	X59945.1
	2	GU196309.1
	3	NC_004280.1

The REOC1 qPCR set was designed to specifically amplify a sample that tested positive by conventional PCR using the REOL3 primer set (95). This sample was sequenced and compared to NCBI's BLAST database with the results described below in Table 2.4.

Table 2.4 NCBI BLAST results from REOL3 positive isolate search.

Accession Number	Total Score	Query Coverage	E-Value	Identity
AF325768.1	383	78%	1e-102	94%
AY494858.1	337	80%	9e-89	90%
KM820746.1	316	100%	1e-82	85%
KM820756.1	300	100%	1e-77	84%
AF325766.1	239	75%	3e-59	85%
AF325764.1	228	75%	6e-56	84%

Integrated DNA Technologies (IDT) synthesized all qPCR and conventional PCR primers at a concentration of 25 nM. The TaqMan® qPCR probes were labeled with FAM and NED detector dyes with Tamara quencher dye and Minor Groove Binder (MGB) were obtained from Applied Biosystems (ABI, USA) in a volume of 200 µL at a concentration of 100 µM.

The qPCR primers were first tested using a LightCycler qPCR Thermal Cycler in conjunction with SYBR Green®. SYBR Green® is a fluorescent chemical dye that is a known intercalating agent (6,49,116). SYBR Green® was also used to detect the expected 83 base pair PCR product. PCR was performed for 95^{o}C for 10 minutes; followed by 45 cycles of 95^{o}C for 10 seconds, 60^{o}C for five seconds, and 72^{o}C for five seconds; followed by melting temperature calling at 95^{o}C for zero seconds, 75^{o}C for 30 seconds, and 95^{O}C for zero seconds; with a final extension at 40^{o}C for 30 seconds. Next, the PCR product was verified by running the PCR product on a 3% agarose gel to verify the size of the fragment (80 base pairs).

After the qPCR primers were demonstrated to be capable of detecting reovirus, the primers and probe were tested using RNA extracted from sequence-confirmed wastewater reovirus positives and TaqMan® master mix in an Applied Biosystems 7500 Fast Real-Time PCR machine. The qPCR conditions are as follows: 50^{o}C for two minutes, 95^{o}C for 10 minutes, followed by 45 cycles of 95^{o}C for 15 seconds and 58^{o}C for one minute.

2.2.4 Two Step Reverse Transcription Real-Time PCR (qRT-PCR)

2.2.4a Reverse Transcription

Two-step qRT-PCR (reverse transcription [cDNA] followed separately by real-time PCR) was performed in this experiment in an effort to decrease reagent cost and the amount of time spent for preparing samples. Additionally, since the designed qPCR fragments are too short to provide valuable sequence reads in the event of positive results, production of cDNA provides the template needed for any downstream conventional PCR reactions for sequencing purposes. Reverse transcription was performed on an Applied Biosystems 2720 Thermal Cycler (ABI, USA).

5 µL of sample extracted and purified (environmental surface water, wastewater, or clinical stool) by Kingfisher™Magnetic Particle Processor was added to a 0.2 mL reaction tube and heated to 97^{o}C for 5 minutes for the initial denaturation. The samples were then immediately placed in an ice bath for 5 minutes, then spun for 10 seconds on a microcentrifuge, and returned to the ice bath. Finally, 15 µL of master mix containing 4 µL of 5x First Strand buffer (1x), 1.0 µL of DTT (5 mM), 3.0 µL of dNTP (0.375 mM of each of the four nucleotides), 2.0 µL of random reverse transcription primer (300 ng/µL),

0.5 µL of RNAseOut™ (40U/µL), 0.5 µL SuperScript II™ (25U), and 4.0 µL of dH_2O to yield a finished volume of 20 µL of complementary DNA (cDNA).

2.2.4b Real-Time PCR

qPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (ABI, USA) under the following cycle conditions: 50°C for 2.0 minutes; an initial denaturation at 95°C for 10 minutes; followed by 45 cycles of 95°C for 15 seconds, 60°C for 1.0 minute; finished with a final hold at 4°C. The qPCR assay utilized Applied Biosystems TaqMan® Universal PCR Master Mix with 10 µM of forward PCR primer, 10 µM of reverse PCR primer, and 5 µM of qPCR probe. In addition to the reagents present, 5 µL of cDNA produced from reverse transcribed environmental surface water, wastewater, or clinical fecal sample was analyzed. The assay was developed using ABI's Primer Express® 3.0 and followed the universal thermal cycling guidelines set out by ABI's "Chemistry Guide" (6).

2.2.5 Construction of Standard Controls and Establishment of Standard Curve

As there are two different qPCR assays being used in this study, two different molecular standards were created to analyze the assays' sensitivity.

The REOM1 primer/probe set was tested using a molecular standard. The standard was produced from a PCR and sequence confirmed positive reovirus sample obtained from wastewater using conventional PCR primers REOX1F1/REOX1R1, with the newly created amplicon containing the REOM1 qPCR sequence internally.

The C1 assay was tested using a molecular standard created from a PCR and sequence positive clinical isolate which most closely resembled reovirus strain RVH (Total Score: 383, Query Coverage: 78%, and Identity: 94%) (Accession Number:

AF325768.1). The REOC1 molecular standard was produced using conventional PCR primers REOL3F/REOL3R and had an expected PCR amplicon of 320 base pairs (101). These primers are summarized in Table 2.5.

Table 2.5 Conventional PCR primers used to construct molecular standard for each of the newly designed qPCR assays.

Primer Name	Gene Fragment	Location	Sequence (5'-3')
REOL3F	L3	3164 – 3183	CAG TCG ACA CAT TTG TGG TC
REOL3R	L3	3483 – 3464	GCG TAC TGA CGT GGA TCA TA
REOX1F1	M1	1403 – 1422	TAC AAA GGG ATT GCT GGC GT
REOX1R1	M1	1780 – 1761	TCC AAA CCA TTT AGG CTG CG

To create a molecular standard, the wastewater and clinical isolate samples were first extracted and purified by Kingfisher™Magnetic Particle Processor. Next, the NA was subjected to reverse-transcription PCR (RT-PCR) to produce a cDNA PCR amplicon with a size of 320 nucleotides for the L3 gene fragment qPCR and 375 for the M1 fragment directed qPCR.

RT-PCR was performed using 5 µL of NA and ingredients from Qiagen's One-Step RT-PCR Kits: 1x One-Step RT-PCR buffer, 1x Q-solution, 400 µM of each of the four dNTPs, 300 nM of forward PCR primer, 300 nM of reverse primer, RNAse free water, and 2 µL of One-Step RT-PCR Enzyme Mix (Qiagen, USA). RT-PCR was performed in an Applied Biosystems 2720 Thermocycler with an initial heat at 50^{o}C for 30 minutes; followed by an initial denaturation at 95^{o}C for 10 minutes; 40 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 55^{o}C for 1 minute, and extension at 72^{o}C for 30 seconds; with a final denaturation at 72^{o}C for 5 minutes and a finished temperature of 4^{o}C.

The two PCR amplicons were then electrophoresed and visualized on a 2% agarose gel using RedSafe™ visualizing dye to ensure each band was present with their expected size. The visualized bands representing the new PCR amplicons were then excised by hand for further use. The PCR amplicons were extracted from the agarose gel slice in a final volume of 50 µL. From here, the purified PCR amplicons each underwent ten repeat readings using a Thermo Scientific NanoDrop 1000 spectrophotometer at an absorbance of 260 nm to determine the concentration of cDNA present in the purified PCR fragment. The newly created PCR amplicons were calculated as known copy number per µL then serially diluted 10-fold from neat to approximately 1 genetic copy/µL (approximately 5 copies per qPCR reaction or approximately 1000 copies/mL). These known copy number positive controls were aliquoted to smaller volume and stored at -70^{o}C until they were used for setting up an external standard curve for quantification of reovirus in the water samples.

The external standard curves were generated using a series of 10-fold dilutions (1 to 10^{10} copies/ µL) of known copy number PCR products containing qPCR target regions described above.

2.2.6 Validation of qRT-PCR Assays for Detection of Reovirus

2.2.6a Sensitivity

In order to assess the sensitivities of both qPCR primer/probe sets, molecular standards with known copy number were produced from known reovirus positives that contained the sequences of interest (Section 2.2.5). The dilution series produced for these two fragments (320 bp for the C1 assay and 375 bp for the M1 assay) were then screened in triplicate for each dilution factor to assess the assays' sensitivities. Three triplicate runs

were completed for each dilution factor for a total of nine repeated samples. The limit of detection (LOD) was determined by observing the lowest copy number dilution factor: the lowest factor that was observed as registering a positive qPCR result in ≥95% of samples was used as the LOD. A probability distribution was used in conjunction with the positive or negative results obtained from the nine repeats of each dilution factor in order to estimate the LOD. The probability of successfully obtaining a positive result is described using the number of trials (N), the number of positives (n), and the probability mass equation: $P = {}_NC_n/2^N$. Therefore, to be able to detect reoviruses at a confidence level of 95%, reovirus would have to be observed in all nine repeats for the given dilution factor.

2.2.6b Specificity

To assess the specificity of the newly created assay, the primers and TaqMan® probe were used to screen PCR and sequence-confirmed positives of other enteric viruses obtained from wastewater and clinical stool. For this experiment, strains of single-strand RNA viruses, double-stranded RNA viruses, and double-stranded DNA viruses were used; specifically, rotavirus, norovirus, coxsackievirus, sapovirus, adenovirus, and echovirus were screened with the new assay. Rotavirus and norovirus were from clinical stool samples obtained from patients suffering from gastroenteritis. Coxsackie B virus and echovirus 30 were obtained from the Provincial Laboratory for Public Health (Calgary). Adenovirus 41 was obtained from the American Type Culture Collection (ATCC). The control viruses were screened using the REOM1 and REOC1 primer/probe sets with the qPCR conditions described previously in Section 2.2.4b.

2.2.6c Reproducibility

In order to determine the reproducibility of the two qPCR assays, a control sample with same dilution factor was included in every qPCR run. In the case of the REOM1 qPCR primer and probe set, the positive control used for every run was prepared from the molecular standard stock: the 10^{-5} dilution of prepared molecular standard (containing approximately 5 x 10^5) was used for every run and the variation of the C_T values obtained from each run were compared and analyzed against each other. In the case of the REOC1 qPCR primer and probe set, the 10^{-4} dilution of the prepared molecular standard (corresponding to approximately 5 x 10^6 copies per qPCR reaction) was utilized as both a positive control and a means of determining reproducibility.

2.3 Results

2.3.1 Assay Development and Primer Design

The qPCR primers and probes were designed to be capable of broad detection of all three reovirus serotypes. The relative locations of the two qPCR primers and probes set are illustrated on Figure 2.2, showing the lack of any visible overlap between the primer/probe sets.

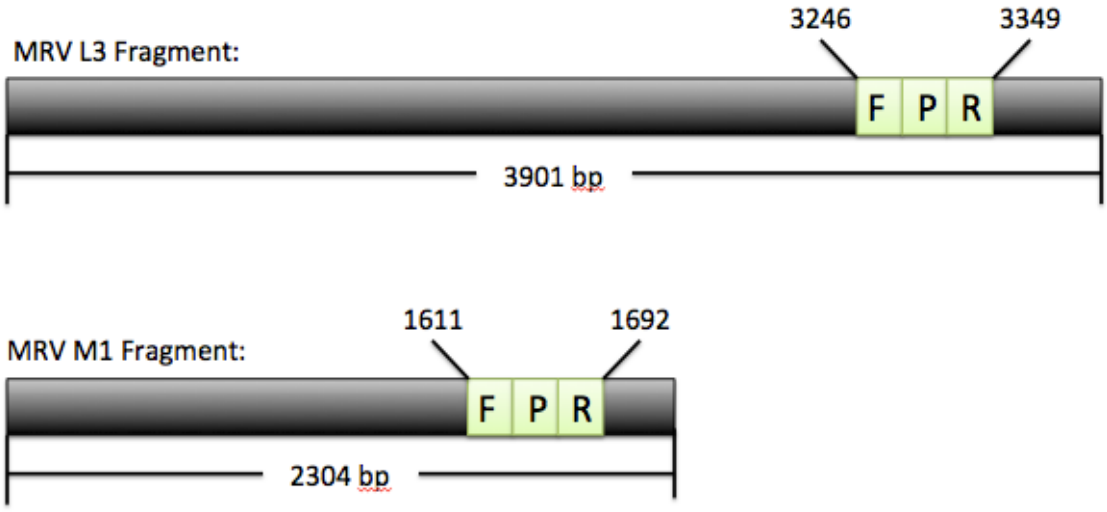

Figure 2.2 Relative physical locations of REOM1 and REOC1 qPCR primer and probe sets on their respective (M1 and L3) genomic fragments (F: forward primer location; P: probe location; R: reverse primer location).

Since two sets of primers and probes were targeted to two different fragments, two different 5' fluorescent reporter dyes were used to help differentiate the different targets. NED™ and FAM™ were chosen as the two fluorescent reporter dyes for this study as they emit their light signals at two different wavelengths (FAM™ at 517-522 nm and NED™ at 575 nm). Using two different wavelength profiles for fluorescent reporter dyes is essential when performing multiplex qPCR in order to allow positive qPCR results to be associated with appropriate qPCR probe. As previously discussed, both fluorescent qPCR probes utilized the same 3' quencher: a non-fluorescent quencher (NFQ) with a minor groove binder (MGB) moiety (4). The use of an MGB-NFQ 3' quencher allowed for the production of a shorter fluorescent molecular probe than would be possible with the use of duel-labeled probes as the NFQ portion of the probe allows for lower background interference and the MGB portion allows increased stabilization and melting point.

The assay was designed to be capable of detecting all serotypes of reovirus. REOM1 was shown to have a detection range of 5 to 5 x 10^{10} copies/PCR. The reactions had a demonstrated efficiency of >90%, having a slope of -3.512 and a $-R^2$ value of 0.994. All previously known REOL3-primed PCR-positive samples obtained from wastewater were detected by the REOM1 qPCR set. REOC1 was shown to have a detection range of 50 to 5 x 10^{10} copies/PCR with an efficiency of >88% and a linear slope of -3.641 and corresponding $-R^2$ value of 0.996. The previously described clinical isolates were detected by the REOC1 qPCR set.

2.3.2 Sensitivity of REOM1 and REOC1 qPCR Assays

The sensitivity of both qPCR assays was determined against prepared molecular standards. The M1 assay was observed to have a limit of detection (LOD) of five copies per reaction volume with a dynamic range of detection varying from five copies to 5 x 10^{10} copies per PCR. The C1 assay was determined to have a limit of detection of 50 copies per reaction volume and a dynamic range of 50 copies to 5 x 10^{10} genetic copies per PCR. This indicates a high sensitivity for REOM1 and a slightly lower sensitivity for REOC1. The replicate screening of molecular standards for determining the LOD is shown in Table 2.6 Additional data regarding the average C_T values for different dilution factors can be found in Table 2.7 for REOM1 and Table 2.8 for REOC1.

Table 2.6 Positive replicates of molecular standard DNA copies for all dilution factors of real time PCR.

Copy number of molecular standard (per qPCR)	Number of replicates	Number of positive replicates (% of positives)	
		REOM1 Assay	REOC1 Assay
5 x 10^{10}	9	9 (100%)	9 (100%)
5 x 10^{9}	9	9 (100%)	9 (100%)
5 x 10^{8}	9	9 (100%)	9 (100%)
5 x 10^{7}	9	9 (100%)	9 (100%)
5 x 10^{6}	9	9 (100%)	9 (100%)
5 x 10^{5}	9	9 (100%)	9 (100%)
5 x 10^{4}	9	9 (100%)	9 (100%)
5 x 10^{3}	9	9 (100%)	9 (100%)
5 x 10^{2}	9	9 (100%)	9 (100%)
50	9	9 (100%)	9 (100%)

5	9	9 (100%)	3 (33.3%)
0.5	9	0 (0%)	0 (0%)

Table 2.7 Performance of REOM1 TaqMan® qPCR assay against custom prepared molecular standard.

Copy Number/qPCR	Average Ct REOM1 Assay
5×10^{10}	5.55
5×10^{9}	5.82
5×10^{8}	9.81
5×10^{7}	14.06
5×10^{6}	17.54
5×10^{5}	21.23
5×10^{4}	24.90
5×10^{3}	28.60
5×10^{2}	32.17
50	35.45
5	39.25
0.5	Negative

Negative = no amplification for any replicate

Table 2.8 Performance of REOC1 TaqMan® qPCR assay against custom prepared molecular standard.

Copy Number/qPCR	Average C_T REOC1 Assay
5×10^{10}	-
5×10^{9}	7.37
5×10^{8}	11.95
5×10^{7}	16.14
5×10^{6}	19.58
5×10^{5}	24.15
5×10^{4}	27.65
5×10^{3}	30.20
5×10^{2}	33.19
50	38.10
5	40.74*
0.5	Negative

Negative = no amplification for any replicate
* = REOC1 detected 3/9 replicates

An example of a standard curve and corresponding amplification plot for the REOM1 set against its molecular standard can be found in Figures 2.3 and 2.4 respectively. The different coloured curves in Figure 2.4 correspond to the different dilution factors of the molecular standard.

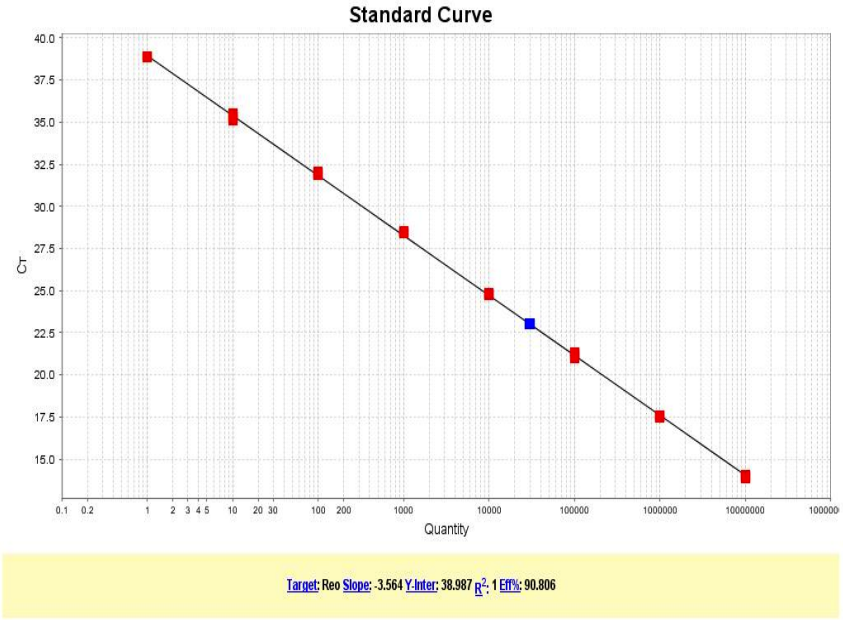

Figure 2.3 Example of a standard curve produced from purified REOX1F1/R1 conventional PCR product for use with the REOM1 qPCR primer/probe set.

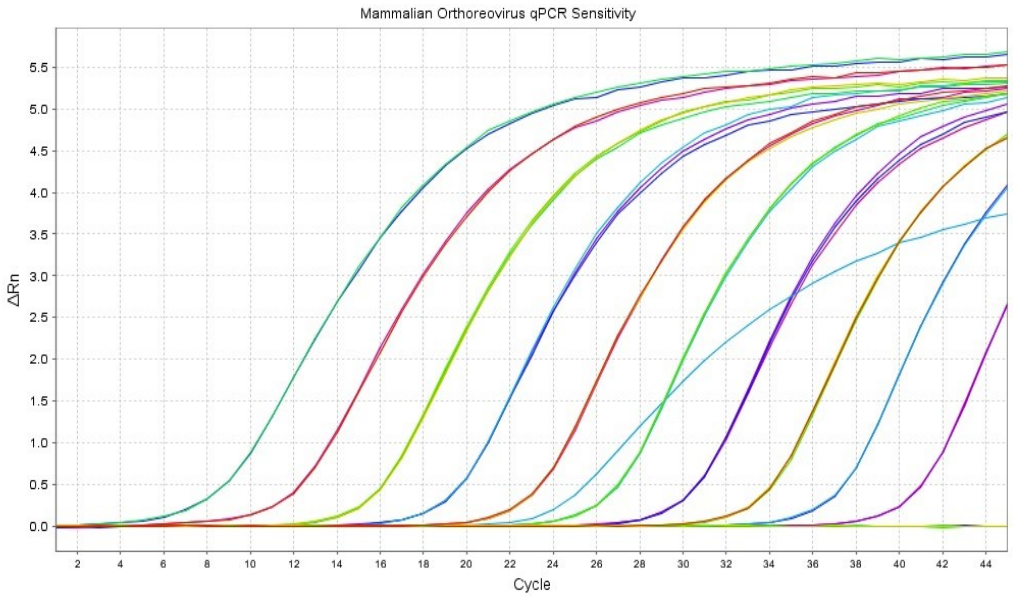

Figure 2.4 Amplification plot of 10-fold serial dilution (starting at 5 x 10^{10} copies per reaction) of REOX1F1/R1 conventional PCR product by REOM1 qPCR primer/probe set with a positive sample obtained from wastewater (light blue).

2.3.3 Specificity

Upon screening the other viruses with the REOM1 primer/probe set, it was observed that the REOM1 set had no cross-reactivity with any other viruses selected. However, a conventional PCR confirmed reovirus sample obtained from a previous clinical stool sample did not yield a qPCR positive when the REOM1 qPCR primer/probe set was used. A second set of qPCR primers and probe (REOC1F1, REOC1R1, and REOC1P1) was designed for detecting the clinical samples. A positive conventional PCR result was obtained with an existing REOL3F/R conventional primer set. The amplicon was isolated, purified, and sent for sequencing. The second set of primers and probe was designed using the L3 gene sequence of the clinical sample to create a highly specific qPCR primer/probe set. The second qPCR set was screened against the same controls to determine its specificity as well.

Table 2.9 Summary table indicating the specificity of the REOM1 and REOC1 qPCR primer and probe sets against mammalian orthoreovirus positive control and other enteric viruses.

Virus	REOM1 qPCR Result	REOC1 qPCR Result
Rotavirus	Negative	Negative
Norovirus	Negative	Negative
Coxsackie B Virus	Negative	Negative
Echovirus 30	Negative	Negative
Sapovirus	Negative	Negative
Adenovirus 41	Negative	Negative
Wastewater Reovirus Serotype 1	Positive	Negative
Wastewater Reovirus Serotype 2	Positive	Negative
Wastewater Reovirus Serotype 3	Positive	Negative
Clinical Mammalian Orthoreovirus	Negative	Positive

As noted above in Table 2.9, the second qPCR primer/probe set (REOC1) was shown to be specific only to the clinical isolate, with none of the other reovirus positive samples being detected.

In summary, two sets of qPCR primers and probes were used during the study: REOM1 was targeted to the M1 gene fragment and REOC1 to the L3 gene fragment specifically. Both sets of qPCR primers and probes were used for all samples throughout the study.

2.3.4 Reproducibility

In order to determine the reproducibility of each qPCR primer and probe set, the individual C_T values of the positive controls were compiled and the mean, standard deviation, and coefficient of variation were analyzed for all PCR reactions using both the REOM1 and the REOC1 qPCR primer and probe sets.

For the REOM1 qPCR assay, the mean C_T value obtained from 18 different qPCR runs was 22.11 with a standard deviation of 1.20 (22.11 ± 1.20). The coefficient of variation calculated from the observed C_T values obtained throughout the study was 0.06. For the REOC1 qPCR assay, the mean C_T value obtained from 20 qPCR runs was 19.9 with a calculated standard deviation of 1.41 (19.9 ± 1.4). Furthermore, the coefficient of variation for the REOC1 assay was 0.07. As such, it was observed that both sets of qPCR primers and probes showed excellent reproducibility.

2.4 Discussion

2.4.1 qPCR Primer and Probe Design

The initial goal of the project was to develop a novel assay or to identify a previously existing assay that was capable of detection of reoviruses broadly from a variety of water matrices. Careful examination of scientific literature regarding the use of molecular methods for the detection of reoviruses yielded several interesting results regarding the use of conventional PCR rather and real-time PCR assays for detection of reoviruses in a variety of samples. However, conventional PCR assays could not be used because of the large amplicon size and the lack of a TaqMan® reporter probe. Additionally, the qRT-PCR assays described in the literature focused on amplifying and reporting a single reovirus serotype at a time, as opposed to broad detection. Furthermore, several qPCR assays were designed to be capable of detecting only a particular strain of reovirus, that being used for its oncolytic properties (15, 60). As such, a new qPCR assay was developed using two different qPCR targets based on reovirus' genetic sequences. To avoid increasing costs and potential interference between primers and probes, an effort was made to design qPCR primers and probes that were capable of the gross

detection of all serotypes of reoviruses. Primers and probes for the gross detection of reoviruses were designed successfully using sequences obtained from all three MRV serotypes on GenBank in this phase of method development: the primers and probes designed were found to exclusively return records for only MRVs when entered as BLAST queries.

The major challenge during the assay development stage was to design a single set of qPCR primers and probe to meet with the initial goal. After the first primer and probe set was designed to be capable of broad detection, it was observed that this set was incapable of detecting the clinical isolate provided for the study. As such, the second primer and probe set was introduced in an effort to detect all potential reoviruses present in the tested samples. There exists several conventional PCR assays capable of broad detection of the reovirus serotypes, but the majority of published work regarding qPCR for detection relies on the use of multiple sets of qPCR primers and probes for the individual serotypes (36).

2.4.2 Sensitivity

Conventional PCR assays for the detection of mammalian orthoreoviruses show a range of sensitivity from 0.3 to 30 PFU, while sensitivities of up to 1.16±0.13 viral particles/PCR (35, 51, 53). The sensitivity of the assays are believed to be acceptable as they are capable of detection down to 5 copies/PCR, while retaining the ability to detect all serotypes with only two sets of primers and probes. Therefore, it is expected that the assay will be capable of screening a variety of sample types for the presence of the virus, including both cultured and pre-culture concentrated samples.

2.4.3 Specificity

Initial investigations of the specificity of the new assays involved screening GenBank with the sequences of each individual primer and probe sequence to look for any cross-reactivity. The sequences for the forward primers (REOM1F1 and REOC1F1), reverse primers (REOM1R2 and REOC1R1), and probes (REOM1P1 and REOC1P1) were used in individual BLAST searches against the entire Nucleotide record bank in order to infer the specificity of each individual primer and probe. BLAST queries were restricted to 100 total target sequences and all of the returned sequences from the BLAST searches that yielded homology and query coverage scores of greater than 90% were derived from MRV and its subtypes, leading to the conclusion that the primers and probes designed to be specific for only reoviruses are likely to be highly specific to MRVs.

Next, the new assays were used to screen against a variety of other enteric viruses that are known to cohabitate with reoviruses. Each sample that was screened with the new qPCR assays previously tested positive by qPCR using primers and probes that were specifically designed for their respective species. Upon screening both new assays against these samples, it was observed that the two qPCR assays did not yield a positive result for rotavirus, norovirus, coxsackievirus, echovirus, or adenovirus, but did provide positive results for reovirus. This is extremely important when it comes to the testing of environmental surface water, wastewater, and clinical stool samples as these samples all contain a wide variety of potential contaminants. For example, surface waters have been shown to play host to a variety of enteric viruses (69, 105). As such, it is important to create a diagnostic assay capable of the specific amplification and detection of reoviruses.

The use of qPCR primers and probes in this study added to the specificity of the assay when compared to PCR: conventional PCR only utilizes a forward and reverse primer, allowing for amplification of any and all sequences that contain both regions of interest. However, real-time TaqMan® PCR requires the use of an internal probe. The use of two PCR primers and an internal probe thusly requires a given sample to contain three identical sequences of interest in order to return a positive result. This increased specificity is observable when discussing the length of qPCR amplicons as well: real-time PCR fragments typically range in length from 50 – 150 base pairs. Given that each primer and probe sequence typically range in length from 15 – 25 nucleotides, in order for a given sample to contain all three sequences of interest it must be extremely similar to the target sequence in order to return a positive result. As such, it is believed that ICC-qPCR increases specificity over other previously utilized detection methods.

3.0 Detection and Sequencing of Reoviruses in Environmental Surface Water, Wastewater, and Clinical Stool Samples

3.1 Introduction

Reoviruses have previously been observed in the environment in a variety of different matrices, including, but not limited to seawater (73, 74), surface water (58, 69, 101), wastewater (49, 69, 97, 105), and groundwater (2, 34). Additionally, reovirus has been frequently detected in a variety of human and animal samples. Specifically, reoviruses have been observed in swine, dogs, and human samples (23, 55, 85, 89 - 95). In terms of humans, the virus has been repeatedly observed in fecal samples (89 - 95). However, while reoviruses have been described and detected in various mammalian and aqueous sources around the world, the presence of reoviruses in Alberta's environmental waters has not been previously investigated in great depth.

Previous attempts at detection of reovirus in both human and environmental samples have focused on the use of viral culture, conventional PCR, or a combination of the techniques (1, 2, 23, 36, 93). In terms of clinical stool, a variety of detection methods for reoviruses have previously been attempted. Initial studies of the virus in the late 1950s and early 1960s focused primarily on the use of cell line culturing from fecal swabs (89 - 93). Conventional culturing utilizes incubation on several cell lines including MDCK, BGM, and MA-104 (36, 39, 56, 101). A major drawback of the use of cell line culturing alone is that culturing is non-specific and may result in multiple viruses infecting the cell line at once (101).

More recent studies have focused on the use of RT-PCR, qRT-PCR, and combinations of PCR with culturing (ICC-RT-PCR). A study in 2001 in the United States

focused on creating an ICC-PCR for detecting reoviruses from surface water using a single primer set for the L3 genetic fragment (101). This single PCR primer set was unique in that it was capable of detecting all three main serotypes of reoviruses based on the primer sequences. The internal portion of the PCR amplicons, however, varied according to serotype, allowing for sequencing of the amplicon to provide insight into the serotype as well. 251 total surface water samples were obtained and cultured on BGM media, producing a total of 26 CPE positive cultures. The 26 CPE positives were then screened by PCR, producing 5 positives (101). All of the sequenced environmental samples contained genetic differences from the reovirus reference strains. Furthermore, multiple strains of reoviruses were obtained and sequenced from one site (101). Ultimately, the surface water samples detected in this study were found to be most similar to L3 sequences observed in the published reference strains (101).

One study published in the United States used three serotype-specific primer/probe sets in an effort to screen treated and untreated wastewater sludge for the presence of reoviruses (36). This study utilized primers and probes targeted to the M2 genetic fragment and sought to compare culture-based detection methods with ICC-qRT-PCR. In the end, reoviruses were observed by ICC-qRT-PCR in all three states tested in 13/24 sludge samples (12/15 untreated sludge samples tested positive and an addition 1/9 treated slude samples) while none (0/24) of the samples exhibited CPE by plaque assay (36). Ultimately, strains of serotypes 1 and 3 were observed in the three states that were screened (New Hampshire, Pennsylvania, and Texas), but these strains were serotyped by ICC-qRT-PCR, not by sequencing. The findings described in the paper indicate that the ICC-qRT-PCR assay that was developed was more sensitive than a plaque assay (36). An

advantage of using three different sets of primers and probes is the rapid differentiation of the reovirus serotypes. However, this leads to triple the cost of using a single primer/probe set. As such, the novel qPCR assays described in Chapter 2 was used to screen for the presence of reoviruses in Alberta's surface water, wastewater, and clinical fecal samples, allowing for two primer/probe sets instead of three, resulting in a diminished cost. Additional qRT-PCR assays for reovirus were designed specifically to serotype 3 Dearing in an effort to track the distribution of the drug ReoLysin® in laboratory animals and are not applicable to the current study (47).

It was believed that reovirus would be detected in Alberta's environmental surface water, wastewater, and clinical fecal samples. Furthermore, it was expected that reoviruses from all serotypes would be observed in all sample types, as the reovirus serotypes are known to coexist in the environment (101).

3.2 Materials and Methods

3.2.1 Study Design

Samples were collected from surface water sites across Alberta, Gold Bar wastewater treatment plant in Edmonton, and from clinical fecal specimens collected from patients in Alberta suffering from gastroenteritis. These samples were all evaluated using the new qRT-PCR assays and samples that would eventually yield a positive qRT-PCR result were subjected to further downstream conventional PCR analysis and sequencing.

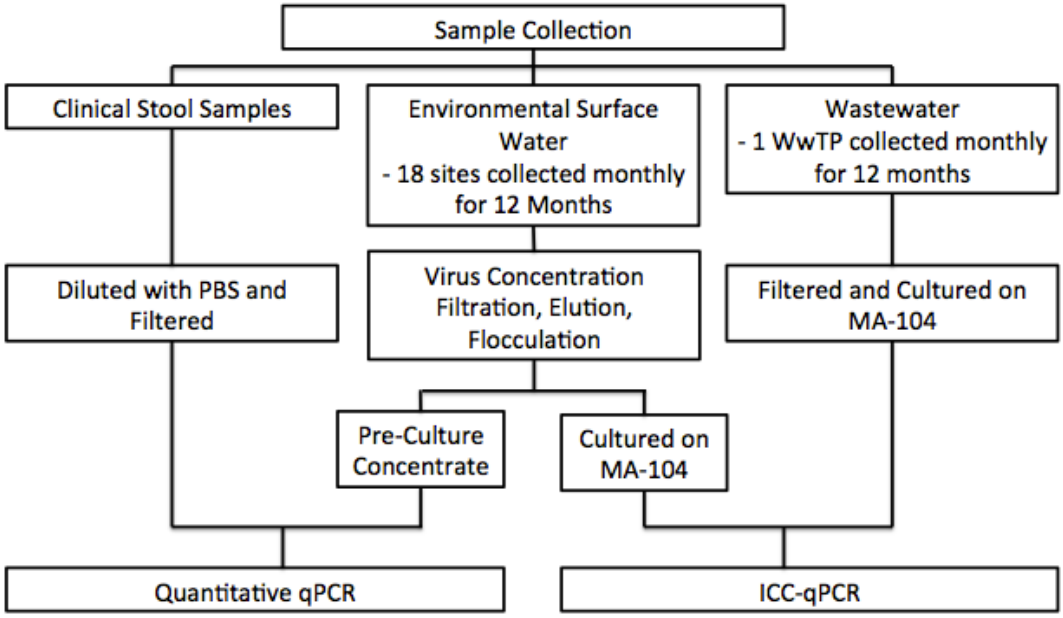

Figure 3.1 Study design for collecting and screening various sample types for the presence of reovirus.

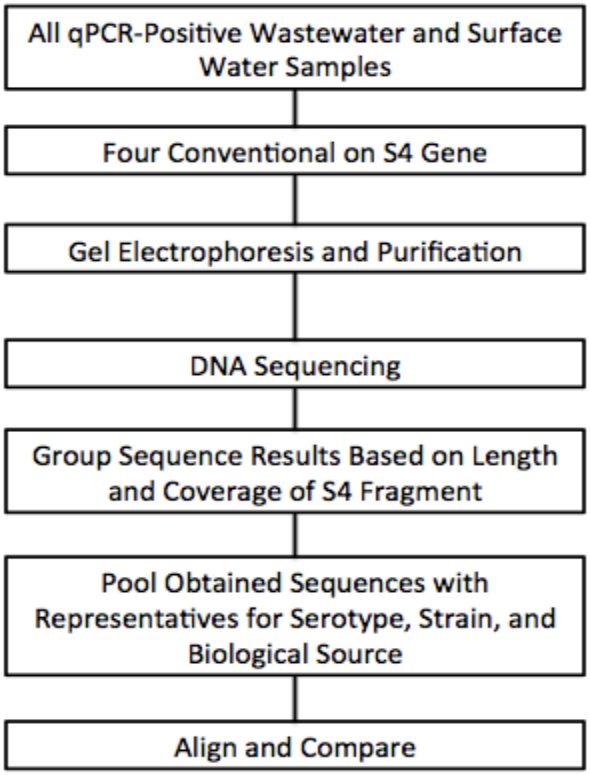

Figure 3.2 Overview of the sequencing process for reovirus-positive samples obtained from screening environmental surface water, wastewater, and clinical fecal samples by qPCR.

3.2.2 Sample Collection

3.2.2a Surface Water Samples

Surface water samples were collected from 18 sites across Alberta (Table 3.1 and Figure 3.3) monthly over the span of one year (June 2012 – May 2013), yielding a total of 216 surface water samples. Ten to twenty litres of water was collected in each site and shipped in a cooler with icepacks to the ProvLab research laboratory in Edmonton, Alberta. The water samples were processed within 48 hours.

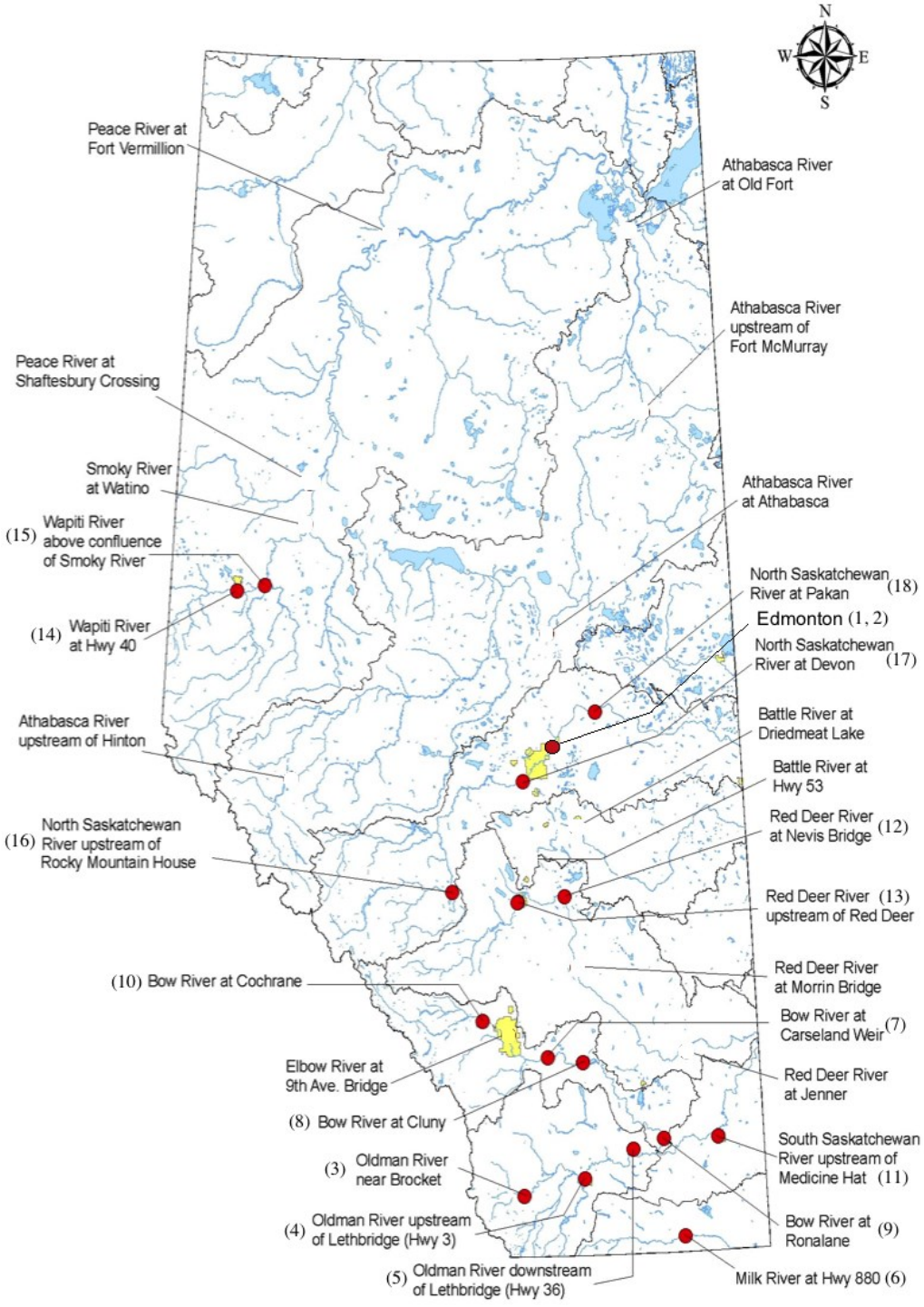

Figure 3.3 Geographic locations of the 18 sites sampled.

Table 3.1 List of surface water sampling locations in Alberta.

Site Location	Sampling Location	Site Number
Edmonton	Epcor Wastewater Treatment Plant 1	1
	Epcor Wastewater Treatment Plant 2	2
Oldman River	Brocket	3
	Highway 3 Bridge	4
	Highway 36 Bridge	5
Milk River	Highway 880 Bridge	6
Bow River	Carseland Weir	7
	Cluny	8
	Ronalane	9
	Cochrane	10
Medicine Hat	South Saskatchewan	11
Red Deer River	Nevis Bridge	12
	Highway 2 Bridge	13
Wapiti River	Highway 40	14
	Smoky River	15
North Saskatchewan River	Clear Water River	16
	Devon	17
	Pakan	18

3.2.2b Wastewater Samples

Wastewater samples were collected from Gold Bar Wastewater Treatment Plant from June 2010 to September 2011. One litre of primary sedimentation effluent (PSE) consisting of gravity setting and scum removal was collected monthly and a total of 16 wastewater samples were included in this study. Samples were shipped to Provlab and processed within 24 h.

3.2.2c Clinical Stool Samples

460 clinical stool samples collected from patients who were suffering from gastroenteritis and submitted to the ProvLab for enteric virus tests during January and April 2013 were used in this study. These samples were analyzed by qualitative real-time PCR using both the REOC1 and the REOM1 assays described in Section 2.2.4b.

3.2.3 Viral Concentration by Filtration, Elution, and Flocculation

The filtration, elution, and flocculation steps followed were followed precisely as described by Pang (76). Clinical stool samples were diluted in 10% phosphate buffered saline (PBS) then filtered using a 0.45 μM Millipore® low protein-binding filter (Sigma-Aldrich, USA).

In order to prevent clogging of the NanoCeram® filter, raw water samples were pre-filtered using a pre-filtering apparatus and filtered using a laminated 90 mm NanoCeram® filter (Argonide Corp., USA). The pre-filtering apparatus was constructed using a cone of four standard-sized White Swan® paper towels inserted into the centre of a 10 mm polypropylene cartridge filter (Parker Hannifin Corporation, USA). The entire cartridge filter was then wrapped with Fisherbrand® chromatography paper (Thermo Fisher Scientific, USA) (82). The pre-filtering apparatus filtered samples using nitrogen gas at a positive pressure of 10 – 18 PSI until the entirety of the sample has passed through. The sample was then filtered under the same conditions as the pre-filtration step through the 90 mm NanoCeram® filter. The pre-filtration apparatus and the NanoCeram® filter were then rinsed with 2 L of sterile water under the pressure and conditions described above to avoid loss of viruses present in solid particulates. Elution was achieved using 1.8 litres of 1.5% beef extract (BE) adjusted to a pH of 9.6 ± 0.1 under nitrogen gas at 4-8 PSI for 2 to 30 minutes. Flocculation was achieved using 1N hydrochloric acid and 0.5M ferric chloride were to adjust the pH of the resulting eluent to 3.5. The sample was then slowly stirred at room temperature for 30 minutes to allow flocculation to occur. Next, the samples were decanted equally into four 750 mL centrifuge bottles and centrifuged at 4^{o}C at 3000-x *g* for 15 minutes on a Beckman

Coulter J6-HC centrifuge (Beckman Coulter, Inc., USA), with the supernatant discarded afterward. The remaining pellets were combined and 10 mL of glycine buffered medium at a pH of 9 was added to aid in dissolving the newly formed pellets. The dissolved pellet solution was transferred to a 50 mL conical flask. The samples were then brought to a finished volume of 30 mL using Eagle's 1X minimal essential media (MEM) with antibiotics (Sigma Aldrich, USA). The pH was again readjusted to 7.2 ± 0.2 and finally decanted to individual volumes of 1 mL in 2 mL microcentrifuge tubes. Samples were then stored in the -70^{o}C freezer until extraction or culturing, the specific effect of storage of samples at -70^{o}C was not specifically tested..

To avoid false positives caused by residual virus or viral genetic material left in the filtering apparatus, the filtering apparatus was decontaminated in a 6% sodium hypochlorite bleach solution for 30 minutes. The filtering apparatus was then rinsed using tap water and sodium thiosulfate solution, with a final rinse using sterile water for 15 minutes.

3.2.4 Viral Culture and sub-culturing of Surface Water and Wastewater Samples

The MA-104 cell line was purchased from the European Collection of Cell Cultures (ECACC). Both cell lines were grown on Eagle's MEM supplemented with 1% non-essential amino acids, 2 mM I-glutamine, and 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37^{o}C (Sigma Aldrich, USA).

One mL of thawed concentrate was added to the MA-104-MEM culture; these cultures were then incubated on a rocking platform at 37^{o}C for 1 hour (Cole-Parmer®, Canada). The original inoculum was removed from the culture flask and replaced with 10 mL of Eagle's MEM. The cultures were incubated for a further 10 days. After the first

day of incubation, the cultures were examined under a microscope for the presence of CPE. After the initial examination of the cultures after the first day, the cell cultures were investigated every 48 to 72 hours for the duration of the 10 days for CPE. Upon completion of the 10 days, three freeze-thaw cycles were performed on each sample: each sample was frozen in a -70^{o}C freezer then incubated in a water bath to a temperature of 37^{o}C three times in an effort to lyse the cells and free the viral particles. The media was then transferred to a conical collection flask and centrifuged for 10 minutes at 200 x *g* to remove debris. One mL of the resulting supernatant from centrifugation was used as inoculum for a second round of culturing with all of the previously described steps being repeated.

3.2.5 Total RNA Extraction

Surface water samples were extracted and screened at two different stages: samples were extracted immediately after filtration, concentration, and flocculation, as well as after culturing with BGM. Wastewater samples however, were extracted and screened after the culturing stage only. Finally, the majority of clinical samples were not cultured for this study: clinical stool samples underwent dilution in a 10% solution of phosphate-buffered solution (PBS) (pH 7.0) prior to the extraction.

Total RNA was extracted from 200 µL of water or stool sample using MagaZorb® Total RNA Mini-Prep Kits under the manufacturer's instructions without deviation (Promega, USA). Total RNA was eluted to a final volume of 50 µL using an automated nucleic acid extraction and purification system - the KingFisher™ mL Magnetic Particle Processors under the manufacturer's instructions (Thermo Scientific,

USA). After elution to 50 µL, samples were transferred to clean 1 mL microcentrifuge tubes. Extracted samples were then stored at in a freezer at -70^{o}C until needed for use.

3.2.6 Two-Step qRT-PCR

The previously described methods for two-step qRT-PCR described in Section 2.2.4 were followed exactly throughout the study. In addition, prepared external standard curves were established for quantification using the prepared serial dilutions of the DNA fragments prepared from reoviruses described in Section 2.2.6a. A series of 10 fold dilutions (5 to 5 x 10^{10} copies/PCR) of standard controls described in section 2.2.5 was used for quantification of the reoviruses.

3.2.7 Calculating the Viral Load of Reovirus-Positive Concentrated Water Samples Based on Collected Volume of Different Water Matrices.

To convert from the amount of copies present per PCR reaction to the amount of copies present per litre, the following equation was used:

$$Concentration_{Copies/L} = A/E*B/F*C/G*D/V$$

$$= ABCD/EFGV$$

Where “A” ” represents the volume of sample after concentration in microlitres (15 000 µ L), “B” represents the eluted volume of each sample after nucleic acid extraction in microlitres (50 µL), “C” represents the total volume of cDNA produced during reverse transcription in microlitres (20 µL), “D” was the number of genetic copies observed by the ABI 7500 software (raw data), “E” represents the amount of concentrated sample used during nucleic acid extraction in microlitres (200 µL), “F” was the volume of nucleic acid used for reverse transcription (5 µL), “G” was the volume of cDNA used for qPCR (5 µL), and “V” was the volume of the initial sample in litres.

3.2.8 DNA Sequencing

In terms of producing sequence data for reovirus-positive samples, the "S" fragments were investigated for use, as these four fragments are known to display a higher degree of variation across serotypes than the "L" and "M" fragments (37). As such, it was believed that sequencing one of the four "S" genes would allow easy serotyping of positive samples.

A review of potential primers indicated that the majority of attempts at amplifying the "S" fragments relied upon the use of serotype-specific PCR primers (14). As such, novel PCR primers capable of amplifying all three serotypes were designed. Complete GenBank records for the four "S" fragments were obtained and aligned using the ClustalW algorithm in MEGA. The resulting alignments indicated that the S4 gene fragment possessed conserved regions at the 5' and 3'-ends that would be suitable for designing a single set of PCR that would be capable of broad detection of the three serotypes. Prior to designing novel primers, the literature was again reviewed to investigate previously designed PCR primers that were capable of amplifying the S4 gene fragment. A study was found that utilized primers designed to amplify a portion of the S4 fragment from serotypes 1 and 3 (54). As this set of PCR primers was not designed using any sequence data for serotype 2, it was discarded in favour of designing novel, broad primers. An additional study sequenced a strain of reovirus isolated from the central nervous system of a child suffering from varicella, oral thrush, diarrhea, and fever in Winnipeg, Manitoba, Canada (46). This study sequenced the four S4 gene fragments using different primer sets for each gene fragment (50). The virus was previously tentatively identified as serotype 2 reovirus based on RNA gel electrophoresis and virus

neutralization studies (46). As the virus was tentatively identified as serotype 2, PCR primers were designed based only on an existing serotype 2 S4 gene fragment sequence (50). As such, these primers are only capable of amplifying serotype 2 and are not useful for other reovirus serotypes and cannot be used to sequence wastewater and environmental surface water isolates of unknown serotype.

All REOM1 and REOC1 qRT-PCR positive samples obtained from surface water and wastewater were amplified using the sets of conventional PCR primers described in Table 3.2. The cDNA for each pre-culture or post-culture sample that yielded a positive qPCR result using either primer set (REOM1, REOC1, or both) were screened by conventional PCR directed towards the reovirus gene fragment S4 (Table 3.2).

Table 3.2 List of conventional PCR primers used for producing PCR amplicons of a suitable length for DNA sequencing from mammalian orthoreovirus cDNA.

Primer Name	Primer Sequence (5'→3')	Primer Location on S4 Fragment
REOS4F	GTT GTC GCA ATG GAG GTG TG	24 – 43
REOS4R2	TGT CCC ACG TCA CAC CAG G	1183 – 1165
REOS4iFS	AAC TGG CTT CAG GTT GAY CC	426 – 445
REOS4iRS	GGY TCA ACC TGA AGC CAG TT	445 – 426
REOS4NF1	AGG GAT GGG ACA AAA CAA TCT CA	121 – 143
REOS4NR1	CCA AGA ATC ATC GGA TCG CCA	1126 – 1106

The six S4 gene fragment primers described above were used in four different primer combinations in an effort to maximize the amount of sequencing data produced from positive samples. PCR primers were used in descending order depicted in Table 3.3 in an effort to create the longest PCR amplicons possible. Several other combinations of PCR primers existed as well (e.g. REOS4NF1/REOS4iRS), however, these combinations

of primers would produce PCR amplicons of insufficient length for DNA sequencing, and thusly were discarded. Combinations of primers and their potential overlap are illustrated in Figure 3.4.

Table 3.3 Combinations of conventional PCR primers used in sequencing of mammalian orthoreovirus positives and their designed fragment lengths.

Forward Primer	Reverse Primer	Amplicon Length (Base Pair)
REOS4F	REOS4R2	1141
REOS4NF1	REOS4NR1	985
REOS4F	REOS4iRS	785
REOS4iFS	REOS4R2	421

All combinations of PCR primers utilized the same conventional PCR conditions with the exception of REOS4NF1/NR1. Five µL of cDNA was added to a 0.2 mL PCR tube containing 45 µL of conventional PCR master mixture containing 1x PCR buffer; 2.0 mM $MgCl_2$; 200 µM of dNTP mixture; 400 nM of forward PCR primer; 400 nM reverse PCR primer; 2 U of *Taq* DNA polymerase; and 29.5 µL distilled H_2O (dH_2O). Samples were amplified on an Applied Biosystems 2720 Thermocycler (ABI, USA) under the following conditions: an initial denaturation at 95^{o}C for 3 minutes; followed by 40 cycles of denaturation at 95^{o}C for 1 minute, annealing at 50^{o}C for 1 minute, and extension at 72^{o}C for 1 minute; a final extension at 72^{o}C for 10 minutes and a final hold at 4^{o}C.

In the case of the use of REOS4NF1/NR1, a nested conventional PCR was performed using the PCR amplicon produced by the REOS4F/R2 PCR as template. 2 µL of the REOS4F/R2 PCR product was used in place of 5 µL of cDNA, the resulting disparity caused by using a decreased volume of template was equalized using dH_2O.

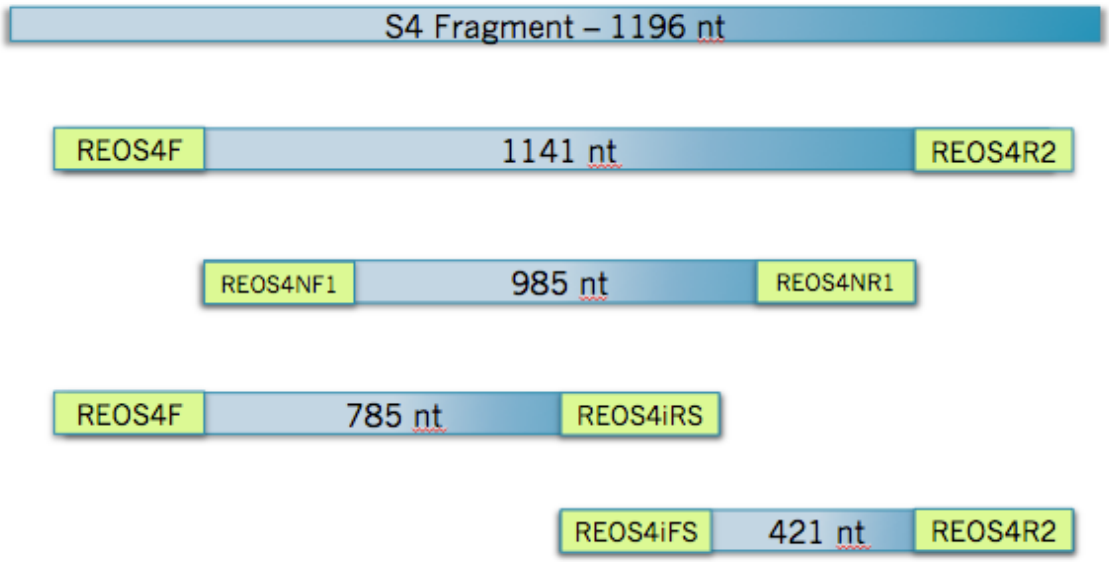

Figure 3.4 Schematic representation of the lengths of the conventional PCR amplicons produced during the sequencing stage of the study. PCR primers are denoted by yellow-green rectangles and are named according to their actual primer name.

In summary, the 35 total qPCR positive samples (20 surface water and 15 wastewater samples) were subjected to conventional PCR for generation of a full-length amplicon of the S4 gene using the primers REOS4F and REOS4R2. Any samples which did not yield a positive PCR result using the primers REOS4F and REOS4R2 were screened by nested PCR using the REOS4NF1 and REOS4NR1 primer set with 2 µL of the 1st PCR products amplified with the REOS4F and REOS4R2. Nested PCR was used in an effort to increase the yield of the initial conventional PCR. If the subsequent nested PCR failed, conventional PCR using an internal PCR primer in both orientations was performed (Figure 3.4).

3.2.9 Gel Electrophoresis and Purification of PCR Products

All conventional PCR products were separated by agarose gel electrophoresis on 2% Invitrogen Ultrapure™ agarose gels containing 5 µL RedSafe™ nucleic acid dye (iNtRON Biotechnology, Inc., Korea) under 85 volts in 1X Tris-Acetic-EDTA (TAE)

buffer (Invitrogen, USA) for a duration of 75 to 85 minutes on a Bio-Rad Power Pac 300 (Bio-Rad, USA). Bands were initially visualized and documented using a Bio-Rad Gel Doc™ UV-transilluminator to examine the presence or absence of a band (Bio-Rad, USA).

Positive bands were then visualized on a transillumination bench and excised by hand. Excised bands were then purified using Qiagen QIAquick Gel Extraction kits under the manufacturer's conditions and instructions (Qiagen, USA). Purified PCR products were then quantified using a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

Purified PCR products obtained throughout this study were commercially sequenced by University Core DNA Services at the Centre for Advanced Technology within the Faculty of Medicine at the University of Calgary. Samples were submitted for sequencing in a finished volume of 12 µL and contained 50 – 100 ng of template (PCR amplicon) and 3.2 pmol of primer, as per the company's suggestions. "Premixed/economy" sequencing was used and sequencing was carried out using an Applied Biosystems 3730xl (96 capillary) genetic analyzer (http://www.ucalgary.ca/dnalab/sequencing).

3.2.10 Bioinformatic Analysis

A library of known reovirus samples was created from GenBank using sequences of known biological source, serotype/strain, and geographical location. All of the existing complete records for the S4 gene fragment in NCBI's GenBank were collected and aligned using MEGA version 6.0.5's built-in maximum likelihood and neighbor joining tree-building methods. From this alignment and subsequent maximum likelihood tree,

repeated sequences (sequences with >99% identity and coverage that converge at a single node) were removed to ensure a diverse collection of representatives from a variety of serotypes, strains, source organisms, and geographic locations.

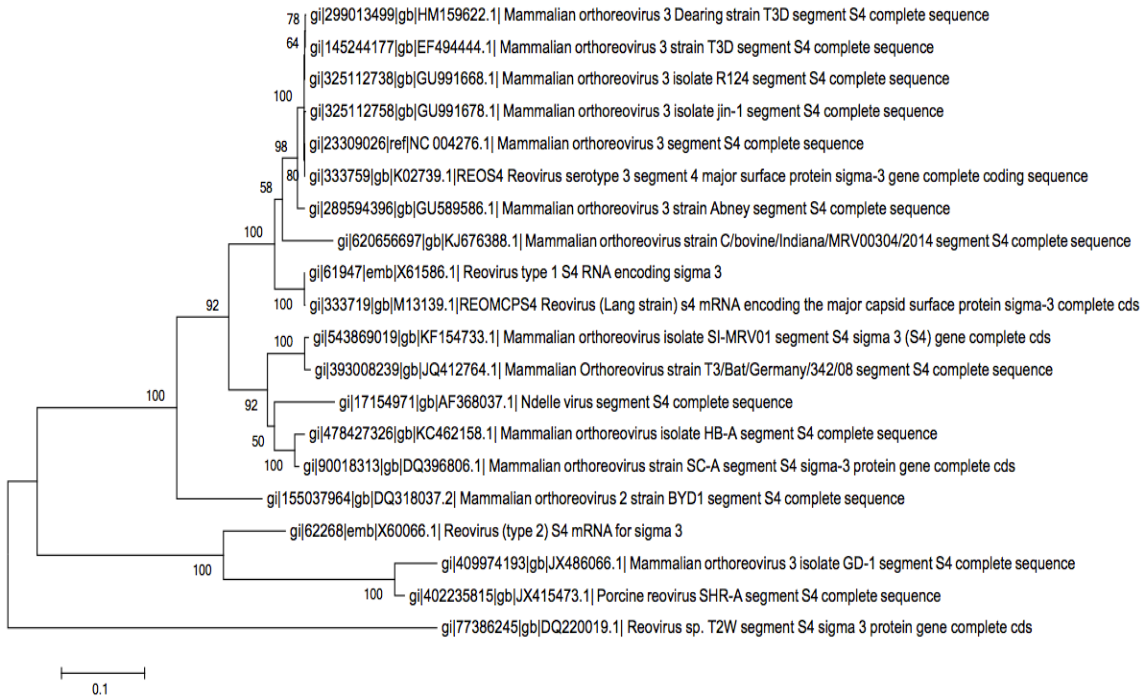

Figure 3.5 Full length S4 gene MEGA maximum likelihood (ML) tree of all complete mammalian orthoreovirus S4 gene fragments from NCBI's GenBank.

Eleven reference sequences featured in Table 3.4 were selected from the alignments created using all complete S4 gene fragments. Representative sequences were selected for each distinct node found on the phylogenetic tree illustrated in Figure 3.5.

Table 3.4 The eleven mammalian orthoreovirus reference sequences taken from NCBI GenBank records representing distinct serotypes and strains.

Accession Number	MRV Serotype/Strain	Source Organism	Year of Publishing	Coded Entry for Tree Building
DQ396806.1	Strain SCA	Swine	2006	SCA/Sw/Ch/06
AF368037.1	Ndelle	Murine	2001	T4/Mu/Fr/01
KF154733.1	S1-MRV01	Human	2013	S1/Hu/Sl/13
X61586.1	Serotype 1	Human	2005	T1/Hu/US/05
KJ676388.1	Strain C	Bovine	2014	SC/Bo/US/14
HM159622.1	Serotype 3	Human	2010	T3/Hu/Ca/10
GU589586.1	Serotype 3 (Abney)	Human	2010	T3A/Hu/Ca/10
DQ318037.2	Serotype 2 (BYD1)	Human	2007	T2/U/Ch/07
X60066.1	Serotype 2	Human	2005	T2/Hu/US/05
JX415473.1	Serotype 1 (SRHA)	Porcine	2012	SHRA/Po/Ch/12
DQ220019.1	Serotype 2 (Winnipeg)	Human	2012	T2W/Hu/Ca/12

The chosen reference sequences were then encoded to prevent further phylogenetic trees from being overcrowded with text. Representative sequences were encoded based on their serotype or strain, the initial source of the virus, the country of origin, and the year of publication. The reference strains X61586.1 (serotype 1) and X60066.1 (serotype 2) were reference strains used in previous studies involving sequencing the S4 gene fragment (50).

3.2.11 Sequence Analysis

Sequences obtained by commercial sequencing were first subjected to a GenBank BLAST search with each individual sequence. Sequences were then aligned with representative sequences for each of the three reovirus serotypes using the ClustalW algorithm and further analyzed using Molecular Evolution Genetics Analysis (MEGA) V6.0.5 (MEGA, USA). Environmental surface water samples included on the

phylogenetic trees had the prefix “SW”, followed by an alphanumerical sample identifier that denotes sample location, and two numbers denoting the year of sample collection. Wastewater samples were symbolized in a similar manner with “WW” followed by a numeric identifier and the year of collection.

3.3 Results

3.3.1 Prevalence of Reovirus in Environmental Surface Water

A total of 216 surface water samples taken from 18 different sites across Alberta over the span of 12 months were screened as part of this study. These 216 samples were subjected to pre-culture extraction and screening by two-step qRT-PCR, as well as post-culture extraction and screening by qRT-PCR.

3.3.1a Pre-Culture Surface Water qPCR Results

First, the 216 environmental surface water samples were tested for reovirus using the RT-qPCR method after concentration and prior to cell culturing. This direct screening method is referred to throughout the thesis as the screening of the pre-culture environmental surface water sample. These samples were screened qualitatively initially using the qPCR assay then any positive samples were quantified by using qPCR with a standard curve.

As denoted in Table 3.5, the pre-culture environmental water samples showed a low number of total positives, with only four of the 216 (1.9%) samples testing positive.

Table 3.5 Summary of pre-culture surface water sample REOM1 and REOC1 qRT-PCR results by site and sample location.

Site	Sample Location	Pre-Culture qPCR Result		Total
		Positive	Negative	
Edmonton	Epcor Site 1	0	12	12
Edmonton	Epcor Site 2	1[a]	11	12
Oldman River	Brocket	0	12	12
Oldman River	Hwy 3 Bridge	0	12	12
Oldman River	Hwy 36 Bridge	0	12	12
Milk River	Hwy 880 Bridge	0	12	12
Bow River	Carseland Weir	0	12	12
Bow River	Cluny	0	12	12
Bow River	Ronalane	0	12	12
Bow River	Cochrane	0	12	12
Medicine Hat	South Saskatchewan River	1[b]	11	12
Red Deer River	Nevis Bridge	0	12	12
Red Deer River	Hwy 2 Bridge	2[c]	10	12
Wapiti River	Wapiti R Highway 40	0	12	12
Wapiti River	Wapiti R Smoky	0	12	12
North Saskatchewan River	Clear Water River	0	12	12
North Saskatchewan River	Devon	0	12	12
North Saskatchewan River	Pakan	0	12	12
	Total	4	212	216

[a,b] REOC1 qRT-PCR positive only
[c] Two separate positives, one REOM1 positive and one REOC1 positive

Of the 18 sites screened, reoviruses were detected at three different sites. Positive qPCR results were obtained from Epcor Site 2 in Edmonton, the South Saskatchewan River in Medicine Hat, and the Highway 2 Bridge along the Red Deer River. A visual breakdown of the locations of positive qPCR results from pre-culture environmental water samples can be observed in Table 3.5. The four reovirus positive samples were further quantified using our qPCR with standard curves. Relatively low viral loads were

observed in the samples with copy numbers of 5730, 8145, 3705, and 1290 copies/L respectively.

3.3.1b Cell Culture and ICC-qRT-PCR

The 216 environmental surface water samples that were collected previously and analyzed pre-culture for reoviruses were further subjected to culturing on a MA-104 cell line. For the current study, only the MA-104 cultured results were analyzed, as this cell line is specific to rotaviruses and reoviruses (36). Furthermore, the 216 MA-104 cultured samples were screened with the two new qRT-PCR assays.

Cell culture CPE positives were found in 6.9% (15/216) of the cultured surface water samples (Table 3.6 and 3.7). Of 15 CPE positive samples, 10 (66%) were confirmed as being infected with reovirus by two qPCR assays (3 REOM1 positive only, 2 REOC1 positive only, and 5 REOM1/REOC1 positives). An additional 10 reovirus were detected by qPCR (2 REOM1 positive only and 8 REOM1/REOC1 positives) from the cell culture CPE negative samples therefore a total of 20 (9.3%) reovirus detected all 216 surface water samples (Table 3.6).

Table 3.6 MA-104 cultured environmental surface water samples that indicated the presence of cytopathogenic effect (CPE) after culturing.

Collection Date (dd-mmm-yy)	Site	Sampling Location	Pre-Culture qRT-PCR Result (REOM1/ REOC1)[c]	Observed CPE[a,b]	ICC-qRT-PCR Result (REOM1/REOC1)[d]
16-Apr-13	Epcor	Site 1	-/+	-/++	+/-
28-Oct-12	Epcor	Site 2	-/-	-/±	-/-
17-Dec-12	Epcor	Site 2	-/-	-/++	+/-
23-Jan-13	Epcor	Site 2	-/-	-/+	-/-
16-Apr-13	Epcor	Site 2	-/-	-/-	+/-
17-Jan-13	Oldman River	Hwy 3 Bridge	-/-	-/-	+/-
06-Feb-13	Oldman River	Hwy 3 Bridge	-/-	-/-	+/+
17-Jan-13	Oldman River	Hwy 36 Bridge	-/-	-/++	-/-
27-Nov-12	Medicine Hat	South Saskatchewan River	-/+	-/-	+/+
16-Nov-12	Red Deer River	Nevis Bridge	-/-	-/+++	-/-
13-Apr-13	Red Deer River	Nevis Bridge	-/-	-/-	+/+
19-Nov-12	Red Deer River	Hwy 2 Bridge	-/+	±/+++	-/+
10-Dec-12	Red Deer River	Hwy 2 Bridge	-/-	-/+++	-/+
22-Apr-13	Red Deer River	Hwy 2 Bridge	+/-	-/++	+/+
08-Apr-13	Wapiti River	R Hwy 40	-/-	-/+++	+/+
14-May-13	Wapiti River	R Hwy 40	-/-	-/-	+/+
16-Jul-12	Wapiti River	Smoky River	-/-	-/+	-/-

11-Dec-12	Wapiti River	Smoky River	-/-	-/++	+/+
08-Apr-13	Wapiti River	Smoky River	-/-	-/+	+/+
07-Jan-13	North Saskatchewan River	Devon	-/-	-/-	+/+
08-Apr-13	North Saskatchewan River	Devon	-/-	-/-	+/+
06-May-13	North Saskatchewan River	Devon	-/-	-/-	+/+
13-Dec-12	North Saskatchewan River	Pakan	-/-	-/+++	+/+
13-Mar-13	North Saskatchewan River	Pakan	-/-	-/+	+/-
11-Apr-13	North Saskatchewan River	Pakan	-/-	-/-	+/+

[a] Samples were each put through two passages through MA-104 media.
[b] For observed CPE: -: negative; ±: indeterminate; +: slightly positive; ++: intermediate positive; and +++: very strong positive.
[c,d] For qRT-PCR and ICC-qRT-PCR + denotes a positive result and – denotes a negative result

Table 3.7 Summary of results of MA-104 cultured surface water samples screened with mammalian orthoreovirus qPCR primers.

Reovirus ICC-qPCR Result	**MA104 Cytopathogenic Effect (CPE)**		**Total**
	Positive	**Negative**	
Positive	10	10	20 (9.3%)
Negative	5	191	196 (90.7%)
Total	15 (6.9%)	201 (93.1%)	216

Of the 216 cultured surface water samples, there were reovirus qPCR positive results detected in every month from November 2012 through to May 2013, with no additional positives detected from June 2012 to October 2012. Of the months where reoviruses were detected in the water samples, April 2013 had the peak amount of positives with eight, corresponding to 40% of the total amount of positives. December

2012 contributed a further four qPCR positives (20% of the total amount of positives). Additionally, November 2012, January 2013, and May 2013 each contributed two qPCR positives. Finally, February and March of 2013 each contributed a single reovirus qPCR positive (Figure 3.6). Ultimately however, since only one sample was screened per site per month, it cannot be conclusively stated that reoviruses were more active or more present in the months that they were detected in. Furthermore, in the data presented, all of the results from all of the sites were consolidated and examined on a month-to-month basis.

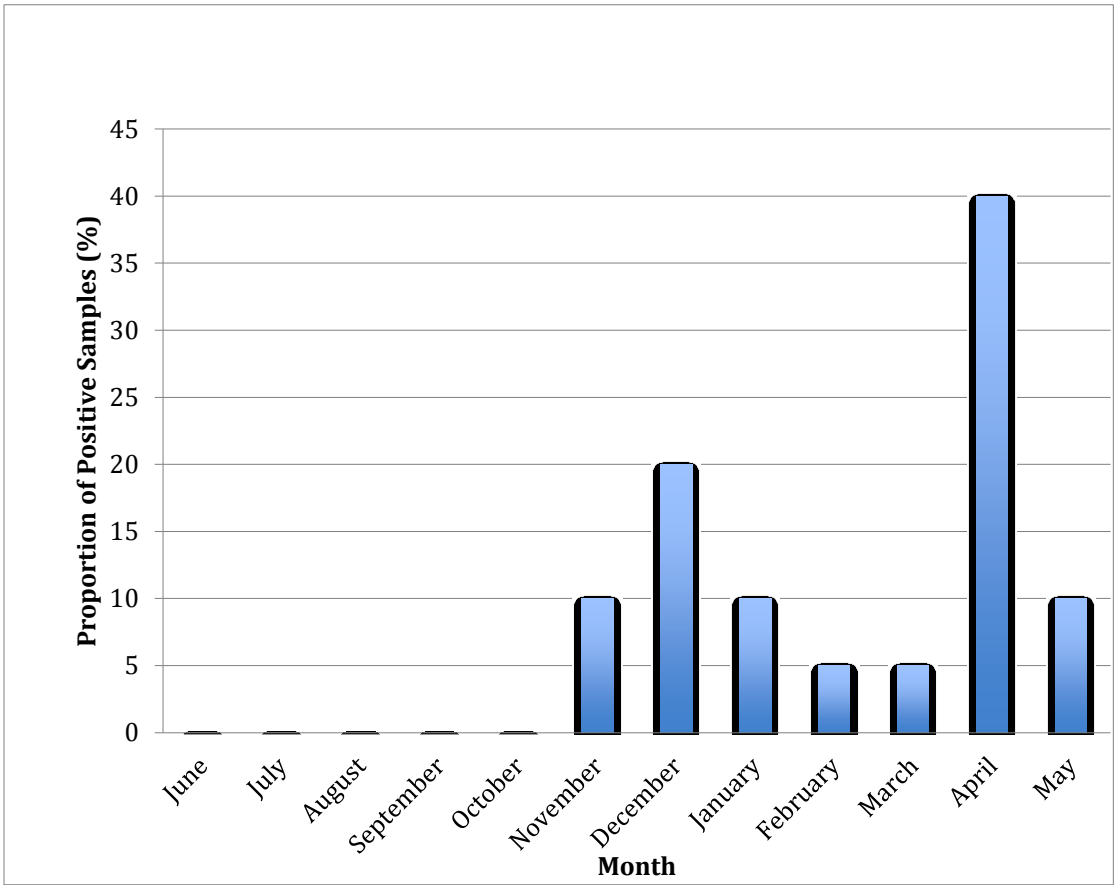

Figure 3.6 Number of reovirus positives detected by ICC-qRT-PCR from all surface water sites by month of sample collection from June 2012 to May 2013.

Of the 18 sites sampled during the study, positive qPCR results for reovirus from cultured environmental surface water were obtained in 10 sites. Of the 18 sites listed at

the beginning of the study, reoviruses were detected at both Epcor Edmonton sites, Highway 3 bridge in Oldman River, the South Saskatchewan River in Medicine Hat, Nevis and Highway 2 bridges along the Red Deer River, Devon and Pakan along the North Saskatchewan River, and Highway 40 and Smoky River along the Wapiti River. As for the amount of positives detected at each site during the study, no one site accounted for more than 3 (15%) of qPCR positives, with Pakan and Devon along the North Saskatchewan River and the Highway 2 Bridge along the Red Deer River each accounting for 3 of the positives. Brocket and the Highway 36 bridge on the Oldman River, the Highway 880 bridge on the Milk River, all four sites along the Bow River (Carseland Weir, Cluny, Ronalane, and Cochrane), and Clear Water River on the North Saskatchewan River each did not contribute any reovirus positives (Figure 3.7).

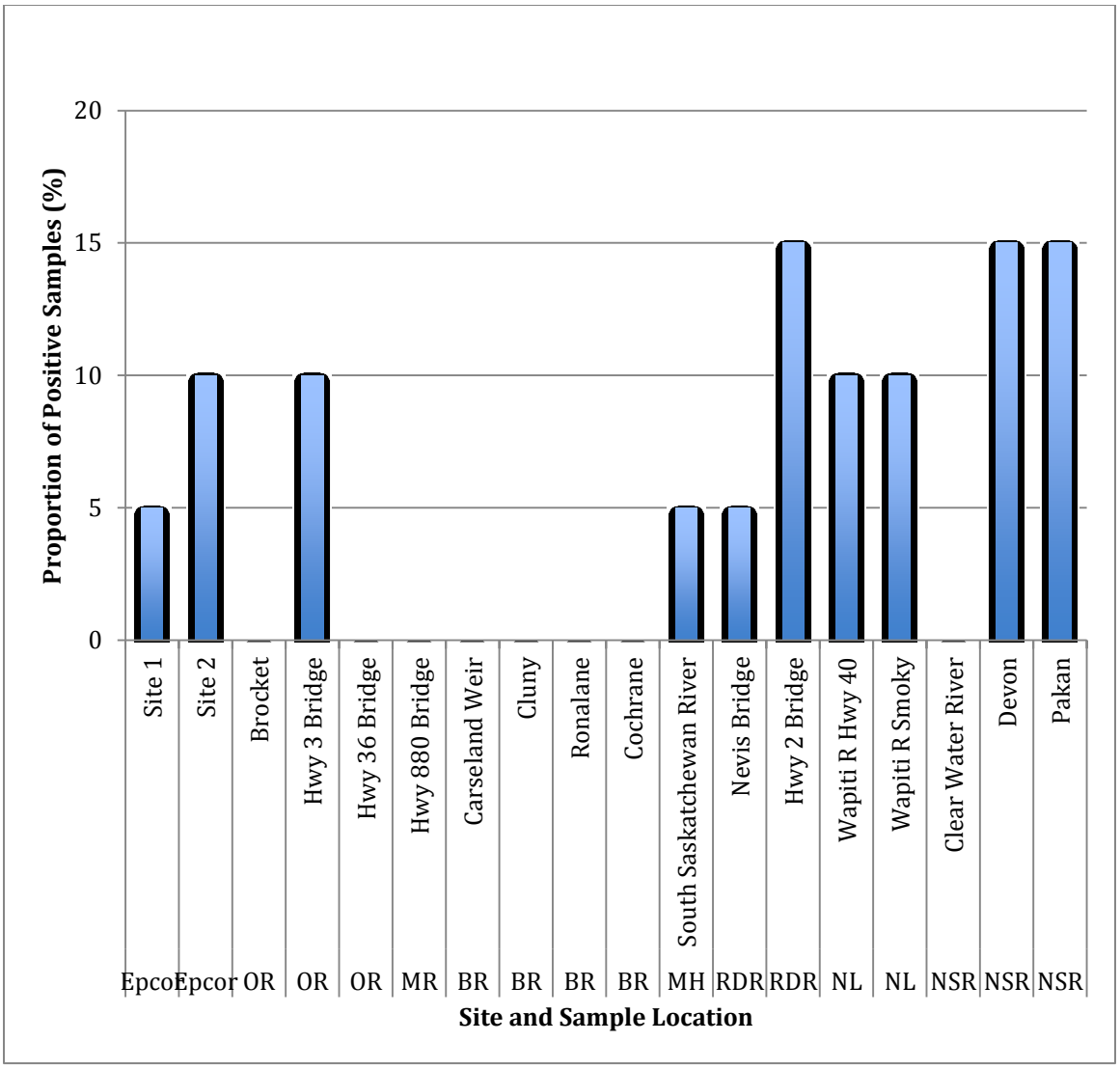

Figure 3.7 Proportion of qPCR positive MA-104-cultured environmental water samples by geographic sampling location in Alberta (n = 12 samples/location).

3.3.1c Concordance Between Pre-Culture and Post-Culture Environmental Surface Water qPCR Results

Of 216 pre-culture environmental surface water samples, reovirus was detected in 4 samples (1.9%) using the C1 assay. All four pre-culture environmental surface water samples that yielded a qPCR positive also yielded a qPCR positive result when the corresponding cultured water sample was screened. Additionally, there were 16 post-culture qPCR positives that did not yield a corresponding pre-culture qPCR positive. Of

the 20 post-culture positives, 12 samples tested positive for reovirus using both qPCR assays, six samples tested positive by only the M1 assay, and two samples tested positive by only the C1 assay.

3.3.2 Reovirus in Wastewater

16 wastewater samples (primary effluent or PE) were subjected to screening by integrated cell culture real-time PCR (ICC-qPCR) using both the REOM1 and REOC1 assays. All 16 (100%) yielded a CPE positive result when cultured and subcultured on the MA104 cell line. Fifteen of 16 CPE positive samples (93.75%) were confirmed as reovirus using the qPCR assays (Table 3.8).

Table 3.8 Summary of wastewater results for all 16 samples taken from Gold Bar Wastewater Treatment Plant in Edmonton, Alberta.

Reovirus ICC-qPCR Result	MA104 Cytopathogenic Effect (CPE)		Total
	Positive	Negative	
Positive	15	0	15 (93.75%)
Negative	1	0	1 (6.25%)
Total	16	0	16

3.3.3 Clinical Gastroenteritis Samples

460 clinical isolates from patients suffering of gastroenteritis were screened by both the REOM1 and REOC1 qPCR assays for the presence of reovirus. Of these 460 samples, 450 were obtained from January 2013 to May 2013 and were screened. The remaining 10 samples were cultured on MA-104 media as previously described. Of the ten cultured samples, only one yielded a CPE positive result. All 460 clinical isolates were screened by qPCR using both the REOM1 and REOC1 assays for reovirus and all samples tested negative. PCR inhibition was controlled for through the use of an internal positive qPCR salmon DNA control.

3.3.4 S4 Gene Fragment Sequencing of Positive Reovirus Samples

The 35 reovirus positive samples (surface sample = 20, and wastewater = 15) were subjected to additional conventional PCR reactions in an attempt to produce PCR amplicons for DNA sequencing. All 15 wastewater samples and 11/20 surface water samples for a total of 26/35 (74.3%) samples were successfully amplified and were submitted for sequencing. A breakdown of which PCR products were produced from the S4 PCRs and sent for sequencing can be found in Table 3.9. Upon sequencing, 13/15 wastewater samples and 9/11 surface water samples provided usable sequence data, with the remaining samples that did not produce usable data either not amplifying at all using any of the combinations of PCR primers or producing sequence reads that contained large gaps throughout the sequence.

Table 3.9 Overall results of the four sequencing conventional PCRs. Each individual qPCR positive was subjected to up to four different conventional PCR primer combinations.

Sample Source	PCR Result	Full-Length (1141 bp)	Nested (985 bp)	Internal Forward (421 p)	Internal Reverse (785 bp)
Surface Water	Positive	2	3	7	4
	Negative	18	17	13	16
Total		20	20	20	20
Wastewater	Positive	4	15		
	Negative	12	1		
Total		16	16		

The primary conventional PCR primer set REOS4F/R2 was used to screen the environmental surface water and wastewater samples in an effort to produce an 1141 bp PCR amplicon for DNA sequencing. Of the 20 qPCR positive surface water samples, only four (20%) would eventually yield a positive conventional PCR result using the full-

length conventional PCR primers or the nested PCR primers. The remaining 16 samples would be screened using the internal PCR primers.

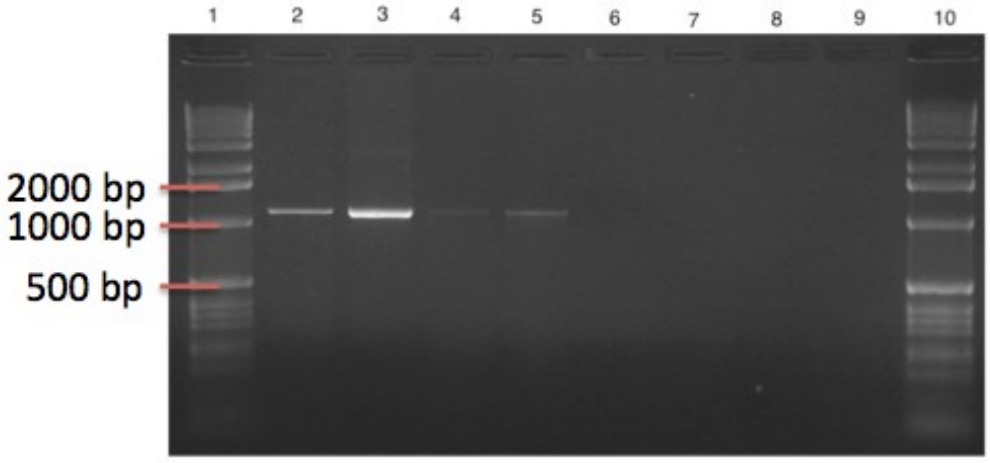

Figure 3.8 Agarose gel showing the four qPCR positive wastewater isolates that indicated a positive result when screened with the full-length S4 primer pair (lanes 2-5). The bands produced by the four samples represent a PCR amplicon of 1141 nucleotides in length. Lanes 1 and 10 represent a 1 kb DNA ladder and lanes 6 – 9 indicate a negative PCR result obtained from additional wastewater samples.

In the event that a qPCR positive sample either did not yield a positive by conventional PCR using the REOS4F/R2 primer set or if a faint band was produced (as in the PCR product in lane 4 of Figure 3.8 demonstrating the conventional PCR products of the primary PCR), the PCR products produced by the initial PCR were used as template for a second nested PCR using the REOS4NF1/NR1 PCR primer set. Four of 16 (25%) wastewater samples yielded a positive conventional PCR result when screened using the REOS4F/R2 full-length S4 PCR primers. As there were originally 15 qPCR positive samples for wastewater and the initial conventional PCR only yielded 4 positives, the samples were subjected to a nested PCR using the product of the first PCR as template. The nested PCR of wastewater samples was performed using the REOS4NF1/NR1 PCR primer set (Figure 3.9). Of the 15 samples that originally yielded a qPCR positive, all 15 (100%) yielded a positive result using the nested PCR primer set. The remaining

wastewater sample in which a CPE effect was observed, but did not yield a qPCR positive result also did not yield a positive result using either of the conventional PCR primer sets, leading to a confirmed negative for reovirus. As all 15 wastewater samples that were qPCR positive yielded a conventional PCR positive by nested PCR, no additional conventional PCRs were performed.

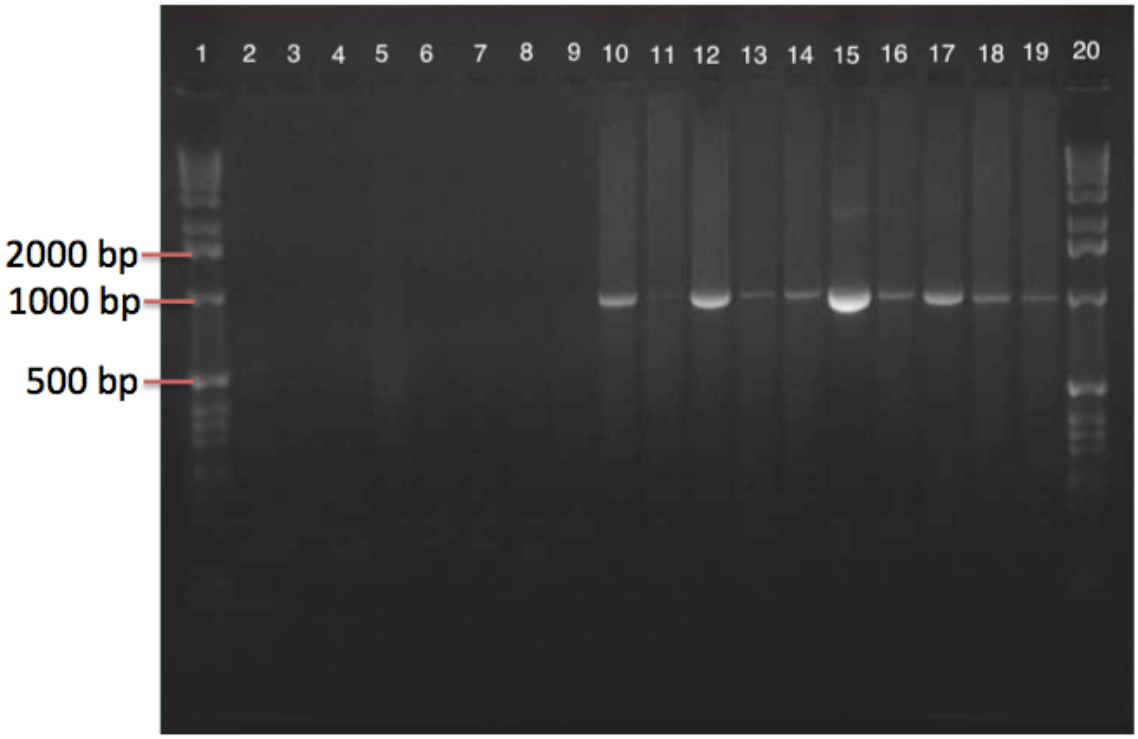

Figure 3.9 Nested PCR products produced from wastewater by the REOS4NF1/NR1 PCR primer pair using the REOS4F/R2 PCR product as template. Gel lanes 1 and 20 present a 1 kb DNA ladder, lanes 2 – 9 represent negative wastewater samples, and lanes 10 – 19 represent 10 wastewater positive nested PCR products.

Finally, if neither of the previous two PCR reactions produced any viable amplicons for sequencing, the samples were screened by two final PCR reactions using an internal primer. The samples were screened using the REOS4F forward primer along with the REOS4iRS reverse primer, as well as with the REOS4iFS forward primer and the REOS4R2 reverse primer. REOS4iFS and REOS4iRS are actually the exact same

primer but are ordered in opposing orientations, producing a portion at the 3'-end of one amplicon and the 5'-end of the second amplicon that overlap with one another. Thus, if both fragments are produced and sequenced the overlapping region can be used to produce one long fragment. These PCR amplicons are depicted in Figure 3.10.

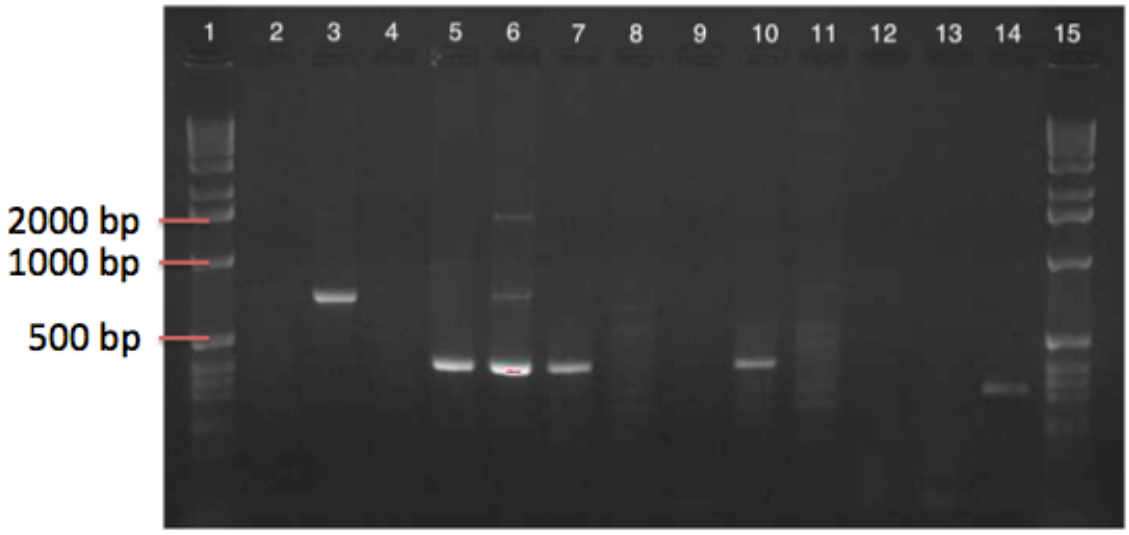

Figure 3.10 PCR products created by using the REOS4iFS/REOS4R2 and REOS4F/REOS4iRS primer pairs. Lanes 1 and 15 represent 1 kb DNA ladders; lane 3 is a single conventional PCR positive using the REOS4F/iRS against environmental surface water, with lanes 2 and 4 corresponding to negative surface water samples; lanes 5 – 7 and 10 indicate positive conventional PCR results using the REOS4iFS/R2 against samples taken from environmental surface water, with lanes 8, 9, and 11 – 12 corresponding to negative samples; lane 13 is a negative control for the REOS4iFS/R2 set; and lane 14 is a positive control using the REOL3F/R primer set.

Phylogenetic trees were created using the sequence alignment and tree-building tools in Molecular Evolutionary Genetics Analysis (MEGA). Alignments were first created using all experimentally produced sequences and representative sequences. Three separate alignments were eventually generated for comparison.

3.3.5 Reference Reovirus Strains for Sequence Comparison

3.3.6 Sequence Analysis of Full-Length of S4 Gene Fragment

The two environmental surface water samples and four wastewater samples that yielded full-length PCR products were analyzed using MEGA 6.0.5. In the end, both surface water samples and two of the four wastewater samples provided full-length sequence reads that were aligned and compared against the reference S4 sequences of reovirus. This alignment was used to create maximum likelihood (Figure 3.11) and neighbour joining phylogenetic trees using 1026 bp of sequence data.

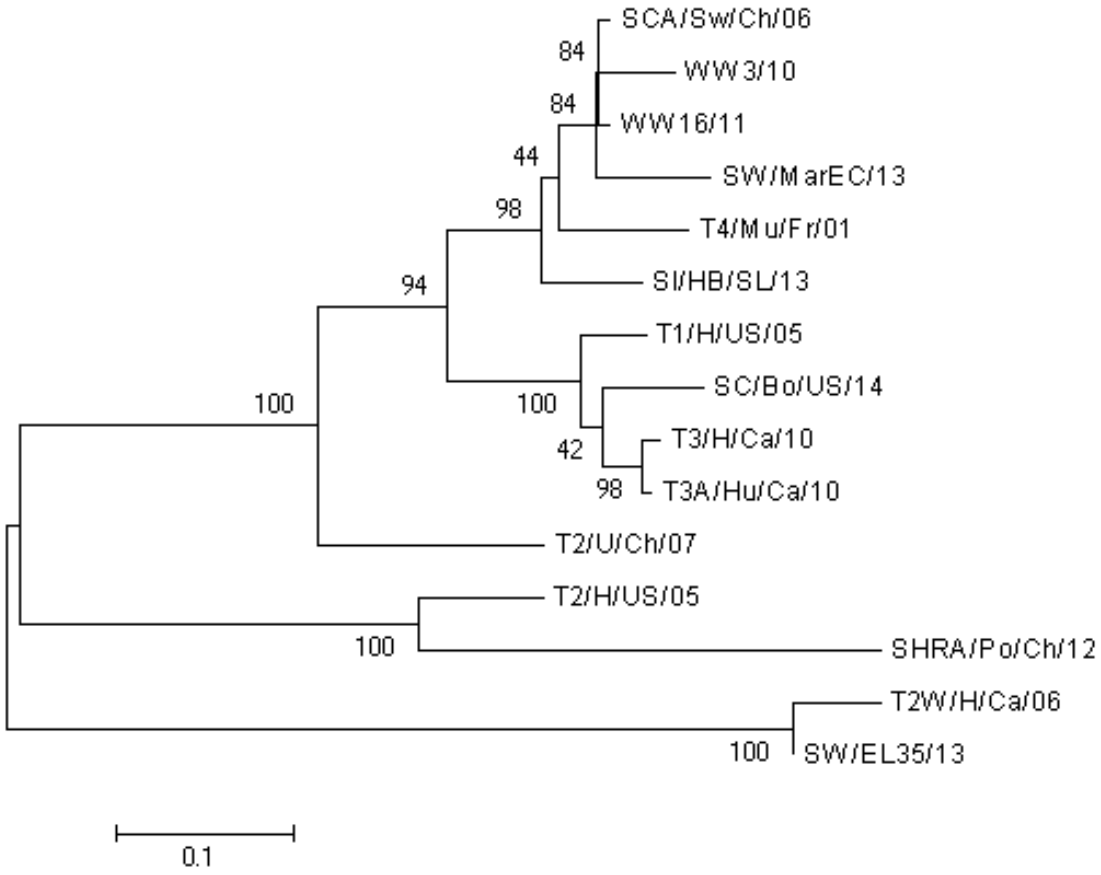

Figure 3.11 Maximum likelihood tree of full-length wastewater and surface water S4 gene fragment reads.

Based on the phylogenetic trees produced using the full-length S4 reference sequences and the two wastewater and two surface water full-length sequence reads, it is

possible to identify clustering patterns which suggest what serotypes and strains the experimental sequences are members of.

The two surface water samples (SW/MarEC/13 and SW/EL35/13) are clustered to two distinct groupings. The first surface water sample (SW/EL35/13) formed a node with a newly described strain of serotype 2 reovirus known as serotype 2 Winnipeg as it was previously discovered in human subjects in Winnipeg, Manitoba (DQ220019.1). Based on the phylogenetic tree produced, the newly identified surface water sample and the serotype 2 Winnipeg strain are distinct enough from the other serotypes that the two sequences create their own separate grouping, distinct from even serotype 2. The second surface water sample SW/MarEC/13 was obtained from the North Saskatchewan River near Pakan in March of 2013 and groups together with both of the wastewater samples (WWPE3 and WWPE16). This group of wastewater samples and the single surface water sample create a distinct grouping with the SCA strain of reovirus obtained from swine in China. Additionally, this grouping of samples aligns well with the newly proposed serotype 4 reovirus (Ndelle), which was initially isolated from laboratory mice.

3.3.7 Sequence Analysis of Nested PCR of S4 Fragment

While 15 wastewater samples would produce a positive result using the nested PCR primers REOS4NF1/REOS4NR1, 6 samples produced sequence reads of 949 bp each and were aligned against the reference strains (Figure 3.12).

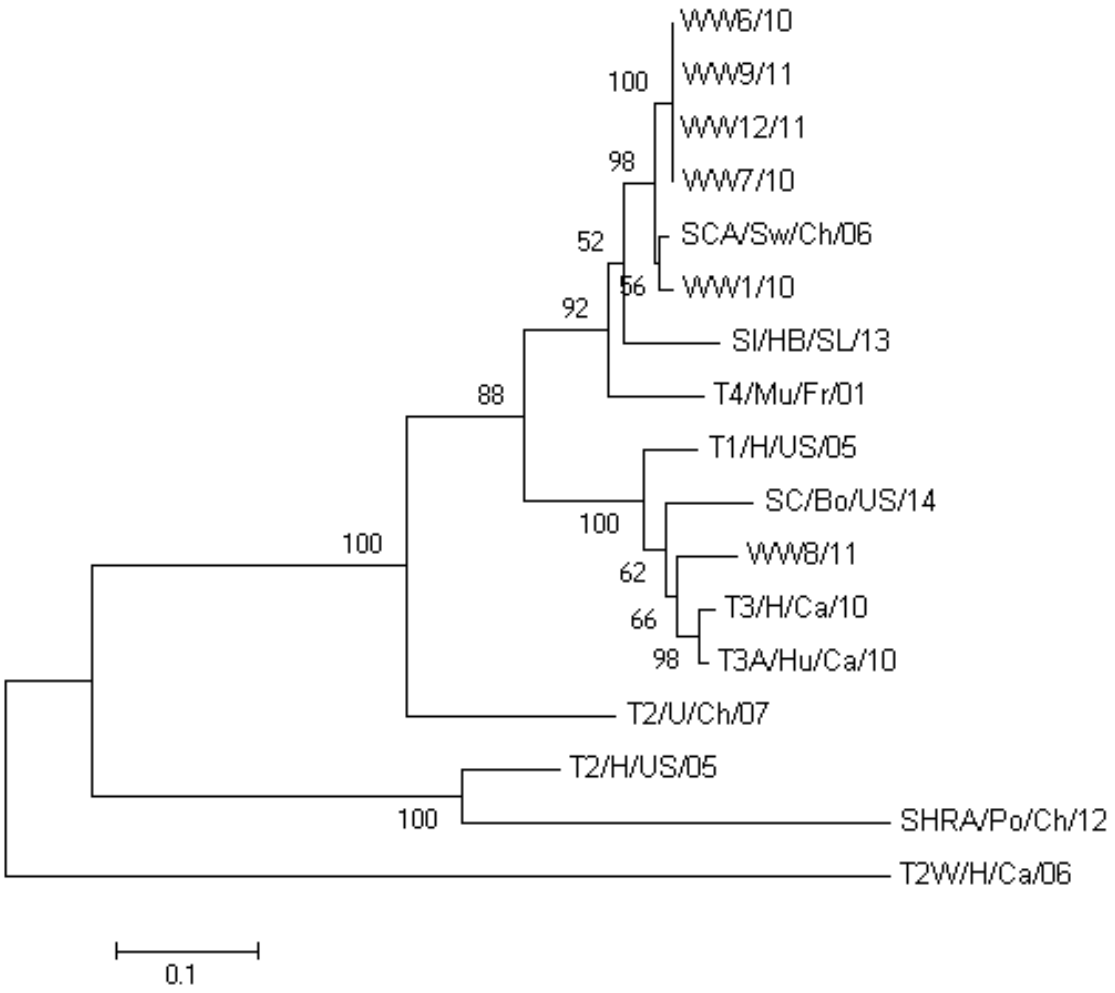

Figure 3.12 Maximum likelihood tree produced from the nested PCR reads produced from environmental surface water and wastewater samples obtained throughout the study.

The wastewater samples WW1, WW6, WW7, WW9, and WW12 all cluster into a single node with the SCA reference strain of reovirus that was obtained from swine in China. These wastewater samples and the wastewater samples WW3 and WW16 as well as the surface water sample MarEC clustered together with this Chinese SCA strain. The final wastewater sample (WW8) aligns closely with both of the serotype 3 reovirus representative sequences used for creating the alignment and phylogenetic tree. Both

serotype 3 mammalian orthoreovirus samples were previously obtained from human subjects in Canada.

3.3.8 5'-Short Length Sequence Analysis of Environmental Surface Water and Wastewater

A second alignment was produced using all of the sequencing data produced for the 5'-region of the S4 genetic fragment (~300 bp) (Figure 3.13). Several samples only produced viable sequencing reads in either the 5' to 3' or 3' to 5' (depending on sequence read direction) orientation, creating increased amounts of sequence data for the ends of the S4 fragment.

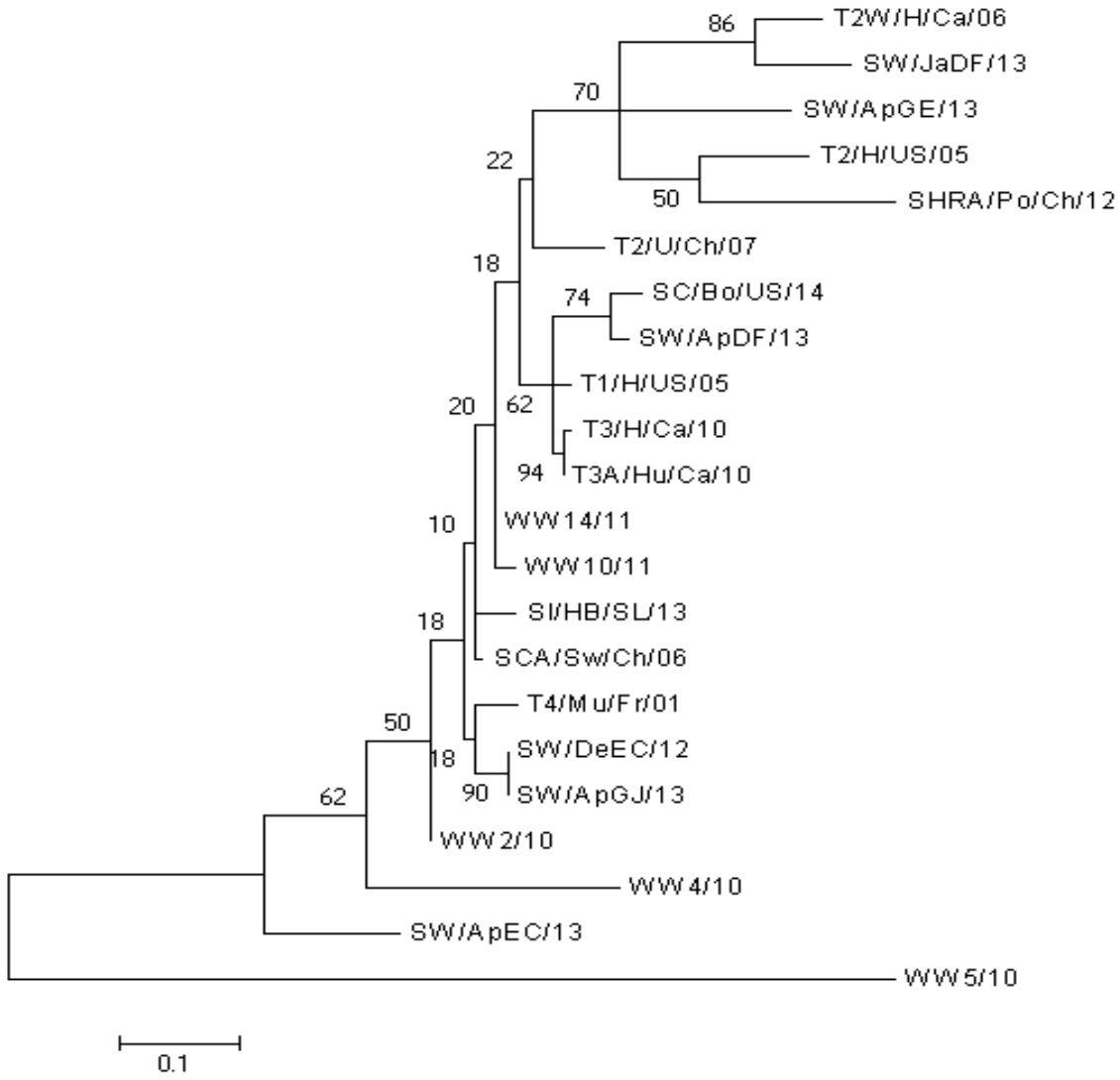

Figure 3.13 Maximum likelihood tree produced from the 5'-end reads produced from sequencing runs of the environmental surface water and wastewater samples obtained throughout the study.

In the case of the 5'-end sequence reads, there was a multitude of sequences that were obtained from surface water and wastewater samples that were short in length. In order to accommodate all of the available sequences, only 312 nucleotides of sequence data was used to create the alignment and phylogenetic tree. The resulting phylogenetic trees showed low bootstrapping values because of the short length of DNA sequence fragments included in the alignments.

3.3.9 3'-Short Length Sequence Analysis of Surface Water and Wastewater Samples

Finally, any samples that did not yield data for the 5' portion of the S4 genetic fragment were aligned with full-length sequences for the S4 genetic fragment obtained from environmental surface water and wastewater, as well as with the representative reovirus sequences (Figure 3.14). In addition to the full-length phylogenetic tree and alignment, two sequences derived from environmental surface water were added.

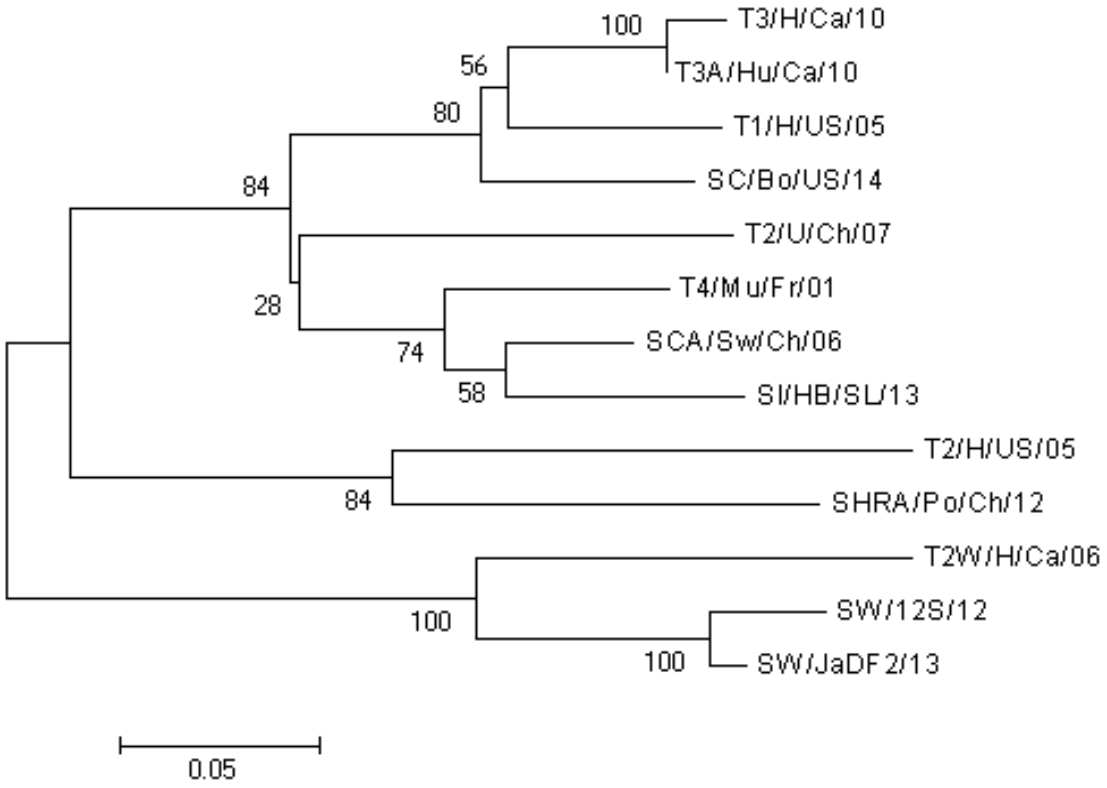

Figure 3.14 Phylogenetic tree produced from the 3'-end reads produced from sequencing runs of the surface water and wastewater samples obtained throughout the study.

In addition to the surface water sample SW/EL35/13, the two newly added environmental surface water samples were grouped together with the serotype 2 Winnipeg representative sequence. SW/12S/12 and SW/JADF2/13 created a new, larger cluster with the two sequences that were previously named to create a distinct node

featuring this newly described serotype 2 subtype. SW/12S/12 was obtained in December 2012 from the Highway 2 Bridge near the Red Deer River while SW/JADF2/13 was procured from the North Saskatchewan River near Devon in January of 2013.

In summary, of the 10 sequenced samples, MarEC, DecEC, AprGJ, and AprDF would all form a distinct cluster with each other and sequences of known serotype 1 origin. Specifically, samples MarEC, DecEC, and AprGJ were aligned closely with a serotype 1 reovirus that was previously isolated from humans in the United States (X61586.1), leading to speculation that these samples belong to serotype 1 and could have originated from a human source. The sequence obtained from AB05DF0010 April aligned more closely to another serotype 1 representative (DQ396806.1) obtained from swine in China. Samples SW-EL-WTPB-35, AB05DF0010 January, and 12SWCB1201 all aligned closely together with a sequence obtained from a strain of serotype 2 known as serotype 2 Winnipeg which was also isolated from humans (DQ220019.1). Additionally, AprGE and AprEC were observed to cluster closely with serotype 2, which had all of its representatives previously isolated from humans. Finally, R1923384 did not cluster with any of the known representative sequences or with other sequences obtained throughout the study.

While these samples clustered most closely with samples that were previously isolated from human and animal sources, the ultimate biological source of contamination cannot be readily determined from the data obtained. The GenBank database contains a limited number of nucleotide records for mammalian orthoreoviruses that is not representative of all strains and variations of reoviruses in the environment. As such, it is

believed that more extensive sequencing of the reovirus genome is required in order to infer the biological source of contamination.

The sequence obtained for sample R1923384 was used as a BLAST query against the nucleotide database and was able to return sequences from reovirus: the sequence most closely matched sequence JN799422.1, which was isolated from swine in Austria, with a maximum identity of 77% and a query coverage of 37%.

An overview of sequenced samples and their proposed serotypes is shown in Table 3.10.

Table 3.10 Breakdown of environmental surface water samples that yielded usable sequencing data and their potential serotypes.

Sample	Date (dd-mmm-yy)	Site	Potential Serotype/Strain
SW/12S/12	10-Dec-12	Red Deer River	Seroype 2
SW/DeEC/12	13-Dec-12	North Saskatchewan River	Seroype 1
SW/JaDF/13	07-Jan-13	North Saskatchewan River	Seroype 2
R1923384	06-Feb-13	Oldman River	Unknown
SW/MarEC/13	14-Mar-13	North Saskatchewan River	Seroype 1
SW/ApDF/13	08-Apr-13	North Saskatchewan River	Seroype 1
SW/ApGE/13	08-Apr-13	Wapiti River	Seroype 2
SW/ApGJ/13	08-Apr-13	Wapiti River	Seroype 1
SW/ApEC/13	11-Apr-13	North Saskatchewan River	Seroype 2
SW/EL35/13	16-Apr-13	Epcor	Seroype 2

In summary, in wastewater samples that yielded a qPCR positive result, there were a total of 13 samples that yielded usable sequencing data (Table 3.11). Of the 13 sequenced wastewater samples, it was observed that 10 of the 13 sequenced samples would ultimate form a tight cluster on the phylogenetic trees with reovirus serotypes 1 and 3. Specifically, sample WWPE8 formed a cluster with representative sequences from MRV serotype 3. Both representative sequences for serotype 3 included in the alignments and phylogenetic trees (GU589586.1 and HM159622.1) were derived from human sources of viral contamination. Therefore, it is possible that the sample WWPE8 was most likely derived from a human source. The remaining samples in this cluster grouped together closely with a particular representative strain sequence (SCA/Sw/Ch/06) obtained from a swine source in 2006 in China. The samples WWPE1, WWPE2, WWPE3, WWPE6, WWPE7, WWPE9, WWPE10, WWPE12, WWPE14, and WWPE16 all created a tight clustering pattern when aligned with the swine strain. Finally, there were two additional wastewater samples, WWPE4 and WWPE5 that created a cluster on the phylogenetic trees with all of the serotype 2 representative sequences which were all previously determined as having a human source.

Table 3.11 Breakdown of the wastewater qPCR positive samples that were analyzed throughout the study and their prospective serotypes.

Sample Name	Date	Potential Serotype (Strain)
WWPE1	08-Jun-10	Serotype 1 (SCA)
WWPE2	12-Jul-10	Serotype 1 (SCA)
WWPE3	17-Aug-10	Serotype 1 (SCA)
WWPE4	13-Sep-10	Serotype 2
WWPE5	13-Oct-10	Serotype 2
WWPE6	15-Nov-10	Serotype 1 (SCA)
WWPE7	13-Dec-10	Serotype 1 (SCA)
WWPE8	17-Jan-11	Serotype 3
WWPE9	14-Feb-11	Serotype 1 (SCA)
WWPE10	07-Mar-11	Serotype 1 (SCA)
WWPE12	10-May-11	Serotype 1 (SCA)
WWPE14	11-Jul-11	Serotype 1 (SCA)
WWPE16	1-Sep-11	Serotype 1 (SCA)

3.4 Discussion

3.4.1 Environmental Surface Water

216 surface water samples were obtained monthly from 18 sites across Alberta for one year. The surface water samples were subjected to qPCR screening for reovirus immediately after filtration, flocculation, and concentration (pre-culture) and after two passages through viral culture using MA104 cell lines (post-culture), yielding results from 216 pre-culture and corresponding post-culture samples. Reoviruses were observed in 1.9% (4/216) pre-culture samples and in 9.3% (20/216) post-culture samples. This finding coincides with previous studies that compared the use of conventional PCR and ICC-PCR (51). It was shown that the use of culturing on a cell line prior to qPCR offered enhanced detection of reoviruses over the use of qPCR alone.

In the 20 reovirus positive samples (ICC-qPCR positive), cytopathogenic effect (CPE) in MA-104 cells was observed in 10 samples (50%) after culture, indicating a reasonably high correlation between detection of the viruses by ICC-qPCR and the

infectivity of live viral particles. As the MA-104 cell line has been previously shown to be susceptible to reoviruses, enteroviruses, and rotavirus, it was anticipated that positive CPE in MA-104 cells could be attributed to the presence and replication of either reoviruses or other infectious enteric viruses (56). Inversely, there were 10 samples that were ICC-qPCR positive but CPE negative. It is believed that the CPE negative/ICC-qPCR positive samples could be attributed to either viable, non-cultivable reoviruses or other enteric viruses without virulence. It is also possible that the viruses present in the samples are viable and infectious, but are slow growing and not forming CPE.

Among the 20 qPCR positives for reovirus in post-culture samples, at least one positive was detected monthly from November 2012 through to May 2013. It was observed that there were no reovirus positives in surface water from June to October during 2012. The data provides evidence of the seasonality of reovirus in the environment in Alberta. These findings correspond exactly with previous studies of the seasonality of reovirus in Japan: the study indicated that reovirus is most active in surface water during the colder winter months of November to March (105). In both studies it can be noted that reoviruses were observed much more frequently during the winter than in warmer summer months.

In this one-year study, it was observed that reovirus was detected in at least one sample at 10 of the 18 (55.6%) sampling locations. The findings obtained suggest a widespread, albeit seasonal presence of reovirus in the surface water found in rivers and waterways across Alberta. Since previous work into the geographical distribution of reovirus in Alberta has not been previously examined, the results put forward here are the first step at investigating the widespread distribution of this virus.

In terms of the source of the reovirus contamination, it was observed that there were viruses that originated in both human and animal sources. While it was expected that the majority of positive samples would most closely resemble animal sources, it is consistent with previous findings regarding reoviruses in surface water (56, 101).

3.4.2 Wastewater Treatment Plant Samples

Sixteen wastewater samples in total were collected from the Gold Bar wastewater treatment plant in Edmonton, Alberta over the course of sixteen months. These 16 samples were collected after the primary treatment of wastewater (primary effluent), which involves gravity settling of wastewater and the removal of top layer solids. Of the 16 wastewater samples screened in the study, the presence of reovirus was detected in 15 of 16 samples by ICC-qPCR, while cytopathogenic effect (CPE) was observed in all 16 of wastewater samples in MA-104 cell culture.

With regards to the single sample in which reovirus was not detected by ICC-qPCR, but CPE was seen, it is anticipated that the CPE was caused by the presence of viable, cultivable rotavirus and/or enterovirus (56). The presence of both rotavirus and enterovirus in the sample was verified by specific qPCR testing, indicating that either individual virus or a combination of both viruses could have resulted in CPE.

The finding of reoviruses in wastewater is consistent with previous findings described in Australia and the United States (49, 97). Reovirus has previously been observed at all different stages of wastewater treatment, during all months of the year, as the virus is described as being difficult to remove from wastewater (49). While it was expected that the reoviruses observed in wastewater would be mostly of human origin, the findings of this study suggest that both humans and animals are contributing to the

presence of reovirus found in wastewater. Also, it has previously been stated that reovirus likely enters wastewater from a variety of sources, as wastewater often pulls in contaminants as it moves along to the treatment facilities, which is consistent with the results reported here (49, 105).

3.4.3 Clinical Stool Samples

A total of 460 clinical stool samples from patients suffering from gastroenteritis were screened by qPCR for reovirus and all samples returned negative results for reovirus. This finding corresponds to previous research indicating that reovirus either does not exist in the gastrointestinal tract of the patients who had gastroenteritis associated with other enteric viruses or it is not present naturally in human gastrointestinal tract at all (38). Previous studies have focused on the presence of the virus in the gastrointestinal tract of children hospitalized with gastroenteritis and this previous work has indicated that reovirus is likely not a major cause of gastroenteritis (38).

It is interesting that 15/16 wastewater samples tested positive for reovirus, while none of the 460 clinical stool samples tested positive. This is of interest, as it would be expected that clinical stool samples and wastewater samples would correspond to one another due to the presence of human fecal samples. However, it is believed that the presence of reovirus in wastewater is likely due to the amalgamation of a variety of source waters into the wastewater treatment facility. Therefore, it is likely that reoviruses from both human and animal sources are present in wastewater.

The findings discussed here coincide with other studies regarding human fecal samples collected from patients suffering from gastroenteritis. It has previously been

indicated that reovirus likely does not have as high of a presence in humans as once thought (38). Further study is needed to explore when and where human reoviruses are present and its clinical significance.

3.4.4 Sampling Error

A potential cause for the diminished number of observed qPCR positive reovirus samples detected in the environmental surface water as well as the clinical fecal samples might possibly stem from sampling error. In those samples that gave negative qPCR results for reovirus, it is possible that the samples did originally contain either complete viral particles of reovirus or even long fragments of genome of the virus that is an adequate target for PCR amplification. When the environmental surface water samples were processed for qPCR detection, each individual sample was tested by qPCR at two different times: concentrated water (pre-culture) and cell culture/concentrate mix (post-culture). The potential sampling error could be introduced during the repeated sample mixing during the concentration procedure of the surface water and wastewater samples, and during dilution of clinical stool samples. Small aliquots of prepared samples may not contain the targeted fragments of reovirus when prepared for reverse transcription. In addition, the samples that yielded a negative qPCR result for reovirus could have potentially contained a very small amount of virus in a large volume of water, which may have been lost during sample preparation process.

3.4.5 DNA Sequencing of Reovirus-Positive Surface Water and Wastewater Samples

Reoviruses have been shown to be present in a variety of mammalian sources including, but not limited to, humans, sea lions, dogs, and sheep (23, 59, 80, 90, 91, 111). In addition to being found in a wide variety of host species, reoviruses have been detected

in a wide variety of geographical locations across the world. Reoviruses have been detected and sequenced in North America, Europe, and Asia (23, 88, 89, 121). However, the sequencing database in NCBI for reoviruses remains relatively less informative than that for other enteric viruses.

A primary goal of this research project was to characterize all of the reoviruses found in qPCR positive samples collected for one year, including serotypes, geographic and biological sources. The S4 genomic fragment of reovirus was chosen for sequencing target for a number of reasons. Firstly, the short length of S4 (1196 nucleotides) allows for the majority of the fragment to be amplified with a single PCR, avoiding the need for multiple PCR reactions. Second, the fragment itself displays a high degree of conservation at the 5' and 3' ends, allowing for a single set of PCR primers to be created at each end of the fragment for amplification of the interior portion of the fragment. Finally, the GenBank record for the S4 genetic fragment contains a variety of sourced organisms, serotypes, and the majority of records are of the complete fragments.

Among all 35 qPCR positive samples of reovirus from surface water and wastewater, 10 surface water samples and 13 wastewater samples would eventually yield positive results by conventional PCR for downstream sequencing.

3.4.6 Source Determination of Reovirus in Environmental Surface Water and Wastewater Samples

In order to make assumptions about the source of the reoviruses discovered in environmental surface water and wastewater samples in the study, S4 genetic fragment sequences obtained from the samples were compared to reference reovirus DNA sequences deposited in GenBank with known serotype, geographic and biological origin.

In the initial hypothesis, it was expected that the predominant source of reoviruses in wastewater would be human strains and serotypes since municipal wastewater being treated at the treatment plants contains mainly human excreta such as human feces (46,91,95,98). It was also expected that reoviruses in environmental surface water could be derived from different mammals including humans and animals, as the surface water contains water from a variety of intakes, tributaries, and sources.

Thirteen total wastewater samples contributed usable sequencing information. Experimental results demonstrated that a variety of sources of reoviruses were present in wastewater. Nine samples containing reovirus clustered closely with serotype 1 Lang were most closely related to a swine source; 2 samples clustered with serotype 2 were likely to be derived from a human source; and a single sample clustered with serotype 3 was likely originated from human; a final wastewater sample (WW5/10) did not cluster well with any known sequences. This finding was slightly deviated from our hypothesis since a majority of sequencing results of reoviruses in wastewater (9/13 or 69%) were determined as the swine origin. However, it was not a surprise because wastewater could come from industrial sources, such as packing plant or slaughterhouse, and rain/storm water carrying contaminates from animals. Reoviruses in rest of wastewater samples (3/13 or 23%) were identified by sequence analysis as a human source. This finding reveals that diverse sources of reoviruses are present in wastewater and the viruses still possess the infectivity after primary treatment.

Of 20 environmental surface water samples with detectable reoviruses, 10 sequences were successfully obtained and used for analysis against known sequences of reoviruses deposited in Genbank. Of the 10 strains of reovirus found in environmental

surface water samples, 4 are clustered closely with serotype 1 Lang, 3 of these samples are aligned closely to the human strain with the remaining clustering closely with the swine strain. 5 additional strains were clustered with serotype 2 Jones, aligning with human strains isolated in Winnipeg, Manitoba (DQ220019.1). The remaining sequence data derived from the sample R1923384 was not clustered with any reference sequences for reovirus in GenBank and other sequences obtained from the study. It is believed that this sequence was derived from a reovirus strain that likely has undergone either extensive reassortment and/or mutation. Sequence analysis in this study confirmed that a majority of reoviruses (8/10 or 80%) in environmental surface water samples were identified as human strains based their proximity to sequence data previously published from other reovirus strains. This finding differs from the initial hypothesis that a variety of sources of reoviruses would have been present in the surface water. Further sequence analysis on a large number of samples may provide a definitive answer as to why the discrepancy between our expectation and the true distribution of reoviruses found in environmental surface water.

A study performed in Slovenia on stool samples taken from a 17-month old male suffering from gastroenteritis yielded a novel strain of mammalian orthoreovirus. Whole genome ion torrent sequencing of this reovirus indicated that 8/10 of the genomic fragments showed a high degree of homology with a type 2 reovirus isolated from bats in Germany while the remaining 2 fragments (S1 and S2) showed a higher degree of homology with a serotype 3 reovirus, also isolated from a bat (103).

As such, it is believed that sequencing of more than a single genetic fragment is required in order to infer a sample's serotype. Therefore, whole genome sequencing (or,

at the very least, sequencing of multiple genomic fragments) is required in order to provide more meaningful speculation of the serotype of the virus present.

3.4.7 Enhanced Detection of ICC-qPCR Over qPCR

All 216 environmental surface water samples were analyzed pre-culture (qPCR) and post-culture (ICC-qPCR). The pre-culture samples would ultimately contribute 4/216 qPCR positives (1.9%) while the corresponding post-culture screening uncovered 20/216 positives (9.3%). It can be seen that using a culture method in conjunction with qPCR leads to enhanced detection over qPCR alone. This finding corresponds to several previous studies in which reovirus is demonstrated as being an easily cultured virus (56). These finding also correspond to other studies that combined the use of cell line culturing with PCR (36, 39, 56, 86).

3.5 Summary of Findings

It was observed that reoviruses are present in Alberta's surface water in a variety of sites. A complete breakdown of pre-culture qRT-PCR, ICC-qRT-PCR, and CPE data can be seen in Table 3.12.

3.5.1 Surface Water Samples Obtained from 18 Sites Across Alberta

Table 3.12 Summary of findings for pre-culture qPCR, cytopathogenic effect (CPE), and integrated cell culture qPCR (ICC-qPCR) for environmental surface water samples taken from 18 sites across Alberta.

Sample Location	Pre-Culture qPCR		Cytopathogenic Effect (MA104)		Post-Culture qPCR (ICC-qPCR)		Total Samples
	Positive	Negative	Positive	Negative	Positive	Negative	
Epcor Site 1	0	12	1	11	1	15	16
Epcor Site 2	1	11	3	9	2	14	16
Brocket	0	12	0	12	0	16	16
Hwy 3 Bridge	1	11	0	12	2	14	16
Hwy 36 Bridge	0	12	1	11	0	16	16
Hwy 880 Bridge	0	12	0	12	0	16	16
Carseland Weir	0	12	0	12	0	16	16
Cluny	0	12	0	12	0	16	16
Ronalane	0	12	0	12	0	16	16
Cochrane	0	12	0	12	0	16	16
South Saskatchewan	2	10	0	12	1	15	16
Nevis Bridge	1	11	1	11	1	15	16
Hwy 2 Bridge	2	10	3	9	3	13	16
Wapiti R Hwy 40	0	12	1	11	2	14	16
Wapiti R Smoky	0	12	2	10	2	14	16
Clear Water River	0	12	0	12	0	16	16
Devon	0	12	0	12	3	13	16
Pakan	0	12	2	10	3	13	16
Total	7	209	14	202	20	196	216

All 20 qPCR positives obtained from cultured surface water samples and one additional qPCR positive from a pre-culture water sample were further subjected to conventional PCR to produce an amplicon of sufficient size for sequencing.

3.5.2 Wastewater Samples Obtained from Gold Bar Wastewater Treatment Plant

Of the 16 cultured water samples obtained from Gold Bar Wastewater Treatment Plant in Edmonton, Alberta, all 16 yielded a positive CPE culture result. Of the 16 CPE positives, 15 samples would yield a positive qPCR result for reovirus. All 15 qPCR positives yielded a positive conventional PCR result and were subjected to sequencing.

3.5.3 Clinical Fecal Samples from Patients Suffering from Gastroenteritis

460 clinical isolates taken from patients suffering from gastroenteritis were screened by qPCR for the presence of reovirus. None of the 460 clinical isolates screened yielded a positive result for reovirus.

3.5.4 Sequencing of Reovirus-Positive Environmental Surface Water and Wastewater Samples

All qPCR positive results obtained throughout the entire study were subjected to conventional PCR using primers directed at the S4 gene fragment. Any amplicons obtained during the conventional PCR runs were extracted, purified, and subjected to DNA sequencing. Ten of the original 21 environmental surface water samples would yield a positive conventional PCR amplicon using one or more of the conventional PCR primer sets. All 15 qPCR positive wastewater samples would yield positive conventional PCR amplicons using the nested PCR primer set. These resulting 25 PCR positives were sequenced and compared to known representative sequences.

4.0 Summary of Project and Future Directions

4.1 Summary of Findings

In this study, reoviruses from a diverse group of serotypes and strains were detected and observed in samples collected across Alberta in both environmental surface water and municipal wastewater. Two novel sets of qPCR primers and probes were designed and used for screening environmental water, wastewater, and clinical stool samples for the presence of reoviruses.

Two hundred sixteen environmental water samples were collected monthly from 18 sites across Alberta over the span of this study. These 216 environmental surface water samples were concentrated and then cultured with the MA-104 cell line then screened using qPCR with the REOM1 and REOC1 qPCR primer/probe sets immediately after concentration (pre-culture), as well as after culturing (post-culture). The results showed that 4 of 216 (1.9%) pre-culture samples tested positive by qPCR positive for either REOM1 or REOC1 and 20 of 216 (9.3%) of post-culture surface water samples tested positive by qPCR for one or both of the qPCR primer sets.

It was observed that there was a strong correlation between detection of reovirus in pre-culture concentrates and a positive post-culture qPCR result in environmental surface water samples. In cultured environmental surface water samples, it was observed that there was also a strong correlation between cytopathogenic effect (CPE) of cells and a positive qPCR result for reovirus. Furthermore, reoviruses were observed in environmental surface water from November 2012 to May 2013, however, no positive qPCR results were recorded from June 2012 to October 2012. Reovirus was detected at 10 of the 18 (55.6%) sites that were screened throughout the study, suggesting that the

virus is widespread in surface water across Alberta. Sequence data obtained from the surface water samples was compared against a variety of sequences from GenBank of known source origin. The sequence data showed several different clusters with the sequences of known origin and it is believed that there were positive samples obtained from all three serotypes of reovirus from GenBank. Particularly of note, there was a surface water sample that was closely matched with a sequence obtained from the proposed serotype 4 (Ndelle) reovirus. There were three positive samples that created a distinct group that was similar to a variant of the seroype 2 serotype of reovirus known as serotype 2 Winnipeg.

16 cultured wastewater samples were obtained from primary effluent at a wastewater treatment plant in Edmonton, Alberta and cultured in cell line then screened by qPCR. 15 of the 16 samples would provide evidence of reovirus, which was expected as previous studies have investigated the link between reovirus and human fecal matter. The sequence data obtained for the majority of wastewater samples showed that the viruses present closely resemble a strain of reovirus previously obtained from a swine sample. However, some viruses in the wastewater clustered to two strains of serotype 3 reovirus that were derived from human fecal matter.

In conclusion, reoviruses were observed in both pre-culture concentrate and post-culture environmental surface water samples obtained from various sites across Alberta, and in wastewater primary effluent obtained from the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta. Interestingly, reoviruses were not observed in the clinical stool samples included in this study. The reovirus samples detected by qPCR were derived from a variety of human and animal sources, with a variety of different serotypes

and strains. Therefore, while reoviruses were detected in both environmental surface water and wastewater, the source of the viral contamination is not always originating from strains of reovirus that have been previously isolated from humans.

It is believed that more extensive sequencing of the viral positive samples from environmental surface water and wastewater samples using whole genome sequencing or targeted fragment sequencing approaches would be helpful to further distinguish the origin and sources of reoviruses. Furthermore, sequencing data would enable investigations into genetic reassortment within the reovirus population.

This study also indicates that further study into the relationships between reoviruses existing in the environment and the strains of the virus present in humans is necessary and encouraged.

4.2 Future Directions

The new qPCR assay for reoviruses can be applied to clinical specimens taken from children who have been hospitalized for liver transplants. One of the major causes of liver failure in newborn children is biliary atresia (79,105). Additionally, reoviruses been isolated from tissue and anal swab samples from children diagnosed with biliary atresia (40,79,105). Therefore, it is believed that tissue samples removed from affected children can be screened using the qPCR and culture test to detect the presence or absence of reoviruses. Furthermore, this assay could potentially be used to screen fecal samples from patients undergoing oncolytic chemotherapy with reovirus to determine how long the virus is staying within the patient's system. In addition to being detected in humans in all stages of health, reoviruses have also found use in the field of oncology. serotype 3 Dearing reovirus has been used to treat a variety of cancers as a delivery

system in conjunction with several chemotherapeutic agents (45, 48, 52, 63, 76, 119, 120). This is due to serotype 3 reovirus' ability to preferentially infect transformed cells (24, 42, 45, 48). Reoviruses have been used successfully against head and neck carcinomas, colorectal, breast, cervical, and prostate cancers (48, 63, 120). Reovirus seroype 3 Dearing has been used as a delivery system for docetaxel in human tumours (45).

As reoviruses were detected in both environmental surface water and wastewater, it would be interesting to continue surveillance of these water matrices to determine if the number of positive samples varies over time or by location. In the case of primary effluent wastewater samples, reovirus was detected in 15 of 16 samples from across all seasons. As was seen in the surface water data presented however, reoviruses in Alberta's surface water were most prevalent during the winter months, with no positive samples being detected in the warmer months of June and July. Further screening of samples obtained during the summer months may eventually yield positive reovirus samples or continue to indicate a diminished presence of the virus in the environment in the summer.

Furthermore, as only one genetic fragment was sequenced and analyzed as part of this study, it would be interesting to look at the sequences of the only genetic fragments of reovirus. Sequencing of other fragments or the whole genome of each positive sample would provide a greater deal of information to use when performing sequence alignment and comparison. Increased sequence data would serve to provide increased insight into the differences found between samples found at different sampling locations compared to sequences of known origin already present in the literature. Additionally, increased sequence data would allow for more absolute assignment of positive samples to known

genotypes, as it would be possible to compare more than a single genomic fragment. In the case of using whole genome sequencing, all of the sequence data for every positive sample would be available for in-depth sequence comparison and characterization. As was the case with sequencing multiple genomic fragments compared to a single fragment, sequencing the entire genome of each positive sample would only serve to increase the certainty with which each sample could be assigned to a specific genotype or strain. In addition, increased sequence data obtained from surface water and wastewater could be used to bolster the already existing sequence database for reovirus.

In addition, further sequencing of positive reovirus samples from wastewater would also serve to illustrate a stronger or weaker link between the samples and strains of known human origin. As it was initially believed that reoviruses detected in the wastewater would most likely resemble strains previously isolated from humans, increased sequencing data would serve to strengthen a link between the positive samples and the source of their strains.

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Appendix A – NCBI GenBank Accession Numbers Used for Designing qRT-PCR Primers and Probes

Table A.1 Accession numbers of GenBank sequences used for development of qPCR primers and probes.

Genetic Fragment	GenBank Accession Numbers	
L1	DQ664184.1	EF494435.1
	KC462149.1	KJ676379.1
	KF154724.1	JN799426.1
	GQ468266.1	DQ997719.1
	KF791261.1	AF368033.1
	M31058.1	JX486057.1
	M31057.1	GU991669.1
	M24734.1	GU991659.1
	HM159613.1	JQ412755.1
	KM820754.1	JQ599140.1
	KM820744.1	GU196306.1
	GU589577.1	JX204738.1
	NC_013225.1	KP208814.1
	NC_004282.4	KP208804.1
	KM087105.1	JX415466.1
L2	DQ664185.1	EF494436.1
	KC462150.1	KJ676380.1
	KF154725.1	JN799427.1
	GQ468267.1	DQ885990.2
	AF378010.1	J03488.1
	AF378009.1	HQ642770.1
	AF378008.1	JX486058.1
	AF378007.1	GU991670.1
	AF378006.1	GU991660.1
	AF378005.1	JQ599139.1
	AF378004.1	JQ412756.1
	AF378003.1	GU196307.1
	HM159614.1	JX204739.1
	GU589578.1	KP208815.1
	NC_013226.1	KP208805.1
	KM820755.1	JX415467.1
	KM820745.1	JQ599141.1
	KM087106.1	JQ599140.1
	NC_004275.1	JQ599138.1
L3	DQ664186.1	AF129822.1
	KC462151.1	AF129821.1
	KF154726.1	AF129820.1
	EF029088.1	JX486059.1
	GQ468270.1	GU991671.1
	HM159615.1	GU991661.1
	KM820756.1	JQ412757.1

	KM820746.1	JQ599141.1
	GU589579.1	GU196308.1
	NC_013229.1	KJ806993.1
	KM087107.1	JX204740.1
	EF494437.1	KP208816.1
	NC_004274.1	KP208806.1
	KJ676381.1	JX415472.1
	JN799428.1	
M1	DQ664187.1	AY428877.1
	KC462152.1	AY428876.1
	KF154727.1	AY428875.1
	AY551083.1	AY428874.1
	GQ468268.1	AY428873.1
	AF461684.1	AY428872.1
	AF461683.1	AY428871.1
	AF461682.1	AY428870.1
	AF124519.1	NC_004281.1
	HM159616.1	M27261.1
	KM820757.1	JX486060.1
	KM820747.1	GU991672.1
	GU589580.1	GU991662.1
	NC_013227.1	JQ412758.1
	KM087108.1	GU196309.1
	EF494438.1	M27262.1
	NC_004280.1	JX204741.1
	KJ676382.1	KP208817.1
	JN799423.1	KP208807.1
	DQ396804.2	JX415468.1
	AY428878.1	
M2	U24260.1	M19355.1
	DQ664188.1	AF368034.1
	KC462153.1	M19408.1
	KF154728.1	M19407.1
	GQ468269.1	M19345.1
	DQ482462.1	HQ642773.1
	AF490617.1	JX486061.1
	HM159617.1	GU991663.2
	GU589581.1	GU991673.1
	NC_013228.1	JQ412759.1
	KM820758.1	GU196310.1
	KM820748.1	M20161.1
	KM087109.1	JX204742.1
	EF494439.1	KP208818.1
	NC_004278.1	KP208808.1
	KJ676383.1	JX415471.1
	JN799424.1	

M3	DQ664189.1	AF174383.1
	KC462154.1	AF174382.1
	KF154729.1	NC_004280.1
	GQ468271.1	M27262.1
	HM159618.1	JX486062.1
	KM820759.1	GU991674.1
	KM820749.1	GU991664.1
	GU589582.1	JQ412760.1
	NC_013230.1	GU196314.1
	KM087110.1	AF174384.1
	EF494440.1	M27261.1
	NC_004281.1	JX204743.1
	KJ676384.1	KP208819.1
	JN799425.1	KP208809.1
	DQ403254.1	JX415474.1
S1	KC462155.1	KJ676385.1
	KF154730.1	DQ312301.1
	GQ468272.1	JN799419.1
	JF829222.1	AY302467.1
	JF829221.1	DQ911244.1
	JF829220.1	EF494445.1
	JF829219.1	AF368035.1
	JF829218.1	EU049607.1
	JF829217.1	EU049606.1
	JF829216.1	EU049605.1
	JF829215.1	EU049604.1
	JF829214.1	EU049603.1
	JF829213.1	AY862138.1
	U74293.1	AY862137.1
	U74292.1	AY862136.1
	M35964.1	AY862135.1
	M35963.1	AY862134.1
	HM159619.1	AY862133.1
	KM820750.1	DQ220017.1
	GU589583.1	M10261.1
	NC_013231.1	M10260.1
	JQ979285.1	M14779.1
	JQ979284.1	M10262.1
	JQ979283.1	JX486063.1
	JQ979282.1	KM820760.1
	JQ979281.1	GU991675.1
	JQ979280.1	GU991665.1
	JQ979279.1	NC_004277.1
	JQ979278.1	JQ599138.1
	JQ979277.1	JQ412761.1
	JQ979276.1	GU196315.1

	JQ979275.1	KJ806994.1
	JQ979274.1	JX204737.1
	JQ979273.1	KP208820.1
	JQ979272.1	KP208810.1
	JQ979271.1	JX415469.1
	KF013857.1	JQ599141.1
	KF013855.1	JQ599140.1
	KM087111.1	JQ599139.1
	EF494441.1	
S2	DQ664190.1	KJ676386.1
	KC462156.1	JN799420.1
	KF154731.1	DQ396805.1
	GQ468273.1	AF368036.1
	L19776.1	M17598.1
	L19775.1	DQ220020.1
	L19774.1	JX486064.1
	HM159620.1	GU991676.1
	KM820761.1	GU991666.1
	KM820751.1	JQ412762.1
	GU589584.1	GU196311.1
	NC_013232.1	JX204744.1
	KM087112.1	KP208821.1
	EF494442.1	KP208811.1
	NC_004279.1	JX415465.1
S3	DQ664191.1	DQ411553.1
	KC462157.1	DQ220018.1
	KF154732.1	M18390.1
	GQ468274.1	M18389.1
	HM159621.1	M14325.1
	KM820762.1	JX486065.1
	KM820752.1	GU991677.1
	GU589585.1	GU991667.1
	NC_013233.1	JQ412763.1
	KF013858.1	GU196312.1
	KF013856.1	AF076293.1
	KM087113.1	JX204745.1
	EF494443.1	KP208822.1
	NC_004283.1	KP208812.1
	KJ676387.1	JX415470.1
	JN799421.1	
S4	KC462158.1	DQ396806.1
	KF154733.1	AF368037.1
	GQ468275.1	DQ220019.1
	HM159622.1	M13139.1
	KM820763.1	JX486066.1
	KM820753.1	GU991678.1

	GU589586.1	GU991668.1
	NC_013234.1	JQ412764.1
	KM087114.1	GU196313.1
	EF494444.1	K02739.1
	NC_004276.1	JX204746.1
	KJ676388.1	KP208823.1
	JN799422.1	KP208813.1
	DQ318037.2	JX415473.1

Appendix B – Spectrophotometry Readings and Calculations for Production of Molecular Standards for REOM1 and REOC1 qPCR Primer and Probe Sets

The conventional PCR fragments that were designed for use as molecular standard were amplified, electrophoresed on 2% agarose gels, extracted, and purified. Each purified PCR product was then analyzed using a spectrophotometer to identify the concentration of DNA present in the sample.

REOM1 Molecular Standard (REOX1 Conventional PCR Product)

The REOX1 conventional PCR fragment is an expected 375 base pairs in length and is produced by the REOX1F1 forward PCR primer and the REOX1R1 reverse PCR primer.

Table B.1 Spectrophotometry readings of purified conventional REOX1 PCR product used for producing molecular standard for the REOM1 qPCR primer and probe set. The PCR fragment was analyzed 10 times and the average reading was calculated.

Sample	Reading (ng/µL)
1	8.74
2	6.57
3	6.33
4	6.95
5	6.24
6	6.48
7	6.47
8	7.01
9	7.33
10	7.25
Mean	6.94

Molecular Weight (MW) = 660 Da/base pair x Length of Fragment
= (660 Da/base pair)(375 base pairs)
= 2.48×10^{5} Da

1 Da ≈ 1.65×10^{-24} grams ≈ 1.65×10^{-18} µg

Molecular Weight of REOX1 Fragment = $[(1.65 \times 10^{-18}\ \mu g)(2.48 \times 10^{5}\ Da)]/1\ Da$
= 4.09×10^{-13} µg
= 4.09×10^{-10} ng

Therefore, one copy of the REOX1 amplicon has a mass of approximately 4.09 x 10^{-10} ng or, 4.09 ng of REOX1 PCR amplicon contains approximately 10^{10} copies of REOX1.

Number of REOX1 Copies/µL = Mean Concentration x 10^{10} Copies / 4.09 ng
= (6.94 ng/µL x 10^{10} Copies)/4.09 ng
= 1.70 x 10^{10} copies/µL

According to the calculations, a 10^{-10} dilution of purified REOX1 conventional PCR product should yield a concentration of approximately 1 copy of REOX1 amplicon per µL.

REOC1 Molecular Standard (REOL3 Conventional PCR Product)

The REOL3 conventional PCR primer set was used to create a PCR amplicon for use as a molecular standard for the REOC1 qPCR primer and probe set. The REOL3 conventional PCR amplicon is 320 base pairs in length.

Table B.2 Spectrophotometry readings of purified conventional REOL3 PCR product used for producing molecular standard for the REOC1 qPCR primer and probe set. The PCR fragment was analyzed 10 times and the average reading was calculated.

Sample	Reading (ng/µL)
1	3.49
2	4.20
3	4.28
4	4.26
5	3.20
6	3.73
7	3.64
8	4.39
9	5.11
10	4.76
Mean	4.11

Molecular Weight (MW) = 660 Da/base pair x Length of Fragment
= (660 Da/base pair)(320 base pairs)
= 2.11 x 10^{5} Da

1 Da ≈ 1.65×10^{-24} grams ≈ 1.65×10^{-18} μg

Molecular Weight of REOC1 Fragment = $[(1.65 \times 10^{-18}\ \mu g)(2.11 \times 10^{5}\ Da)]/1\ Da$
$= 3.48 \times 10^{-13}\ \mu g$
$= 3.48 \times 10^{-10}$ ng

Therefore, one copy of the REOC1 amplicon has a mass of approximately 3.48×10^{-10} ng or, 3.48 ng of REOC1 PCR amplicon contains approximately 10^{10} copies of REOC1.

Number of REOC1 Copies/μL = Mean Concentration x 10^{10} Copies / 3.48 ng
$= (4.11\ ng/\mu L \times 10^{10}\ Copies)/3.48\ ng$
$= 1.18 \times 10^{10}$ copies/μL

According to the calculations, a 10^{-10} dilution of purified REOX1 conventional PCR product should yield a concentration of approximately 1 copy of REOX1 amplicon per μL.

Appendix C. Complete qPCR and Culture Results for Environmental Surface Water Samples.

Table C.1 Complete list of every environmental surface water sample collected throughout the study and the corresponding cytopathogenic effect (CPE) results after culturing on MA-104 media; "negative" corresponds to two consecutive negative CPE results, "-" denotes a complete lack of CPE in the first passage, "+" corresponds to some observed CPE, "++" denotes moderately prevalent CPE throughout the culture, and "+++" denotes complete and widespread CPE.

Sample Site – Sampling Location	Date	Sample Name	CPE (1^{st}/2^{nd} Passage)
Epcor - Site 1	25-Jun-12	SW-EL-WTPB-25	Negative
	23-Jul-12	SW-EL-WTPB-26	Negative
	30-Aug-12	SW-EL-WTPB-27	Negative
	23-Sep-12	SW-EL-WTPB-28	Negative
	28-Oct-12	SW-EL-WTPB-29	Negative
	23-Nov-12	SW-EL-WTPB-30	Negative
	17-Dec-12	SW-EL-WTPB-31	Negative
	23-Jan-13	SW-EL-WTPB-32	Negative
	26-Feb-13	SW-EL-WTPB-33	Negative
	20-Mar-13	SW-EL-WTPB-34	Negative
	16-Apr-13	SW-EL-WTPB-35	-/++
	21-May-13	SW-EL-WTPB-36	Negative
Epcor – Site 2	25-Jun-12	SW-R-WTP-25	Negative
	23-Jul-12	SW-R-WTP-26	Negative
	30-Aug-12	SW-R-WTP-27	Negative
	23-Sep-12	SW-R-WTP-28	Negative
	28-Oct-12	SW-R-WTP-29	-/±
	23-Nov-12	SW-R-WTP-30	Negative
	17-Dec-12	SW-R-WTP-31	-/++
	23-Jan-13	SW-R-WTP-32	-/+
	26-Feb-13	SW-R-WTP-33	Negative
	20-Mar-13	SW-R-WTP-34	Negative
	16-Apr-13	SW-R-WTP-35	Negative
	21-May-13	SW-R-WTP-36	Negative
Oldman River - Brocket	28-Jun-12	1484191	Negative
	25-Jul-12	1787828	Negative
	23-Aug-12	1787861	Negative
	19-Sep-12	R1923271	Negative
	18-Oct-12	R1923301	Negative
	8-Nov-12	R1923311	Negative
	12-Dec-12	R1923368	Negative
	17-Jan-13	R1923341	Negative
	6-Feb-13	R1923361	Negative
	5-Mar-13	R1923334	Negative

	17-Apr-13	R2012639	Negative
	27-May-13	R2012666	Negative
Oldman River – Highway 3 Bridge	28-Jun-12	1484195	Negative
	25-Jul-12	1787829	Negative
	23-Aug-12	1787865	Negative
	19-Sep-12	R8800058	Negative
	18-Oct-12	R1923305	Negative
	8-Nov-12	R1923317	Negative
	12-Dec-12	R1923372	Negative
	17-Jan-13	R1923342	Negative
	6-Feb-13	R1923384	Negative
	5-Mar-13	R1923355	Negative
	17-Apr-13	R2012643	Negative
	27-May-13	R2012673	Negative
Oldman River – Highway 36 Bridge	28-Jun-12	1484200	Negative
	25-Jul-12	1787830	Negative
	23-Aug-12	1787870	Negative
	19-Sep-12	R1923280	Negative
	18-Oct-12	R1923270	Negative
	1-Nov-12	R1923379-2	Negative
	12-Dec-12	R1923375	Negative
	17-Jan-13	R1923343	-/++
	6-Feb-13	R1923387	Negative
	20-Mar-13	R1962540	Negative
	17-Apr-13	R2012646	Negative
	27-May-13	R2012676	Negative
Milk River – Highway 880 Bridge	1481138	28-Jun-12	Negative
	1787531	25-Jul-12	Negative
	1787872	25-Aug-12	Negative
	R8825830	19-Sep-12	Negative
	R1787884	18-Oct-12	Negative
	R1923378	8-Nov-12	Negative
	R1923374	12-Dec-12	Negative
	R1923323	17-Jan-13	Negative
	R1923386	6-Feb-13	Negative
	R1923357	20-Mar-13	Negative
	R2012647	17-Apr-13	Negative
	R2012665	9-May-13	Negative
Bow River – Carseland Weir	1923213	26-Jun-12	Negative
	1923233	1-Jul-12	Negative
	1923264	30-Aug-12	Negative

	R1351292	3-Sep-12	Negative
	R1351318	24-Oct-12	Negative
	R1962553	27-Nov-12	Negative
	R1962558	17-Dec-12	Negative
	R2012612	24-Jan-13	Negative
	R1962570	20-Feb-13	Negative
	R1962527	19-Mar-13	Negative
	R2012627	13-Apr-13	Negative
	R2012665	13-May-13	Negative
Bow River - Cluny	1923214	26-Jun-12	Negative
	1923234	1-Jul-12	Negative
	1923263	30-Aug-12	Negative
	R1351291	3-Sep-12	Negative
	R1351319	23-Oct-12	Negative
	R1962552	27-Nov-12	Negative
	R1962557	18-Dec-12	Negative
	R2012613	24-Jan-13	Negative
	R1962571	20-Feb-13	Negative
	R1962541	19-Mar-13	Negative
	R2012628	13-Apr-13	Negative
	R2012659	13-May-13	Negative
Bow River - Ronalane	1923202	18-Jun-12	Negative
	1923237	19-Jul-12	Negative
	8800057	28-Aug-12	Negative
	R1351290	3-Sep-12	Negative
	R1351320	23-Oct-12	Negative
	1962551	28-Nov-12	Negative
	R1962556	18-Dec-12	Negative
	R2012614	24-Jan-13	Negative
	R1962572	20-Feb-13	Negative
	R1962542	15-Mar-13	Negative
	R2012629	13-Apr-13	Negative
	R2012660	13-May-13	Negative
Bow River - Cochrane	1923211	25-Jun-12	Negative
	1923235	31-Jul-12	Negative
	1923262	30-Aug-12	Negative
	R1351294	20-Sep-12	Negative
	R1351316	17-Oct-12	Negative
	R1962555	14-Nov-12	Negative
	R1962569	17-Dec-12	Negative
	R2012610	22-Jan-13	Negative
	R1923391	21-Feb-13	Negative
	R1962528	12-Mar-13	Negative
	R2012625	13-Apr-13	Negative

	R2012656	13-May-13	Negative
Medicine Hat – South Saskatchewan River	1923204	18-Jun-12	Negative
	1923238	19-Jul-12	Negative
	8807876	28-Aug-12	Negative
	R1351289	27-Sep-12	Negative
	R1351321	18-Oct-12	Negative
	R1351322	27-Nov-12	Negative
	R1962559	18-Dec-12	Negative
	R2012609	28-Jan-13	Negative
	R1962573	20-Feb-13	Negative
	R1962543	15-Mar-13	Negative
	R2012630	17-Apr-13	Negative
	R2012661	9-May-13	Negative
Red Deer River – Nevis Bridge	AB05CD0250	18-Jun-12	Negative
	1850993	18-Jul-12	Negative
	1787878	14-Aug-12	Negative
	R8800060	19-Sep-12	Negative
	R1351295	22-Oct-12	Negative
	R1962577	16-Nov-12	-/+++
	R1962583	4-Dec-12	Negative
	R1923383	22-Jan-13	Negative
	R1923400	20-Feb-13	Negative
	R1923394	19-Mar-13	Negative
	R2012634	13-Apr-13	Negative
	R2012652	13-May-13	Negative
Red Deer River – Highway 2 Bridge	AB05CC0010	18-Jun-12	Negative
	1850992	18-Jul-12	Negative
	1787879	14-Aug-12	Negative
	R8818028	19-Sep-12	Negative
	R1351302	22-Oct-12	Negative
	R1962581	19-Nov-12	±/+++
	12SWCB1201	10-Dec-12	-/+++
	R1923382	22-Jan-13	Negative
	R1923351	20-Feb-13	Negative
	R1923393	19-Mar-13	Negative
	R2012635	13-Apr-13	-/++
	R2012651	13-May-13	Negative
Wapiti – Highway 40	AB07GE0020	14-Jun-12	Negative
	AB07GE0020	16-Jul-12	Negative
	AB07GE0020	20-Aug-12	Negative

	AB07GE0020	25-Sep-12	Negative
	AB07GE0020	17-Oct-12	Negative
	AB07GE0020	13-Nov-12	Negative
	AB07GE0020	11-Dec-12	Negative
	AB07GE0020	14-Jan-13	Negative
	AB07GE0020	12-Feb-13	Negative
	AB07GE0020	11-Mar-13	Negative
	AB07GE0020	13-Apr-13	-/+++
	AB07GE0020	13-May-13	Negative
Wapiti – Smoky River	AB07GJ0030	14-Jun-12	Negative
	AB07GJ0030	16-Jul-12	-/+
	AB07GJ0030	20-Aug-12	Negative
	AB07GJ0030	25-Sep-12	Negative
	AB07GJ0030	17-Oct-12	Negative
	AB07GJ0030	13-Nov-12	Negative
	AB07GJ0030	11-Dec-12	-/++
	AB07GJ0030	14-Jan-13	Negative
	AB07GJ0030	12-Feb-13	Negative
	AB07GJ0030	11-Mar-13	Negative
	AB07GJ0030	13-Apr-13	-/+
	AB07GJ0030	13-May-13	Negative
North Saskatchewan River – Clear Water River	AB05DC0050	7-Jun-12	Negative
	AB05DC0050	5-Jul-12	Negative
	AB05DC0050	2-Aug-12	Negative
	AB05DC0050	6-Sep-12	Negative
	AB05DC0050	11-Oct-12	Negative
	AB05DC0050	1-Nov-12	Negative
	AB05DC0050	5-Dec-12	Negative
	AB05DC0050	3-Jan-13	Negative
	AB05DC0050	7-Feb-13	Negative
	AB05DC0050	7-Mar-13	Negative
	AB05DC0050	13-Apr-13	Negative
	AB05DC0050	13-May-13	Negative
North Saskatchewan River - Devon	AB05DF0010	11-Jun-12	Negative
	AB05DF0010	8-Jul-12	Negative
	AB05DF0010	7-Aug-12	Negative
	AB05DF0010	10-Sep-12	Negative
	AB05DF0010	9-Oct-12	Negative
	AB05DF0010	5-Nov-12	Negative
	AB05DF0010	10-Dec-12	Negative

	AB05DF0010	7-Jan-13	Negative
	AB05DF0010	11-Feb-13	Negative
	AB05DF0010	11-Mar-13	Negative
	AB05DF0010	13-Apr-13	Negative
	AB05DF0010	13-May-13	Negative
North Saskatchewan River - Pakan	AB05EC0010	14-Jun-12	Negative
	AB05EC0010	10-Jul-12	Negative
	AB05EC0010	9-Aug-12	Negative
	AB05EC0010	13-Sep-12	Negative
	AB05EC0010	11-Oct-12	Negative
	AB05EC0010	14-Nov-12	Negative
	AB05EC0010	13-Dec-12	-/+++
	AB05EC0010	10-Jan-13	Negative
	AB05EC0010	14-Feb-13	Negative
	AB05EC0010	14-Mar-13	-/+
	AB05EC0010	13-Apr-13	Negative
	AB05EC0010	13-May-13	Negative
		Total CPE Positives	15

Table C.2 Complete list of all environmental surface water samples collected from across Alberta and their corresponding pre-culture and post-culture qPCR results after screening with the REOM1 and REOC1 qPCR primer and probe sets.

			Pre-Culture qPCR		**Post-Culture qPCR**	
Sample Site – Sampling Location	Date	Sample Name	REOM1	REOC1	REOM1	REOC1
Epcor - Site 1	25-Jun-12	SW-EL-WTPB-25	Negative	Negative	Negative	Negative
	23-Jul-12	SW-EL-WTPB-26	Negative	Negative	Negative	Negative
	30-Aug-12	SW-EL-WTPB-27	Negative	Negative	Negative	Negative
	23-Sep-12	SW-EL-WTPB-28	Negative	Negative	Negative	Negative
	28-Oct-12	SW-EL-WTPB-29	Negative	Negative	Negative	Negative
	23-Nov-12	SW-EL-WTPB-30	Negative	Negative	Negative	Negative
	17-Dec-12	SW-EL-WTPB-31	Negative	Negative	Negative	Negative
	23-Jan-13	SW-EL-WTPB-32	Negative	Negative	Negative	Negative

	26-Feb-13	SW-EL-WTPB-33	Negative	Negative	Negative	Negative
	20-Mar-13	SW-EL-WTPB-34	Negative	Negative	Negative	Negative
	16-Apr-13	SW-EL-WTPB-35	Negative	Negative	38.22	Negative
	21-May-13	SW-EL-WTPB-36	Negative	Negative	Negative	Negative
Epcor – Site 2	25-Jun-12	SW-R-WTP-25	Negative	Negative	Negative	Negative
	23-Jul-12	SW-R-WTP-26	Negative	Negative	Negative	Negative
	30-Aug-12	SW-R-WTP-27	Negative	Negative	Negative	Negative
	23-Sep-12	SW-R-WTP-28	Negative	Negative	Negative	Negative
	28-Oct-12	SW-R-WTP-29	Negative	Negative	Negative	Negative
	23-Nov-12	SW-R-WTP-30	Negative	Negative	Negative	Negative
	17-Dec-12	SW-R-WTP-31	Negative	Negative	26.04	Negative
	23-Jan-13	SW-R-WTP-32	Negative	Negative	Negative	Negative
	26-Feb-13	SW-R-WTP-33	Negative	Negative	Negative	Negative
	20-Mar-13	SW-R-WTP-34	Negative	Negative	Negative	Negative
	16-Apr-13	SW-R-WTP-35	Negative	38.96	32.91	Negative
	21-May-13	SW-R-WTP-36	Negative	Negative	Negative	Negative
Oldman River - Brocket	28-Jun-12	1484191	Negative	Negative	Negative	Negative
	25-Jul-12	1787828	Negative	Negative	Negative	Negative
	23-Aug-12	1787861	Negative	Negative	Negative	Negative
	19-Sep-12	R1923271	Negative	Negative	Negative	Negative
	18-Oct-12	R1923301	Negative	Negative	Negative	Negative
	8-Nov-12	R1923311	Negative	Negative	Negative	Negative
	12-Dec-12	R1923368	Negative	Negative	Negative	Negative

	17-Jan-13	R1923341	Negative	Negative	Negative	Negative
	6-Feb-13	R1923361	Negative	Negative	Negative	Negative
	5-Mar-13	R1923334	Negative	Negative	Negative	Negative
	17-Apr-13	R2012639	Negative	Negative	Negative	Negative
	27-May-13	R2012666	Negative	Negative	Negative	Negative
Oldman River – Highway 3 Bridge	28-Jun-12	1484195	Negative	Negative	Negative	Negative
	25-Jul-12	1787829	Negative	Negative	Negative	Negative
	23-Aug-12	1787865	Negative	Negative	Negative	Negative
	19-Sep-12	R8800058	Negative	Negative	Negative	Negative
	18-Oct-12	R1923305	Negative	Negative	Negative	Negative
	8-Nov-12	R1923317	Negative	Negative	Negative	Negative
	12-Dec-12	R1923372	Negative	Negative	Negative	Negative
	17-Jan-13	R1923342	Negative	Negative	39.12	Negative
	6-Feb-13	R1923384	Negative	Negative	41.4	40.49
	5-Mar-13	R1923355	Negative	Negative	Negative	Negative
	17-Apr-13	R2012643	Negative	Negative	Negative	Negative
	27-May-13	R2012673	Negative	Negative	Negative	Negative
Oldman River – Highway 36 Bridge	28-Jun-12	1484200	Negative	Negative	Negative	Negative
	25-Jul-12	1787830	Negative	Negative	Negative	Negative
	23-Aug-12	1787870	Negative	Negative	Negative	Negative
	19-Sep-12	R1923280	Negative	Negative	Negative	Negative
	18-Oct-12	R1923270	Negative	Negative	Negative	Negative
	1-Nov-12	R1923379-2	Negative	Negative	Negative	Negative

	12-Dec-12	R1923375	Negative	Negative	Negative	Negative
	17-Jan-13	R1923343	Negative	Negative	Negative	Negative
	6-Feb-13	R1923387	Negative	Negative	Negative	Negative
	20-Mar-13	R1962540	Negative	Negative	Negative	Negative
	17-Apr-13	R2012646	Negative	Negative	Negative	Negative
	27-May-13	R2012676	Negative	Negative	Negative	Negative
Milk River – Highway 880 Bridge	1481138	28-Jun-12	Negative	Negative	Negative	Negative
	1787531	25-Jul-12	Negative	Negative	Negative	Negative
	1787872	25-Aug-12	Negative	Negative	Negative	Negative
	R8825830	19-Sep-12	Negative	Negative	Negative	Negative
	R1787884	18-Oct-12	Negative	Negative	Negative	Negative
	R1923378	8-Nov-12	Negative	Negative	Negative	Negative
	R1923374	12-Dec-12	Negative	Negative	Negative	Negative
	R1923323	17-Jan-13	Negative	Negative	Negative	Negative
	R1923386	6-Feb-13	Negative	Negative	Negative	Negative
	R1923357	20-Mar-13	Negative	Negative	Negative	Negative
	R2012647	17-Apr-13	Negative	Negative	Negative	Negative
	R2012665	9-May-13	Negative	Negative	Negative	Negative
Bow River – Carseland Weir	1923213	26-Jun-12	Negative	Negative	Negative	Negative
	1923233	1-Jul-12	Negative	Negative	Negative	Negative
	1923264	30-Aug-12	Negative	Negative	Negative	Negative
	R1351292	3-Sep-12	Negative	Negative	Negative	Negative
	R1351318	24-Oct-12	Negative	Negative	Negative	Negative

	R1962553	27-Nov-12	Negative	Negative	Negative	Negative
	R1962558	17-Dec-12	Negative	Negative	Negative	Negative
	R2012612	24-Jan-13	Negative	Negative	Negative	Negative
	R1962570	20-Feb-13	Negative	Negative	Negative	Negative
	R1962527	19-Mar-13	Negative	Negative	Negative	Negative
	R2012627	13-Apr-13	Negative	Negative	Negative	Negative
	R2012665	13-May-13	Negative	Negative	Negative	Negative
Bow River - Cluny	1923214	26-Jun-12	Negative	Negative	Negative	Negative
	1923234	1-Jul-12	Negative	Negative	Negative	Negative
	1923263	30-Aug-12	Negative	Negative	Negative	Negative
	R1351291	3-Sep-12	Negative	Negative	Negative	Negative
	R1351319	23-Oct-12	Negative	Negative	Negative	Negative
	R1962552	27-Nov-12	Negative	Negative	Negative	Negative
	R1962557	18-Dec-12	Negative	Negative	Negative	Negative
	R2012613	24-Jan-13	Negative	Negative	Negative	Negative
	R1962571	20-Feb-13	Negative	Negative	Negative	Negative
	R1962541	19-Mar-13	Negative	Negative	Negative	Negative
	R2012628	13-Apr-13	Negative	Negative	Negative	Negative
	R2012659	13-May-13	Negative	Negative	Negative	Negative
Bow River - Ronalane	1923202	18-Jun-12	Negative	Negative	Negative	Negative
	1923237	19-Jul-12	Negative	Negative	Negative	Negative
	8800057	28-Aug-12	Negative	Negative	Negative	Negative
	R1351290	3-Sep-12	Negative	Negative	Negative	Negative

	R1351320	23-Oct-12	Negative	Negative	Negative	Negative
	1962551	28-Nov-12	Negative	Negative	Negative	Negative
	R1962556	18-Dec-12	Negative	Negative	Negative	Negative
	R2012614	24-Jan-13	Negative	Negative	Negative	Negative
	R1962572	20-Feb-13	Negative	Negative	Negative	Negative
	R1962542	15-Mar-13	Negative	Negative	Negative	Negative
	R2012629	13-Apr-13	Negative	Negative	Negative	Negative
	R2012660	13-May-13	Negative	Negative	Negative	Negative
Bow River - Cochrane	1923211	25-Jun-12	Negative	Negative	Negative	Negative
	1923235	31-Jul-12	Negative	Negative	Negative	Negative
	1923262	30-Aug-12	Negative	Negative	Negative	Negative
	R1351294	20-Sep-12	Negative	Negative	Negative	Negative
	R1351316	17-Oct-12	Negative	Negative	Negative	Negative
	R1962555	14-Nov-12	Negative	Negative	Negative	Negative
	R1962569	17-Dec-12	Negative	Negative	Negative	Negative
	R2012610	22-Jan-13	Negative	Negative	Negative	Negative
	R1923391	21-Feb-13	Negative	Negative	Negative	Negative
	R1962528	12-Mar-13	Negative	Negative	Negative	Negative
	R2012625	13-Apr-13	Negative	Negative	Negative	Negative
	R2012656	13-May-13	Negative	Negative	Negative	Negative
Medicine Hat – South Saskatchewan River	1923204	18-Jun-12	Negative	Negative	Negative	Negative
	1923238	19-Jul-12	Negative	Negative	Negative	Negative
	8807876	28-Aug-12	Negative	Negative	Negative	Negative

	R1351289	27-Sep-12	Negative	Negative	Negative	Negative
	R1351321	18-Oct-12	Negative	Negative	Negative	Negative
	R1351322	27-Nov-12	Negative	Negative	34.79	39.53
	R1962559	18-Dec-12	Negative	Negative	Negative	Negative
	R2012609	28-Jan-13	Negative	Negative	Negative	Negative
	R1962573	20-Feb-13	Negative	Negative	Negative	Negative
	R1962543	15-Mar-13	Negative	Negative	Negative	Negative
	R2012630	17-Apr-13	Negative	Negative	Negative	Negative
	R2012661	9-May-13	Negative	Negative	Negative	Negative
Red Deer River – Nevis Bridge	AB05CD0250	18-Jun-12	Negative	Negative	Negative	Negative
	1850993	18-Jul-12	Negative	Negative	Negative	Negative
	1787878	14-Aug-12	Negative	Negative	Negative	Negative
	R8800060	19-Sep-12	Negative	Negative	Negative	Negative
	R1351295	22-Oct-12	Negative	Negative	Negative	Negative
	R1962577	16-Nov-12	Negative	Negative	Negative	Negative
	R1962583	4-Dec-12	Negative	Negative	Negative	Negative
	R1923383	22-Jan-13	Negative	Negative	Negative	Negative
	R1923400	20-Feb-13	Negative	Negative	Negative	Negative
	R1923394	19-Mar-13	Negative	Negative	Negative	Negative
	R2012634	13-Apr-13	Negative	40.24	38.87	38.42
	R2012652	13-May-13	Negative	Negative	Negative	Negative
Red Deer River – Highway 2 Bridge	AB05CC0010	18-Jun-12	Negative	Negative	Negative	Negative
	1850992	18-Jul-12	Negative	Negative	Negative	Negative

	1787879	14-Aug-12	Negative	Negative	Negative	Negative
	R8818028	19-Sep-12	Negative	Negative	Negative	Negative
	R1351302	22-Oct-12	Negative	Negative	Negative	Negative
	R1962581	19-Nov-12	Negative	41.98	Negative	28.55
	12SWCB1201	10-Dec-12	Negative	Negative	Negative	19.09
	R1923382	22-Jan-13	Negative	Negative	Negative	Negative
	R1923351	20-Feb-13	Negative	Negative	Negative	Negative
	R1923393	19-Mar-13	Negative	Negative	Negative	Negative
	R2012635	13-Apr-13	Negative	39.54	39.47	37.5
	R2012651	13-May-13	Negative	Negative	Negative	Negative
Wapiti – Highway 40	AB07GE0020	14-Jun-12	Negative	Negative	Negative	Negative
	AB07GE0020	16-Jul-12	Negative	Negative	Negative	Negative
	AB07GE0020	20-Aug-12	Negative	Negative	Negative	Negative
	AB07GE0020	25-Sep-12	Negative	Negative	Negative	Negative
	AB07GE0020	17-Oct-12	Negative	Negative	Negative	Negative
	AB07GE0020	13-Nov-12	Negative	Negative	Negative	Negative
	AB07GE0020	11-Dec-12	Negative	Negative	Negative	Negative
	AB07GE0020	14-Jan-13	Negative	Negative	Negative	Negative
	AB07GE0020	12-Feb-13	Negative	Negative	Negative	Negative
	AB07GE0020	11-Mar-13	Negative	Negative	Negative	Negative
	AB07GE0020	13-Apr-13	Negative	Negative	15.24	37.98
	AB07GE0020	13-May-13	Negative	Negative	40.98	40.11
Wapiti – Smoky River	AB07GJ0030	14-Jun-12	Negative	Negative	Negative	Negative

	AB07GJ0030	16-Jul-12	Negative	Negative	Negative	Negative
	AB07GJ0030	20-Aug-12	Negative	Negative	Negative	Negative
	AB07GJ0030	25-Sep-12	Negative	Negative	Negative	Negative
	AB07GJ0030	17-Oct-12	Negative	Negative	Negative	Negative
	AB07GJ0030	13-Nov-12	Negative	Negative	Negative	Negative
	AB07GJ0030	11-Dec-12	Negative	Negative	35.59	24.1
	AB07GJ0030	14-Jan-13	Negative	Negative	Negative	Negative
	AB07GJ0030	12-Feb-13	Negative	Negative	Negative	Negative
	AB07GJ0030	11-Mar-13	Negative	Negative	Negative	Negative
	AB07GJ0030	13-Apr-13	Negative	Negative	18.96	36.39
	AB07GJ0030	13-May-13	Negative	Negative	Negative	Negative
North Saskatchewan River – Clear Water River	AB05DC0050	7-Jun-12	Negative	Negative	Negative	Negative
	AB05DC0050	5-Jul-12	Negative	Negative	Negative	Negative
	AB05DC0050	2-Aug-12	Negative	Negative	Negative	Negative
	AB05DC0050	6-Sep-12	Negative	Negative	Negative	Negative
	AB05DC0050	11-Oct-12	Negative	Negative	Negative	Negative
	AB05DC0050	1-Nov-12	Negative	Negative	Negative	Negative
	AB05DC0050	5-Dec-12	Negative	Negative	Negative	Negative
	AB05DC0050	3-Jan-13	Negative	Negative	Negative	Negative
	AB05DC0050	7-Feb-13	Negative	Negative	Negative	Negative
	AB05DC0050	7-Mar-13	Negative	Negative	Negative	Negative
	AB05DC0050	13-Apr-13	Negative	Negative	Negative	Negative
	AB05DC0050	13-May-13	Negative	Negative	Negative	Negative

North Saskatchewan River - Devon	AB05DF0010	11-Jun-12	Negative	Negative	Negative	Negative
	AB05DF0010	8-Jul-12	Negative	Negative	Negative	Negative
	AB05DF0010	7-Aug-12	Negative	Negative	Negative	Negative
	AB05DF0010	10-Sep-12	Negative	Negative	Negative	Negative
	AB05DF0010	9-Oct-12	Negative	Negative	Negative	Negative
	AB05DF0010	5-Nov-12	Negative	Negative	Negative	Negative
	AB05DF0010	10-Dec-12	Negative	Negative	Negative	Negative
	AB05DF0010	7-Jan-13	Negative	Negative	40.71	21.84
	AB05DF0010	11-Feb-13	Negative	Negative	Negative	Negative
	AB05DF0010	11-Mar-13	Negative	Negative	Negative	Negative
	AB05DF0010	13-Apr-13	Negative	Negative	39.44	39.32
	AB05DF0010	13-May-13	Negative	Negative	41.9	39.32
North Saskatchewan River - Pakan	AB05EC0010	14-Jun-12	Negative	Negative	Negative	Negative
	AB05EC0010	10-Jul-12	Negative	Negative	Negative	Negative
	AB05EC0010	9-Aug-12	Negative	Negative	Negative	Negative
	AB05EC0010	13-Sep-12	Negative	Negative	Negative	Negative
	AB05EC0010	11-Oct-12	Negative	Negative	Negative	Negative
	AB05EC0010	14-Nov-12	Negative	Negative	Negative	Negative
	AB05EC0010	13-Dec-12	Negative	Negative	16.61	38.44
	AB05EC0010	10-Jan-13	Negative	Negative	Negative	Negative
	AB05EC0010	14-Feb-13	Negative	Negative	Negative	Negative
	AB05EC0010	14-Mar-13	Negative	Negative	24.77	Negative
	AB05EC0010	13-Apr-13	Negative	Negative	31.9	40.13

	AB05EC0010	13-May-13	Negative	Negative	Negative	Negative
		Total Positives*	0	4	18	15

*The total positives are representative of the total number of samples that tested positive for mammalian orthoreovirus by each respective qPCR primer and probe set; since some samples yielded a positive result by both primer/probe sets, the actual number of total positives are lower. In reality, there were seven total pre-culture qPCR positives and 20 post-culture qPCR positives.

Appendix D. Encoded Representatives, Environmental Surface Water, and Wastewater Sequences for S4 Genetic Fragment Alignments and Sequencing

Table D.1 Encoded reference sequences for tree-building.

Accession Number	Code (Type or Strain/Source/Country/Year)
HM159622.1	T3/H/Ca/10
KJ676388.1	SC/Bo/US/14
X61586.1	T1/H/US/05
KF154733.1	S1/HB/Sl/13
AF368037.1	T4/Mu/Fr/01
DQ318037.2	T2/U/Ch/07
X60066.1	T2/H/US/05
JX415473.1	SHRA/Po/Ch/12
DQ220019.1	T2W/H/Ca/06
GU589586.1	T3A/H/Ca/10
DQ396806.1	SCA/Sw/Ch/06
WWPE1	WW1/10
WWPE3	WW3/10
WWPE6	WW6/10
WWPE7	WW7/10
WWPE8	WW8/11
WWPE9	WW9/11
WWPE12	WW12/11
WWPE16	WW16/11
WWPE2	WW2/10
WWPE14	WW14/11
WWPE10	WW10/11
WWPE4	WW4/10
WWPE5	WW5/10
WWPE15	WW15/11
AB05EC0010 March	SW/MrEC/13
SW-EL-WTPB-35	SW/E35/13
AB07GE0020 April	SW/ApGE/13
AB05DF0010 January	SW/JaDF/13
AB05EC0010 December	SW/DeEC/12
AB07GJ0030 April	SW/ApGJ/13
AB05DF0010 April	SW/ApDF/13
R1923384	SW/384/13
AB05EC0010 April	SW/ApEC/13
12SWCB1201	SW/12S/12