The assessment of sewage treatment efficacy through the detection and characterisation of enteroviruses

by

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DECLARATION

I, Maxime Muilwijk, declare that this work was not copied or repeated from any other studies either from national or international publications. Procedures were carried out in accordance with the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

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THE ASSESSMENT OF SEWAGE TREATMENT EFFICACY THROUGH THE DETECTION AND CHARACTERISATION OF ENTEROVIRUSES

BY

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SUMMARY

Enteroviruses (EVs) are small RNA viruses that have been responsible for outbreaks of hand, foot and mouth disease, viral meningitis, encephalitis and paralytic poliomyelitis. Water may act as a reservoir for waterborne EVs that are faecally shed by infected populations and subsequently introduced to water sources. The inefficient removal of EVs with wastewater treatment and its discharge into natural water sources may pose as a potential health concern if utilised by the public for domestic, agricultural or recreational purposes. An environmental surveillance (ES) complements the symptomatic surveillance of EVs since EV infections are generally asymptomatic and can, therefore, be detected in sewage as a representative of the infected population. By combining direct reverse transcription – polymerase chain reaction (RT-PCR) and

integrated cell culture-RT-PCR (ICC-RT-PCR) detection and characterisation it provides a broader epidemiological overview of the current EV diversity in South Africa and the survival of specific EVs after wastewater treatment. The aim of this project was to determine the viral removal efficiency of six selected wastewater treatment plants by detecting and characterising EVs in wastewater, wastewater discharge and surface water, with molecular and viral isolation methods. Between April 2015 and March 2016, 156 water samples were collected and viruses were recoverd. The recovered viral suspensions were inoculated onto Buffalo green monkey kidney cells (BGM), L20B murine cells and Primary liver carcinoma cells (PLC/PRF/5). Enteroviruses were detected in 69.2% of the recovered viral suspensions from the water samples with direct RT-nested PCR (RT-nPCR), of which 45.4%, 43.5% and 11.1% were from wastewater, wastewater discharge and surface water, respectively. With ICC-real-time RT-PCR, 52.6% of the harvested cell culture extracts were positive for EV, of which 56.1%, 32.9% and 11% were from wastewater, wastewater discharge and surface water, respectively. The most diverse species were the EV-B species that predominated in cell culture, with echovirus 6, coxsackievirus (CV)-B3 and CV-A13 being most predominantly identified in wastewater discharge after viral amplification in cell culture. The most prevalent species were the EV-C species and predominated in the recovered viral suspensions, with EV-C99, CV-A20 and CV-A22 being most predominantly identified in the wastewater discharge with direct RT-nPCR characterisation. The majority of EVs and EV types were isolated from the PLC/PRF/5 cell line. A combination of viral isolation and molecular detection of EVs enhanced the detection of EVs in water sources and provided a wider epidemiological overview of EVs that are shown to be more resilient to treatment and environmental exposure. To conclude, it is evident that wastewater treatment systems do not efficiently remove EVs, specifically potentially viable EVs, which are subsequently discharged into natural water sources. If natural water sources become a reservoir for a wide diversity of EVs that may be potentially infectious to humans, it might raise a public health concern.

PRESENTATIONS AND PUBLICATIONS

Publications

Muilwijk M, van Zyl WB, Taylor MB. Occurrence and genetic diversity of enteroviruses in wastewater and wastewater effluent using a combination of direct and integrated cell culture RT-PCR (In preparation).

Local presentations

Muilwijk M, van Zyl, WB**,** Taylor MB. The assessment of sewage treatment efficacy through the detection and characterisation of human enteroviruses [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria, South Africa. 18–19 August 2015.

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Muilwijk M, van Zyl, WB, Taylor MB. Molecular epidemiology of human enteroviruses detected from surface and wastewater sources [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria, South Africa. 23-24 August 2016.

National presentations

Muilwijk M, van Zyl WB, Taylor MB. Integrated cell culture and direct RT-PCR detection and molecular characterization of human enteroviruses in wastewater treatment plants in the Vaal catchment area [Presentation]. WISA 2016 Water - The ultimate constraint. Biennial Conference and Exhibition of the Water Institute of Southern Africa. International Conference Centre, Durban, South Africa. 15-19 May 2016.

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Muilwijk M, van Zyl WB, Taylor MB. The detection and characterisation of enteroviruses in wastewater discharge and surface water in Gauteng, South Africa [Poster]. 18th Symposium of Health-Related Water Microbiology, Lisbon, Portugal. 13–18 September 2015.

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ABBREVIATIONS AND SYMBOLS

Abbreviation/Symbol	Word/Phrase
µl	Microlitre
µm	Micrometer
ß	Beta
A549	Human lung epithelial cell line
aa	Amino acid
AFP	Acute flaccid paralysis
BGM	Buffalo green monkey kidney cell line
BLAST	Basic Local Alignment Sequence Tool
Caco-2	Human colorectal adenocarcinoma cell line
cfu	Colony forming units
CNS	Central nervous system
CPE	Cytopathic effect
cPCR	Colony polymerase chain reaction
CSF	Cerebrospinal fluid
CV	Coxsackievirus
cVDPV	Circulating vaccine-derived poliovirus
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
E	Echovirus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E-MEM	Eagle's minimum essential medium
ES	Environmental surveillance
EtBr	Ethidium bromide
EtOH	Ethanol
EV	Enterovirus
g	Gram
GPEI	Global polio eradication initiative
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HeLa	Human cervical cancer cell line
Hep-2	Laryngeal carcinoma cell line
HEV	Human enterovirus
HFMD	Hand, foot and mouth disease
ICC-RT-PCR	Integrated cell culture reverse transcription - polymerase chain reaction
ICC-rtRT-PCR	Integrated cell culture real-time reverse transcription - polymerase chain reaction
IPV	Inactivated polio vaccine
kb	Kilobases
kDa	Kilodalton
L	Litre

L20B	Murine cell line
LLC-MK2	Rhesus monkey kidney cell line
m	Meter
M	Molar
MCF-7	Human mammary gland adenocarcinoma cell line
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimeter
mM	Millimolar
NaAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHP	Non-human primates
nm	Nanometer
nM	Nanomolar
NPEV	Non-polio enterovirus
nt	Nucleotide
OPV	Oral poliovirus vaccine
ORF	Open reading frame
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PLC/PRF/5	Primary liver carcinoma cell line
PMA	Propidium monoazide
PV	Poliovirus
PVK	Primary vervet monkey kidney cell line
qrtRT-PCR	Quantitative real-time reverse transcription - polymerase chain reaction
RD	Rabdomyosarcoma cell line
rtRT-PCR	Real-time reverse transcription - polymerase chain reaction
RT-nPCR	Reverse transcription - nested polymerase chain reaction
RT-PCR	Reverse transcription - polymerase chain reaction
s	Seconds
TCID	Tissue culture infective dose
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
VAPP	Vaccine-associated poliovirus
VDPV	Vaccine-derived poliovirus
Vero	African green monkey cell line
VIRADEL	Viral adsorption-elution filtration
VP	Viral protein
VPg	Viral protein, genome linked
WHO	World Health Organization
WTP	Water treatment plant
WPV	Wild poliovirus
WWTP	Wastewater treatment plant

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Environmental water sources may serve as a natural reservoir for numerous resilient human pathogenic viruses (Rajtar *et al*., 2008). Enteroviruses (EVs) transmit via the faecal-oral route and when inadequately removed/inactivated during wastewater treatment, they may be distributed to natural water sources when effluent is discharged (Okoh *et al*., 2010). Faecally-polluted water has been frequently associated with global waterborne disease outbreaks, of which human EVs (HEVs) are responsible for 23% (Sinclair *et al*., 2009). Human EV infections can range from being asymptomatic or a mild febrile illness to acute haemorrhagic conjunctivitis, hand, foot and mouth disease (HFMD), acute flaccid paralysis (AFP), aseptic meningitis and encephalitis, myocarditis and type 1 diabetes (Nix *et al*., 2006; La Rosa *et al*., 2012). Enteroviruses have an infectious dose ranging between 10 – 100 viral particles and exhibit a high stability in the environment, circulating through their transmission in fomites, food, aerosols, soil, water and sewage (Rodríguez-Lázaro *et al*., 2012; Gibson, 2014). Since symptomatic HEV infection surveillances present inadequate data due to the asymptomatic nature of EVs, an environmental surveillance (ES) of wastewater and other water bodies can provide an epidemiological overview of HEVs circulating within a population (Conell *et al*., 2012; Manor *et al*., 2014; Adeniji and Faleye, 2014). An ES is able to determine the origin of an either former or current outbreak and has been added to the Global Polio Eradication Initiative (GPEI) strategic plan of 2013-2018 to complement the disease-based AFP surveillance in assisting with the identification of residual poliovirus (PV) transmission (Asghar *et al*., 2014; Manor *et al*., 2014). Enteroviruses are more challenging to remove during wastewater treatment than bacterial indicators due to their high resistance to chlorination, filtration and lipid solvents (Fong and Lipp, 2005). This raises a public health concern as the insufficient removal of HEVs in wastewater treatment plants (WWTPs) may lead to its subsequent distribution in a reusable water source (Kitajima *et al*., 2014).

1.2. GENERAL VIROLOGY OF ENTEROVIRUSES

1.2.1. Classification

The *Picornaviridae* family belongs to the order *Picornavirales* and currently consists of 50 species within 29 genera that are typically small single-stranded positive sense RNA viruses (Zhou *et al*., 2016). The genus *Enterovirus* is one of the 29 genera belonging to the *Picornaviridae* family and currently consists of 12 species of which four are HEVs, namely EV-A to D (Knowles *et al*., 2012) (Table 1.1). Other members of the *Enterovirus* genus are the human rhinoviruses A-C and the animal EV-E, F, G, H and J, which include the simian, porcine and bovine EVs (Knowles *et al*., 2012).

Table 1.1. Enterovirus serotypes classified within the human enterovirus species (Knowles *et al*., 2012).

Human enterovirus species	Enterovirus serotypes
EV-A	CV-A2 to A8, CV-A10, CV-A12, CV-A14, CV-A16, EV-A71, EV-A76, EV-A89, EV-A90 to A92, EV-A114, EV-A119, EV-A120, EV-A121, simian EV (SV) 19, SV43, SV46, baboon EV-BA13
EV-B	CV-B1 to B6, CV-A9, E-1, E-2 to E-7, E-9, E-11 to E-21, E-24 to E-27, E-29, E-30 to E-33, EV-B69, EV-B73 to B75, EV-B77 to B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 to B113, SA5
EV-C	PV-1 to 3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19 to A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116 to C118
EV-D	EV-D68, EV-D70, EV-D94, EV-D111, EV-D120

*CV = Coxsackievirus; E = Echovirus; EV = Enterovirus.

Enterovirus classification was traditionally based on physical properties and serological relatedness and classified into four groups based on the type of cytopathic effect (CPE) in cell culture and the clinical manifestation in animal models (Tapparel *et al*., 2013; Lugo and Krogstad, 2016). It included the PVs (poliomyelitis in human and non-human primates [NHP]), Coxsackievirus (CV)-As (herpengina, AFP and other central nervous system [CNS] diseases in suckling mice), CV-Bs (CNS and cardiac disease; spastic paralysis in mice), and the echoviruses (Es) (non-pathogenic in mice) (Lugo and

Krogstad, 2016). However, due to their genetic variability, classification has become more nucleotide (nt) sequence-based (Khetsuriani *et al*., 2010; Tapparel *et al*., 2013). The RNA-dependent RNA polymerase essential for RNA synthesis is error-prone and generates a mutation frequency of 10^{-3} to 10^{-5} per nt (Tapparel *et al*., 2013). Enteroviruses possess recombination mechanisms, such as template switching or "replicative recombination", which allows EVs to recombine with other circulating EVs (Tapparel *et al*., 2013; Dimitriou *et al*., 2014). This type of recombination ensures the stability and variability of the EV genome and contributes to the wide diversity of EVs discovered (Tapparel *et al*., 2013). There are currently more than 110 distinct genotypes known to infect humans (Lugo and Krogstad, 2016). Enterovirus genotypes are classified into EV-A to D according to their genome organisation, sequence similarity and biological properties (Xu *et al*., 2013). Enterovirus serotypes are classified into EV-A to D based on the viral protein (VP), VP1, which is the most immunodominant capsid protein and characterises the EVs based on serotype-specific neutralisation epitopes (Tryfonos *et al*., 2011; Adenij and Faleye, 2015). The correlation between the VP1 sequences and neutralisation epitopes allows for optimal typing of EV serotypes (Blomqvist *et al*., 2008; Adeniji and Faleye, 2015). Enteroviruses can furthermore be targeted at the highly conserved 5'-untranslated region (UTR) of the VP4 region, which is generally used for epidemiological research and clinical aspects (Ayukekbong *et al*., 2013).

The genetic classification of EVs is based on the structural region's sequence divergence, rather than the non-structural region due to recurrent recombination between related viruses (Norder *et al*., 2011). Enterovirus types are classified according to the degree of similarities in nt (>75%) and amino acids (aa) (>88%) in the VP1 region (Smura *et al*., 2014). Strains are classified into different types if the nt and aa similarity is less than 70% and 85%, respectively, where divergent strains with a nt and aa similarity between 70-75% and 85-88% may occur in the 'grey-zone' of EV typing (Smura *et al*., 2014). This event can be regularly observed with some CV-A24 and EV-C99 strains (Smura *et al*., 2014). When the Sabin oral polio vaccine (OPV) strains exhibit more than 1% nt divergence from the original OPV-1 and OPV-3 strains and 0.6% nt divergence from the original OPV-2 strains they are classified as vaccine-derived PVs (VDPVs) (Burns *et al*., 2014).

1.2.2. Morphology

The EV virion has a non-enveloped spherical capsid of 30 nanometer (nm) in diameter enclosing a positive-sense single-stranded RNA genome (Racaniello, 2013). The protein capsid consists of protomers of 80-97 kilodalton (kDa) with 60 copies each of the three external proteins VP1-3 and the internal VP4 (Racaniello, 2013). The structural proteins (VP1-3) are three intertwined surface proteins of 24-41 kDa located on the exterior of the capsid. The internal structural protein, VP4, of 5.5-13.5 kDa is located in the inner capsid surface (Knowles *et al*., 2012). The structural proteins VP1 to 3 do not share any sequence homology, yet they share a similar topology of a wedge-shaped structure constructed from two eight-stranded, anti-parallel ß-barrels (Racaniello, 2013). The ß-barrels are topologically located on the outer surface of the capsid as jellyroll barrels, forming prominent structural "mesa", "canyon" and "propeller" features on the exterior surface of the viral particle (Figure 1.1) (Lin *et al*., 2011).

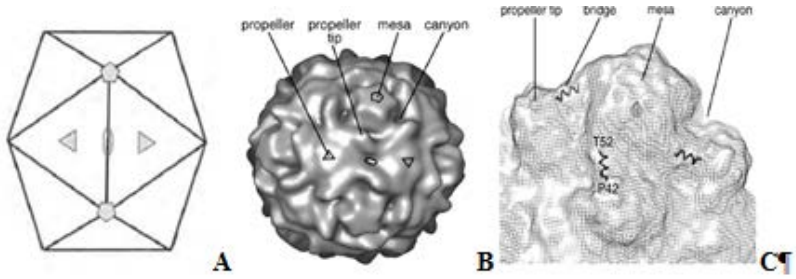


Figure 1.1. Structural features of the external surface of an enterovirus particle. **(A)** Outline of an icosahedral symmetry displaying the fivefold (pentagon), threefold (triangles) and twofold (oval) axes. **(B)** Exterior structural features of the 160S poliovirus particle. **(C)** Cryo-electromagenetic reconstruction of exterior structural features of the 135S poliovirus particle (Lin et al., 2011).

The structural star-shaped "mesa" feature centred on a five-fold symmetry axis is exclusively synthesized by VP1, while VP1, VP2 and VP3 all form the "canyon" and "propeller" features (Lin *et al*., 2011). The canyons represent deep clefts (2 nm deep) in the icosahedron that are around all 12 vertices and comprise the viral attachment sites (Carter and Sanders, 2007). The ß-barrel domain platform is strengthened by a protein-protein network on the interior of the capsid, especially around the fivefold axis and is essential for the stability of the virion (Racaniello, 2013). The N- and C-terminal aa

sequences within the loops extending from the ß-barrel domain are responsible for the distinctive morphology and antigenic characteristics of EVs (Racaniello, 2013).

1.2.3. Viral replication and protein synthesis

The viral protein capsid encloses one molecule of positive-sense single-stranded RNA of 7-8.8 kilobases (kb), comprising a 7500 nt genome (Knowles *et al*., 2012). The RNA genome encodes a single polyprotein in an open reading frame (ORF) that controls the synthesis of a single, large viral polyprotein of 6627 nt (2200 aa), flanked by an extensive 5'-UTR and a shorter 3'UTR with a poly (A) tail (Knowles *et al*., 2012; Lowry *et al*., 2014) (Figure 1.2).

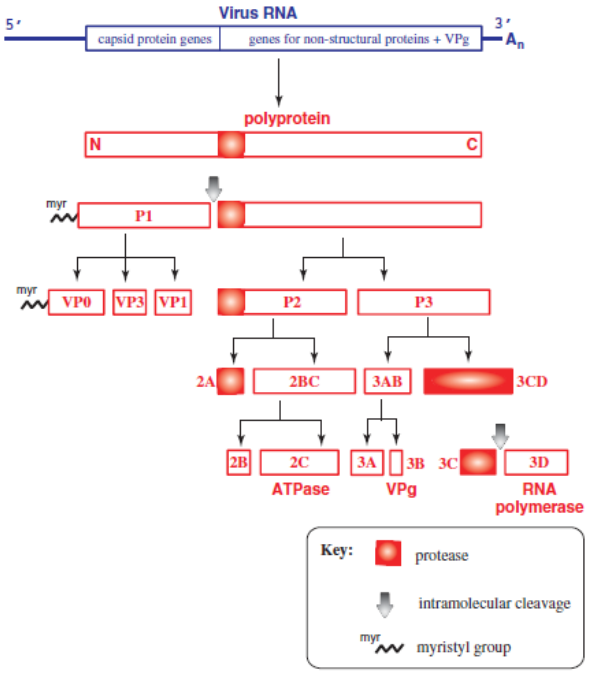


Figure 1.2. Simplified diagram of the viral RNA encoded for a single polyprotein and its precursor proteins. The diagram shows the viral RNA genome as a single large polyprotein with its three domains P1, P2 and P3 that generates the functional polypeptide precursors (Carter and Saunders, 2007).

The poly (A) tail length is vital for the viability of the virus and determines the magnitude of RNA replication and mRNA translation (Kempf *et al*., 2013). The 5'-UTR of 742 nts is highly structured and constitutes of three fundamentals, namely the *cis*-acting structural elements, a 5'-terminal domain necessary for replication and an internal ribosomal entry site to initiate and set forth translation of the viral genome

(Racaniello *et al*., 2013; Tapparel *et al*., 2013). The 5'-end has a covalently linked VPg (viral protein, genome linked) unique to picornavirus RNA and is removed from the virion RNA in order to set forth translation (Racaniello *et al*., 2013). The single polypeptide is proteolytically cleaved by the viral proteases $2A^{pro}$ and $3C^{pro}/3CD^{pro}$, yielding the structural domain (P1) and the non-structural domains (P2, P3) (Jurgens *et al*., 2006). Each intermediate codes for different functional precursors and polypeptides, with P1 yielding the four mature viral capsid proteins (VP1-4) while P2 and P3 gene regions encode for the non-structural proteins (Reuter *et al*., 2012).

The non-structural proteins include the proteases $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$ that are functional for protein processing and the 2B, 2C, 3AB, $3B^{vpg}$, $3CD^{pro}$ and $3D^{pol}$ proteins that are necessary for genome replication (Reuter *et al*., 2012). The cleavage efficiency differs for the various proteins and proteins are subsequently synthesised in unequal quantities (Carter and Saunders, 2007). The viral polymerase, $3D^{pol}$, initiates the transcription of the positive-sense RNA strand into a negative-sense form that subsequently functions as a transcription template for the generation of progeny positive-sense RNA strands (Jurgens *et al*., 2006; Carter and Saunders, 2007). These positive-sense RNA strands can now function as templates for negative strand RNA synthesis, mRNA or as genomes of progeny virions (Jurgens *et al*., 2006; Reuter *et al*., 2012). During negative strand RNA synthesis, a recombination mechanism called "template switching" occurs (Lowry *et al*., 2014). Human EVs therefore have a significant genetic variability due to their viral $3D^{pol}$ that lacks the essential proofreading activities during genomic replication and consequently result in high mutation rates (Tryfonos *et al*., 2011).

1.2.4. Biochemical and biophysical properties

Enteroviruses are insensitive to ether, chloroform and non-ionic detergents and show relative resistance to some common laboratory disinfectants that include 70% ethanol (EtOH), isopropanol, dilute lysol and quaternary ammonium compounds (Knowles *et al*., 2012; Pallansch *et al*., 2013). Enteroviruses are inactivated by formaldehyde, glutaraldehyde, sodium hypochlorite and free residual chlorine (Pallansch *et al*., 2013). Two milligram (mg) per one free chlorine acts on both the genome and the proteins and have shown an inactivation of more than four logs after a viral infectivity assay of

$TCID_{50}$ (tissue culture infective dose which will infect 50% of the cell monolayers) (Leifels *et al*., 2015). Ultraviolet (UV) irradiation is another inactivation treatment against EVs, which has been mainly implemented on decontaminating surfaces (Pallansch *et al*., 2013). Their acid stability is variable, as it can tolerate weak acids at pH 3.0 while it may be inactivated by strong acids (pH 0 to 1), such as hydrochloric acids that completely ionise in solution (Knowles *et al*., 2012; Pallansch *et al*., 2013). Enteroviruses can be inactivated at 42˚C but can be stabilised at 50˚C when sulhydryl reducing agents and magnesium cations are present (Pallansch *et al*., 2013).

1.3. CLINICAL VIROLOGY OF ENTEROVIRUSES

1.3.1. Pathogenesis

Enterovirus attachment and entry into host cells involve the recognition of certain cell-surface receptors by virus attachment proteins, most commonly the CD155 receptor (PV receptor), three integrins (α2ß1, αvß3 and αvß6), decay-accelerating factor (CD55), CV-adenovirus receptor and intracellular adhesion molecule 1 (Solomon *et al*., 2010; Wang and Liu, 2014). The virus-receptor attachment catalyses a conformational change to the cell that allows viral RNA to move into the cytoplasm and is subsequently translated into one large polypeptide (Solomon *et al*., 2010; Strauss *et al*., 2015).

The epithelial cells of the respiratory and gastrointestinal tract as well as the small intestine's lymphoid follicles are the primary sites of EV infection, where replication may occur for weeks to months, creating the possibility of subsequent circulation into the bloodstream (Hovi *et al*., 2007; Pallansch and Oberste, 2009; Igarashi *et al*., 2010). It first reaches a primary viraemic phase, which may contribute to the development of secondary phase organ-specific symptoms (Hovi *et al*., 2007). Another phenomenon recently observed is the association between HEVs and type 1 diabetes. It has been suggested that, since the risk gene for type 1 diabetes, *IFIH1*, is an innate immunesystem receptor for HEVs, certain HEVs contain diabetogenic properties when infecting the pancreas and intestinal mucosa (Tauriainen *et al*., 2011).

1.3.2. Clinical syndromes

Human EVs are responsible for a wide diversity of clinical manifestations ranging from asymptomatic or mild symptoms of fever, upper respiratory tract infection to aseptic meningitis and encephalitis, haemorrhagic conjunctivitis, myocarditis, paralytic poliomyelitis, HFMD as well as type 1 diabetes (Wurtzer *et al*., 2014; Meuhlenbachs *et al*., 2015). The incubation time ranges between 2 and 35 days before the first signs and symptoms occur (Pallansch and Oberste, 2009).

Hand, foot and mouth disease is one of the illnesses primarily caused by the EV-A species: EV-71, CV-A6 and CV-A16 that causes fluid-filled lesions to develop in the mouth, and on the hands and feet (Lugo and Krogstad, 2016). Enterovirus-infected patients can furthermore develop non-cardiogenic pulmonary edema and brain stem encephalitis that have been associated with a high mortality rate in previous disease outbreaks (Lugo and Krogstad, 2016). Neonatal EV infections are generally caused by EV-B species and may develop myocarditis and meningitis with haemorrhagic symptoms that manifests within three to five days after birth of the infant and has a mortality rate ranging between 30-80% (Fuchs *et al*., 2013; Lugo and Krogstad, 2016). Enteroviruses have also been associated with severe respiratory infections, especially in patients with underlying asthma (Horner *et al*., 2015).

Acute flaccid paralysis

Poliomyelitis is an acute illness affecting the lower motor neurons of the spinal cord and brainstem that leads to flaccid, asymmetric paralysis caused by a PV infection (Harvey *et al*., 2013). Before the development of poliomyelitis, a combination of fever, headache, neck and back pain and AFP without sensory loss occurs (Harvey *et al*., 2013). Poliomyelitis develops in an estimated 1% of infected populations and two thirds of the patients that develop poliomyelitis do not recover (Pallansch *et al*., 2012; Harvey *et al*., 2013). Of the 20-30% of patients that do recover fully from poliomyelitis will re-develop muscle weakness, atrophy and fatigue within 25-35 years after the illness, called post-poliomyelitis syndrome (Harvey *et al*., 2013). The majority of individuals infected with PV remain asymptomatic, whilst 4% to 8% of individuals develop symptoms of a mild febrile illness with or without gastroenteritis, also known

as abortive poliomyelitis (Pallansch *et al*., 2012). Infected individuals may present with self-limiting symptoms such as fever, headache and meningeal signs, while non-paralytic CNS infections, such as viral meningitis, occur less frequently (Pallansch *et al*., 2012).

Vaccine-associated paralytic poliomyelitis

Poliovirus genomes evolve at an annual nt substitutions rate of 1% that is suggested to occur more rapidly in the early OPV replication cycle (Diop *et al*., 2015). Continuous replication of the attenuated PV leads to viral alterations such as back mutations, recombination and site suppression mutations that give rise to VDPVs that become highly virulent (Minor, 2009). In an immunocompetent OPV recipient the virus typically accumulates less than five VP1 substitutions in a normal replication cycle (Diop *et al*., 2015). Vaccine-associated paralytic poliomyelitis (VAPP) is the paralytic illness that develops in an estimated 1 in 900 000 children vaccinated with the live attenuated OPV (Burns *et al*., 2014). This illness can occur in immunologically competent vaccine recipients and their contacts, but individuals with primary immunodeficiency disorders, such as B-cell immunodeficiency, are more prone to develop VAPP due to a prolonged infection lasting more than six months (Burns *et al*., 2014; Diop *et al*., 2015). These patients become asymptomatic chronic carriers of the VDPV and are at higher risk of developing VAPP (Minor, 2009).

1.3.3. Laboratory diagnosis

Human EVs can be isolated from various clinical specimens, namely stool, vesicular fluid, cerebrospinal fluid (CSF), nasopharyngeal aspirates, pharyngeal exudates, broncho-alveolar lavages and blood (Ooi *et al*., 2010; Prim *et al*., 2013). The most confirmative diagnosis is made through the testing of specimens from sterile sites, such as vesicular fluid, CSF and serum where it corresponds to the clinical symptoms (Pallansch and Oberste, 2009; Ooi *et al*., 2010).

Viral isolation

The gold standard for HEV diagnosis is viral isolation in cell culture, combining multiple cell lines for optimal isolation, even though molecular techniques are

becoming more preferable (Prim *et al*., 2013). Traditionally used cell lines include the rhabdomyosarcoma cells (RD), laryngeal carcinoma cells (Hep-2), African green monkey epithelial kidney cells (Vero), Buffalo green monkey kidney cells (BGM), human cervical cancer cells (HeLa) and human colorectal adenocarcinoma cells (Caco-2, Skco-1 and HT-29) (Kargar *et al*., 2009). Due to a minimum incubation period of five to 14 days and the high maintenance and supply cost of traditional cell culture, small shell vials were developed where monolayer cells grow at the base of the vial (Mohebi, 2016). The shell vial method with direct immunofluorescence currently remains the most sensitive non-molecular viral detection method, even though the sensitivity and specificity is not as high as with PCR (Garner and Wu, 2014). A confirmative diagnosis is provided with serology or molecular techniques.

Serology

Most serological detection techniques use the detection of IgM antibodies to indicate an acute infection, such as the enzyme-linked immunosorbent assay (ELISA) for EV-specific IgM antibodies (Pallansch *et al*., 2013). Even though ELISA is mainly heterotypic instead of serotype-specific, identifying a generic EV infection, it is widely used as an alternative to neutralisation tests (Pallansch *et al*., 2013). With a neutralising antibody test, an EV diagnosis can be made with an antibody titer comparison between acute and convalescent phase serum specimens, by incubating the antisera-antigen mixture at 36°C for one hour to allow proper binding (Kargar *et al*., 2009; Pallansch *et al*., 2013). Direct or indirect immunofluorescence may also be used to identify specific EV serotypes, namely CV-B (1-6), PV (1-3), EV-70,-71 and E-4, 6, 9, 11, 30 and 34 (Fuchs *et al*., 2013). This makes it an unreliable diagnostic test since antigens/antibodies of limited EV serotypes are being tested for (Fuchs *et al*., 2013).

Molecular techniques

Polymerase chain reaction (PCR) testing is a rapid, more affordable diagnostic approach that requires a low specimen quantity while multiple pathogens can be simultaneously tested for, gradually replacing viral isolation in diagnostic laboratories (Kadambari *et al*., 2014). It has also been found to have a higher sensitivity and specificity for EVs than viral isolation in cell culture, while also delivering a more rapid result within 5-6

hours that reduces hospital stays and unnecessary antimicrobial use (Kadambari *et al*., 2014; Shaker and Abdelhamid, 2015). Another major advantage of reverse transcription-PCR assays (RT-PCR) is that it allows the detection of EVs that cannot be propagated in cell culture (Pallansch *et al*., 2013). It is more common to use primers that amplify the 5'-UTR region of the EV genome, while the VP1 region can also be targeted when focusing on a specific EV serotype since the antigenic properties are in the viral capsid proteins (Pallansch *et al*., 2013). The amplified PCR products can be analysed with agarose gel electrophoresis after conventional RT-PCR or with real-time RT-PCR (rtRT-PCR) (Garner and Wu, 2014). With rtRT-PCR the target amplification and detection occurs simultaneously using fluorescent dyes that emit fluorescent signals, which will generate an amplification plot for each cycle using computer software (Garner and Wu, 2014). The analysis of nucleic acid amplification is reduced since post-PCR processing steps are removed with rtRT-PCR (Garner and Wu, 2014).

1.3.4. Infection prevention and control

Standard precautions for the containment of transmissible infectious EVs are through the control of blood and other body fluids and management by the aid of gloves, environmental surface disinfection, laundry handling guidelines and safe injection principles (Guzman-Cottrill *et al*., 2013). Enterovirus infections can be prevented through handwashing with soap or other detergents after close contact with infected individuals or their objects (Chang *et al*., 2013). It is furthermore recommended to frequently disinfect exposed surfaces (Centres for Disease Control and Prevention [CDC], 2015). The components of a currently available alcohol-based disinfectant in hospitals are 70% isopropanol and 0.5% chlorhexidine gluconate that exhibit high antimicrobial activity against bacteria, fungi and enveloped viruses (Chang *et al*., 2013). However, it is lacking a broadspectrum, virucidal effect against non-enveloped viruses (Chang *et al*., 2013). Even though 70% isopropanol and 0.5% chlorhexidine gluconate does reduce viral titres of non-enveloped rotavirus and calicivirus, it exhibits no antiviral activity against several EVs, including EV-71, PV and CV-B (Chang *et al*., 2013). Ethanol at 95% concentration has been shown to exhibit a virucidal activity against several non-enveloped viruses, including EVs (Chang *et al*., 2013).

Enterovirus-infected individuals who have a deficiency in type-specific antibodies are presented with an increased possibility of developing meningitis, myocarditis and hepatitis with hemorrhagic phenomena (Fuchs *et al*., 2013). Two preventative approaches towards critical non-polio EVs (NPEV) infections in asymptomatic infants or their contacts are the administration of intravenous immunoglobulins and prophylactic intramuscular immune serum globulin (Fuchs *et al*., 2013). No commercial vaccine is yet available to prevent and protect individuals from NPEV infections and only symptomatic treatment is generally necessary as the majority of EV infections are self-limited (Modlin *et al*., 2014). There are, however, two very effective polio vaccines containing all three serotypes, namely the inactivated polio vaccine (IPV) designed by Jonas Salk that is administered via the intramuscular route and the attenuated Sabin live OPV designed by Albert Sabin (Pallansch *et al*., 2013). The trivalent OPV is more beneficial based on its vaccine delivery, lower production costs and providing high mucosal immunity and has therefore been the main tool for the GPEI (Boot *et al*., 2004; Minor, 2009; Diop *et al*., 2015). Nevertheless, low vaccination coverage lead to the interhuman circulation of OPV-derived PVs that underwent viral genetic drift and reversion to neurovirulent PVs, VDPVs, especially in type-B immunodeficient individuals (Razafindratsimandresy *et al*., 2013). Increased VAPP outbreaks of VDPV type 2 (VDPV-2) lead to the globally synchronised replacement of the trivalent OPV with a bivalent OPV containing only Sabin PV-1 and PV-3 that was initiated in April 2016 (Friedrich, 2016). Since EV-71 is the causative agent of severe HFMD with frequent localised epidemics being reported, vaccine development has been implemented but there is no preventative measure currently available (Geoghegan *et al*., 2015). A phase III clinical trial that enrolled ten thousand participants showed that an inactivated whole virus EV-71 vaccine provided significant protection against EV-71-associated HFMD, especially in children between six and 36 months (Wang *et al*., 2014). Another study evaluated its feasibility for co-immunisation with a pentavalent vaccine (PV, *Bordetella pertussis, Haemophilus influenza* type B, diphtheria toxoid and tetanus toxoid) in mice, which demonstrated to be potentially successful for co-immunisation (Wang *et al*., 2014). However, this interaction has not yet been assessed clinically in infants (Wang *et al*., 2014).

Antiviral agents, such as the inhibitor imidazo-pyrazine, primarily target the host-receptor interactions to block EV infections and has been suggested as treatment for EV infections (Tan *et al*., 2014). Imidazo-pyrazine is a potential kinase inhibitor compound that exhibits micromolar activity that was originally directed against hepatitis C virus (HCV) (MacLeod *et al*., 2013). Due to EVs sharing the positive-sense single-stranded RNA genomic characteristic with HCV, EVs have been shown to respond to the imidazo-pyrazine compounds, with the antiviral agent showing a 3-5 fold increase in activity against EVs (MacLeod *et al*., 2013). Other antiviral agents such as highly sulphated suramin and its NF449 analogue can bind to the VP1 protein or disrupt the integrity of the capsid structure of EV-71 (Tan *et al*., 2014). Pleconaril is another viral capsid inhibitor that exhibitis activity against picornaviruses and is undergoing further research to determine whether it is an efficient form of treatment against severe neonatal EV infections (Abzug *et al*., 2015).

1.4. WASTEWATER TREATMENT AND ENTEROVIRUSES

1.4.1. Wastewater treatment systems and enterovirus removal efficiency

The first phase of wastewater treatment is preliminary treatment and involves manual or mechanical operated inlet screening of incoming raw wastewater to remove non-biodegradable floating objects and thus prevents blockage or damage to downstream process equipment (Department of Water Affairs and Forestry, 2002). Anaerobic, conventional and enhanced ponds, and aerated lagoons are one of the simple, low maintenance pond systems used as a primary phase separation method that allows suspended solids to settle at the bottom to be broken down by anaerobic bacteria (Water Research Commission Report No: TT 651/15). Other primary treatment systems are wetlands, settling tanks and flotation (Department of Water Affairs and Forestry, 2002). Biological wastewater treatment is a secondary treatment phase that include suspended growth systems such as activated sludge and attached growth systems such as biofilm trickling filters and rotating biological contractors (Rattier *et al*., 2012). More advanced treatment systems include membrane bioreactors that combines activated sludge treatment with microfiltration or ultrafiltration membranes to separate solids from liquid (Rattier *et al*., 2012). Pathogens and impurities such as chemical oxygen demand, metals and pesticides can be removed using disinfectants such as chlorine gas dosages,

UV irradiation, calcium hypochlorite and a strong oxidising disinfectant such as ozone (The Department of Water Affairs and Forestry, 2002; Zanacic *et al*., 2016). Furthermore, many drinking water treatment plants (WTPs) remove the unpleasant taste, odour and disinfectants by adding activated carbon (Rattier *et al*., 2012).

Wastewater is often not properly processed through correct primary/secondary treatment due to insufficient funds, high treatment costs of conventional treatment systems, poor maintenance and an exceeding capacity of wastewater inflow into the WWTPs (Edokpayi *et al*., 2015). Enteroviruses can be inactivated through sunlight exposure, physico-chemical damage such as heavy metals and oxidising agents as well as enzymes that degrade proteins and nucleic acids (Department of Water Affairs and Forestry, 1996). Sedimentation, adsorption, coagulation and flocculation are several conventional water purification processes that may partially remove but not necessarily inactivate viruses, such as EVs (Department of Water Affairs and Forestry, 2002). Therefore, additional disinfection processes are needed, which include chlorination or through the use of other oxidising agents, and requires cautious process control of the dosage and contact time (Department of Water Affairs and Forestry, 2002).

Effluent from a physico-chemical treatment plant has shown an EV removal rate of 0%, while the removal rate for faecal coliforms was 25% (Payment *et al*., 2001). Enteroviruses have also been detected in membrane reactor effluent, as adsorption to the mixed liquor suspended solids is less efficient for EVs (Miura *et al*., 2015). Research furhtermore suggests that EVs are not removed via sedimentation, but are rather inactivated by sunlight exposure, as there is limited evidence on virus associated with settleable particles (Symonds *et al*., 2014). Co-precipitation into growing aluminum hydroxide is a main viral removal mechanism, where aluminum hydrolyte species were applied to PV-1 and CV-B5 during coagulation (Shirasaki *et al*., 2016). It has furthermore been shown that three-pond treatment systems, facultative ponds and maturation ponds removed culturable EVs with greater efficiency compared to upflow anaerobic sludge blanket reactors with two maturation ponds (Symonds *et al*., 2014).

1.4.2. Wastewater effluent guidelines and indicators of enteroviruses

Water quality is defined as the physical, chemical, biological and aesthetic properties of water that determines its suitability for various water usages and the protection of the health and integrity of aquatic ecosystems (Department of Water and Environmental Affairs, 1998). The South African Water Quality Guidelines policy states that treated effluent is required to be returned to natural water sites for maximum utilization of scarce water resources (Department of Water Affairs and Forestry, 1996). According to the National Water Act (36/1998) it must not exceed 2000 cubic metres of discharge per day with a general faecal coliform limit of 1000 colony forming units (cfu)/100 millilitre (ml).

Faecal contamination in the environment mainly originates from raw and treated sewage matrices that pollute the main source of viral pathogens to downstream surface waters (Hmaïed *et al*., 2016). Several studies have questioned the method of identifying health risks associated with enteric viruses, such as EVs, by monitoring faecal indicator bacteria (Ahmed *et al*., 2015). It has been suggested that coliform bacteria do not necessarily portray the presence of EVs and other pathogenic viruses after disinfection procedures (Ahmed *et al*., 2015; Hmaïed *et al*., 2016). More precise representatives of enteric viruses are the somatic coliphages and the F+ male specific host with the F sex pilus (F-RNA) coliphages as their environmental stability resembles that of human enteric viruses in natural water sources and are also more resistant to chlorine disinfection (Ashbolt *et al*., 2001). Nevertheless, bacteriophages can be shed by both animals and humans, and animal faeces generally contain higher concentrations of coliphages (Ashbolt *et al*., 2001). It has also been found that the quantity of bacteriophages and enteric viruses that are shed by humans do not correlate. It is, therefore, not an absolute model organism for human enteric viruses (Ashbolt *et al*., 2001). Routine virological monitoring for EVs and other enteric viruses is relatively expensive and the majority of South African laboratories do not possess the necessary facilities and expertise (Department of Water Affairs and Forestry, 1996). It has been recommended to only perform virological assays during an enteric virus disease outbreak or any situation of suspicion (Department of Water Affairs and Forestry, 1996).

1.5. ENVIRONMENTAL SURVEILLANCE OF ENTEROVIRUSES

1.5.1 Environmental surveillance

Enteroviruses replicate within the intestinal lymphatic tissue and are continuously shed via the faeces for several weeks, irrespective of whether the infected individual is symptomatic or asymptomatic (Ndiaye *et al*., 2014). There is limited data on the epidemiology of EVs with a symptomatic EV surveillance as the majority of EV infections are asymptomatic (Lu *et al*., 2015). This is especially the case in high-density populations where the clinical AFP surveillance is either ineffective or absent (Asghar *et al*., 2014). An ES is based on faecally shed EVs that are subsequently introduced into wastewater sources, which when sampled can link EVs, especially PVs isolated from unknown individuals, to populations served by the sewage system (Asghar *et al*., 2014). The epidemiological significance of ES is that it is able to elucidate viral distribution trends and variation patterns within an area over a specific period of time (Lu *et al*., 2015). It is an indirect monitoring system that provides information regarding EV circulation and distribution of symptomatic and asymptomatic infections within a community (Aw and Gin, 2010; Ndiaye *et al*., 2014).

Faecal contamination of environmental water sources may occur when EVs are shed and discharged into sewage systems and subsequent natural water sources (Ndiaye *et al*., 2014). Wastewater, river, dam, sea and recreational water matrices can represent as efficient vehicles of viral transmission to humans when EVs are introduced in these water sources (Battistone *et al*., 2014). This includes faecally-contaminated irrigation water that is applied onto vegetables and fruits, posing as a potential threat to the consumers (Battistone *et al*., 2014). It has, therefore, been shown that ES can also contribute to improved wastewater treatment systems as the significance of waterborne diseases increase (Battistone *et al*., 2014).

The GPEI developed a supplementary surveillance system which involves the sampling of sewage wastewater and testing for the presence of wild PVs (WPVs) and NPEVs (Asghar *et al*., 2014). This ES has been included in the GPEI 2013-2018 strategic plan to assist in identifying residual transmission in PV-endemic countries (Asghar *et al*., 2014). Environmental surveillance studies from the GPEI found that WPVs were

detected in the absence of AFP cases during the same time period (Asghar *et al*., 2014). Environmental surveillance in Egypt and India facilitated in documenting their WPV elimination, through monitoring residual WPV transmission, and has also been implemented to monitor VDPVs and importations of WPVs (Asghar *et al*., 2014). Even though the risk of VDPV infection through the environment is low, the public can still utilise the VDPV-contaminated water for domestic, agricultural and recreational purposes and may be directly exposed to these viral pathogens (Pavlov *et al*., 2005). The great concern lies in the silent, continuous circulation of PVs in the environment after the cessation of the PV vaccine in a non-immune population (Pavlov *et al*., 2005).

1.5.2. Laboratory methodology

1.5.2.1. Sampling principles

According to the Guidelines for Environmental Surveillance of Poliovirus Circulation (World Health Organization [WHO], 2003), the grab and trap sampling methods are two principal methods of collecting environmental water samples used for both PV and NPEV analysis purposes. The grab method allows the collection of a 1 litre (L) water sample at a selected sampling site generally during peak hours of household sewage flow and the trap method is where a bag of non-specific absorbing material is hung into a water source stream and taken out after one or more days (WHO, 2003). Sampling is usually done using a sterile labelled glass bottle, where the sample characteristics, such as pH and temperature, are measured at the moment of sampling and then transferred in a 4°C cooler to an analytical laboratory where it is processed (Kargar *et al*., 2009). Due to the low viral load in environmental water matrices it is important that a sample volume of 100 ml - 1 L need to be processed (Ikner *et al*., 2012).

1.5.2.2. Viral recovery

Viral recovery generally involves a two-stage procedure to concentrate the virus to a 10 ml suspension to increase the low detectable viral load (Jones and Sellwood, 2001). General viral concentration techniques include ultrafiltration, adsorption-elution using filters (VIRADEL), membranes, glass wool or glass powder, flocculation, two-phase separation with polymers and monolithic chromatographic columns (Bosch *et al*., 2011). Each recovery technique involves their own advantages and disadvantages and

the viral recovery efficiency may be affected by the physico-chemical water quality such as pH, conductivity, turbidity, presence of particulate matter and organic acids (Bosch *et al*., 2011). No standard method for viral concentration with consistent high viral recovery rates has been established (Jones and Sellwood, 2001; Rutjes *et al*., 2005).

Adsorption-elution filtration

The VIRADEL procedure involves a primary concentration step that uses adsorptive electronegative or electropositive filters to process large volumes of water, which is followed by the virus being eluted from the filters (Rutjes *et al*., 2005; Ikner *et al*., 2012). The most commonly used filter is a microporous filter, such as the electropositive 1 MDS filter, to which the negatively charged viral particle adsorbs and subsequently releases from during elution (Ikner *et al*., 2012). Another more cost-effective alternative is the NanoCeram filters, composed of nanoalumina fiber-coated microglass filaments with an extensive surface area and high isoelectric point (Ikner *et al*., 2011). Oiled sodocalcic positively charged glass wool is another filter that can be used for large volumes of water without pre-conditioning, and is more cost-effective then alternative electropositive filter media (Katayama *et al*., 2002; Ikner *et al*., 2012). Glass wool is packed into stainless steel holders into a desired density, which attributes to its recovery efficiency where the amount of glasswool is proportional to its efficiency (Ikner *et al*., 2012). It has, however, been noted that glass wool allows the passage of organic material that inhibits PCR assays, which has not been observed with glass powder (Ikner *et al*., 2012).

Magnesium chloride is added to the water sample when filtered through a negatively charged membrane, elution is done with sodium hydroxide (NaOH) and further concentrated using centrifugal ultrafiltration or organic flocculation (Rutjes *et al*., 2005; Ahmed *et al*., 2015). Virus recovery efficiency from negatively charged membranes have been found to improve when directly extracting the nucleic acids from the membranes, with no PCR inhibitory effects found after quantitative real-time RT-PCR (qrtRT-PCR) analysis (Ahmed *et al*., 2015). Multivalent salts and acidification is required for virus adsorption when electronegative filters are being used, which complicates large-volume sampling (Ikner *et al*., 2012). It is, therefore, more ideal to

use electropositive filters since no preconditioning is necessary and viruses can be concentrated over a greater pH range (Ikner *et al*., 2012). A novel concentration method has been developed that utilises a pumpless bag-mediated filtration system allowing large environmental water sample volumes of up to 10 L to be collected and filtered on site using ViroCap filters, specific for PVs (Fagnant *et al*., 2014).

The elution solutions that are used to recover the viruses from the filters can be either organic, such as beef extract, or inorganic, such as sodium polyphosphates (Ikner *et al*., 2012). Beef extract is the main elution solution to recover viruses from both electropositive and electronegative filters with a pH ranging between 9.0 and 9.5 (Ikner *et al*., 2012). A non-ionic detergent, Tween 80, is a component that enhances the desorption of the virus from microporous filters and ultrafilters and can be used in combination with chaotropic salts (Ikner *et al*., 2012). Sodium polyphosphates are highly negative, inorganic polyanionic salts that have been used with Tween 80 to elute viruses from hollow-fiber ultrafilters (Ikner *et al*., 2012). The eluate volume depends on the type of filter, where 420 ml is ideal for ultrafilter membranes and NanoCeram filters and up to 1.6 L can be used for 1 MDS filters (Ikner *et al*., 2012).

Ultrafiltration

Soluble ultrafilters of sodium alginate have been previously used to concentrate PVs through size exclusion instead of electrokinetics, but this technique has not been found to be practical for environmental water samples (Ikner *et al*., 2012). The filters were dissolved in sodium citrate and applied to cell culture infectivity assays, but pre-treatment through a membrane filter was required when turbid natural waters needed to be processed (Ikner *et al*., 2012). It is furthermore not cost-effective and not as feasible compared to virus adsorption-elution techniques (Ikner *et al*., 2012).

Ultracentrifugation

Ultracentrifugation at an estimated 100,000 x g pellets the macromolecules and viruses that can be subsequently resuspended in a much smaller volume to concentrate viruses (Ikner *et al*., 2012). When a density gradient, such as sucrose, cesium chloride or glycerol is used it is referred to as isopycnic ultracentrifugation where viral particles are separated according to their size and/or particle density (Ikner *et al*., 2012). It is

generally used as a secondary step for virus concentration, but has a direct recovery efficiency between 66% and 72% in wastewater and recreational waters, respectively (Prata *et al*., 2012). It allows sufficient gravitational force to sediment small viruses and avoids resuspension from a pellet (Lawrence and Stewards, 2010). It does, however, require supplementary stages to remove PCR inhibitors following both primary and secondary precipitation (Lawrence and Steward, 2010).

Two-phase separation

Aqueous polymer two-phase separation was designed to recover viruses from small volumes of water (500 ml), which has also been recommended by the WHO guidelines for environmental surveillance of PV (WHO, 2003; Ikner *et al*., 2012). The water sample is initially centrifuged to separate the EV particles from the solids and the subsequent pellet is stored at 4°C after which viruses are stimulated to move towards an organic solution, such as dextran and polyethylyne glycol (PEG) (Ikner *et al*., 2012). The water/organic solution mixture is shaken vigorously and is left overnight at 4°C in a separation funnel, where the EVs can be collected from the smaller bottom layer and/or at the interphase (WHO, 2003). The initial pellet is added to the concentrate and subsequently treated with chloroform (WHO, 2003).

1.5.2.3. Viral isolation

According to the WHO guidelines for environmental surveillance of PV circulation (WHO, 2003), the L20B murine cell line that is genetically modified with a PV receptor and the RD tumour tissue cells are used for identifying PV and NPEVs (Adeniji and Faleye, 2014). One of the most common cell lines to be used for routine monolayer plaque assays from environmental water samples are the BGM kidney cells (Rutjes *et al*., 2005). Other cell lines available for environmental surveillance of EVs are the rhesus monkey kidney cells (LLC-MK2), human mammary gland adenocarcinoma cells (MCF-7), Hep2, PLC/PRF/5, primary vervet monkey kidney cells (PVK) and human lung epithelial cells (A549) (Grabow *et al*., 1999; Lee *et al*., 2004; Ehlers *et al*., 2005; Amdiouni *et al*., 2012; Adeniji and Faleye, 2014). Shell vial isolation has also been applied in ES studies where it was compared to the traditional viral isolation method,

which revealed a positivity rate of 100% in shell vial culture and 87.5% in traditional viral isolation (Shukla *et al*., 2013).

Plaque-forming tests are time-consuming, not cost-effective and are limited to only a few cultivable EVs (Hmaïed *et al*., 2016). For the detection and enumeration of cultivable EVs the total cultivable virus assay-most-probable-number method can be used, which is based on the expression of CPE in BGM cells (Chapron *et al*., 2000). It is, however, not as efficient since not all EVs produce a CPE (Chapron *et al*., 2000).

1.5.2.4. Viral detection

Molecular techniques such as qrtRT-PCR are highly sensitive and overcome the disadvantages of cell culture techniques (Hmaïed *et al*., 2016). The primers and probe implemented in a rtRT-PCR target the 5'UTR of the EV genome for detection of the genus, while the 3'UTR is targeted for species-specific detection of EVs (Figure 1.3) (Hovi *et al*., 2007; Zhang *et al*., 2014). Real-time RT-PCR can be applied specifically to distinguish between PV serotypes, where the serotype-specific primers are able to amplify a 195 bp sequence that corresponds to the Sabin strain capsid region (Iwai-Itamochi *et al*., 2014).

It has been suggested that a combination of viral isolation in cell culture and rtRT-PCR or RT-semi-nested PCR offers more accuracy and efficiency than the traditional PCR techniques (Wu *et al*., 2013). Viral isolation in cell culture is the gold standard for the determination of viral infectivity, but not all EVs can be propagated in cell culture and there is no single cell line that supports propagation of all cultivable EVs (Hamza *et al*., 2011; Leifels *et al*., 2015). Polymerase chain reaction is preferred over viral isolation in cell culture with regards to its affordability and timesaving manner (Jebri et al., 2014). Nevertheless, PCR assays lack the ability to evaluate the infectivity of the virus and has limited control over the inhibitory substances that interfere with the amplification enzymes (Gerba, 2007; Wurtzer *et al*., 2014).

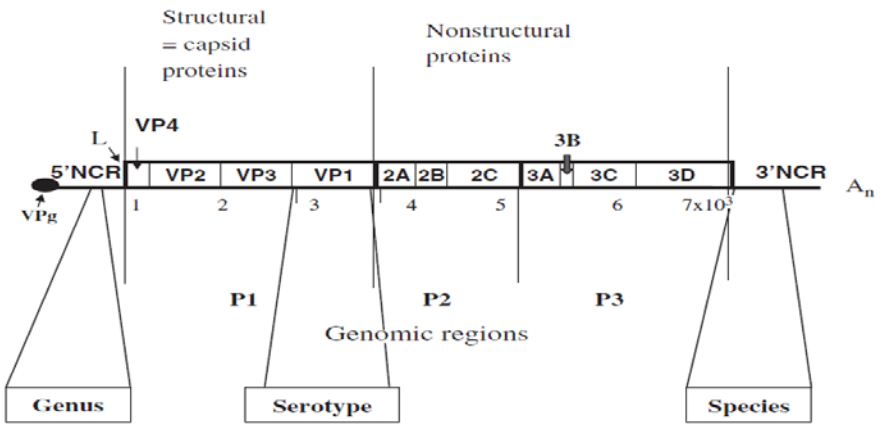


Figure 1.3. Enterovirus genome and its locations for molecular identification of the genus, serotype and species (Hovi *et al*., 2007).

Integrated cell culture RT-PCR (ICC-RT-PCR) is a combination of biological amplification of viral nucleic acid using cell culture techniques and subsequent RT-PCR detection (Hamza *et al*., 2011). This technique overcomes the PCR-based limitation of discriminating between infectious and non-infectious viral particles and allows detection of slow-growing viruses or those that fail to show a CPE (Gensberger and Kostić., 2013; Leifels *et al*., 2015). In addition, inhibitory effects of environmental samples are reduced by passage in cell culture (Gensberger and Kostić., 2013).

Other methods of discriminating between infectious and non-infectious EVs are through the evaluation of viral capsid integrity, which involves pre-treating the water samples using DNA intercalating dyes or nuclease pre-digestion (Wurtzer *et al*., 2014). Propidium monoazide (PMA) is a DNA intercalating dye that can enter viral particles with damaged capsids (Karim *et al*., 2015). A PMA RT-PCR assay has been developed to then distinguish between infectious and non-infectious EVs (Karim *et al*., 2015).

1.5.2.5. Viral characterisation

An initial micro-neutralisation characterisation assay can be done on an isolate, by using PV type-specific rabbit polyclonal antisera as well as pooled horse antisera against Es and CVs that have been most frequently isolated (Zhang *et al*., 2014). In order to characterise the EV genotype, it is amplified by performing an RT-nested PCR (RT-nPCR) where an estimated 888 bp fragment within the VP1 is amplified that functions as a template for the second round which amplifies a 437 bp fragment (Zhang *et al*.,

2014). Nucleotide sequence analysis is done in the forward and reverse directions using an ABI 3730 automatic DNA sequencher, with genotypic identity determined through GenBank Basic Local Alignment Sequence Tool (BLAST) data (Zhang *et al*., 2014). Final confirmation is given through the use of the automated web-based genotyping tool, Enterovirus Typing Tool (Kroneman et al., 2011).

1.6. EPIDEMIOLOGY

1.6.1. General

Enterovirus infections peak in the summer and early autumn months in countries with temperate climates, while no significant seasonality is shown in tropical climates (Lugo and Krogstad, 2016). There is a statistical significance ($p<0.001$) in the difference in average rates between winter and summer, with 1.2 cases per 100,000 being reported during the winter season and 1.9 cases per 100,000 reported during the summer season (Levine *et al*., 2014). In both temperate and tropical climates the EV-B species are generally more prevalent, followed by EV-A species in temperate climates and EV-C species in tropical climates (Apostol *et al*., 2012).

Children, pregnant women, immunocompromised individuals and the elderly are most affected by EV infections where it causes greater morbidity compared to healthy individuals (Gall *et al*., 2015). Children below the age of five are most affected by EV infections, with infants below the age of 12 months being one-third of the cases that are diagnosed (Kadambari *et al*., 2014). Human EVs are generally transmitted via the faecal-oral route either through direct person-to-person transmission or consumption of contaminated food and water (Gerba, 2007; Jebri *et al*., 2014). Direct contact with virus-contained secretions, accidental bloodstream inoculation and vesicular fluid all have the ability to act as vehicles of transmission into the bloodstream, mouth, nose or eyes (Pallansch and Oberste, 2009).

1.6.2. South Africa

Clinical distribution

The majority of clinical EV outbreaks that occur in South Africa involve paediatric patients. Echovirus 4, E-9 and CV-B5 have been previously associated with paediatric

aseptic meningitis cases in Tswane from 2010 to 2011 with E-4 identified in 80% of the cases (Wolfaardt *et al*., 2014). From November 2015 to early 2016, two adults and 40 children below the age of 10 years were reported to have potential aseptic meningitis infection, from which E-6, CV-A9, CV-B2, CV-B4, CV-B5 and E-11 were identified. (National Institute of Communicable Diseases, 2016). Significant South African outbreaks of EVs are shown in Table 1.2.

From 1981 – 1989 there were 11,360 cases of aseptic meningitis in Cape Town of which 3460 cases were of viral aetiology and 91% EVs (Berger, 2016). Major aseptic meningitis outbreaks occurred during the summer season, where E-4 was responsible for two outbreaks, and E-9 and CV-A9 each responsible for one outbreak (Berger, 2016).

Table 1.2. Significant enterovirus outbreaks in South Africa from 1981-2011.

Year	Region	EV Type	Clinical Manifestation	No of cases	Reference
2010–2011	PTA	E-4	Paediatric aseptic meningitis	30	Wolfaardt *et al*., 2014
2001	CPT	E-3	Paediatric Aseptic meningitis	90	Yeats *et al*., 2005
1986-1987	CPT	E-4	Aseptic meningitis	445	McIntyre and Keen, 1993
1985-1986	CPT	CV-A9	Aseptic meningitis	104	McIntyre and Keen, 1993
1984-1986	CPT	E-9	Aseptic meningitis	223	McIntyre and Keen, 1993
1984	JHB	CV-B3	Pleurodynia, meningoencephalitis, myocarditis	142	Schoub *et al*., 1985
1981-1982	CPT	E-4	Aseptic meningitis	706	McIntyre and Keen, 1999

*PTA=Pretoria; CPT=Cape Town; JHB=Johannesburg.

Enterovirus 70 was found responsible for an acute hemorrhagic conjunctivitis outbreak in 1982, followed by CV-B3 that caused pleurodynia and meningitis in patients in Johannesburg in 1984. In 1989 there was an outbreak of hemorrhagic conjunctivits in a mine hospital and 90/130 grade 5 pupils attending a summer camp in 2001 were affected by an aseptic meningitis outbreak of E-3 (Berger, 2016).

Environmental distribution

Environmental surveillance of EVs in South Africa showed that EVs were prevalent in 42.5%, 28.5%, 26.7%, 25.3% and 18.7% of sewage, river-, dam/spring-, borehole-water, and drinking water, respectively (Vivier *et al*., 2004; Ehlers *et al*., 2005). The most predominant EV types identified in environmental water sources were the CV-Bs (Vivier *et al*., 2004; Ehlers *et al*., 2005). Sabin PVs have previously been isolated from environmental water sources, namely sewage and river water, in South Africa, with Sabin PV-1 being most predominantly identified, followed by Sabin PV-3 and PV-2 (Grabow *et* al., 1999; Pavlov *et al*., 2005). Vaccine-derived PVs were also identified, with key mutations being recognized that are associated with reversion to increased neurovirulence in attenuated Sabin PV-1 to 3 strains (Pavlov *et al*., 2005).

1.6.3. Global

Clinical distribution

Non-polio EVs are causing an estimated annual 10 – 15 million infections in the United States of America (USA) alone (Farrel *et al*., 2015). Both widespread epidemics and sporadic outbreaks are occurring due to EV strains that recombine with each other to give rise to new genetically diverse strains (Lugo and Krogstad, 2016). Hand, foot and mouth disease outbreaks of EV-71 generally occur in the Asia-Pacific region (Australia, Japan, Malaysia, Taiwan, Vietnam and China) in a cyclic pattern every 2 - 3 years (Geoghegan *et al*., 2015; Lugo and Krogstad, 2016). Enterovirus 71 and CV-A16 are the most common causative agents of HFMD and are gradually being replaced by CV-A6, CV-A10, E-9 and other EVs (Gopalkrishna *et al*., 2012; Zhou *et al*., 2016). These EVs that are steadily replacing the main HFMD causative agents have increased from 30.0% in 2010 to 83.8% in 2013 as the aetiological agent of HFMD (Gopalkrishna *et al*., 2012; Zhou *et al*., 2016). Several significant outbreaks of EV infections have occurred across the globe and are shown in Table 1.3.

In 2014, a recent respiratory disease outbreak associated with a rare EV-D68 strain in the USA affected mainly children under the age of ten years old, especially those with an underlying asthma condition (Horner *et al*., 2015). Enterovirus D68 was known as the rare subtype responsible for respiratory infections until significant respiratory tract

infection cases were reported between 2008 and 2010 in the Netherlands, Japan, the Philippines, USA and the United Kingdom (Jackson, 2014). Another clinical feature that was noted during this EV-D68 outbreak was an increased reporting of acute flaccid myelitis cases, but it was difficult to establish the causality (Kadambari *et al*., 2014; Horner *et al*., 2015).

Table 1.3. Significant global enterovirus outbreaks from 2000 - 2016.

Country	Year	Month	EV type	Clinical Manifestation	Age Group	Reference
Spain	2016	May	EV-71	Neurological complications	< 15yrs	ECDC, 2016
USA	2014-2015	Aug-Jan	EV-D68	Severe respiratory infection	< 15yrs	CDC, 2015
China	2012	May-June	E-30	Aseptic meningitis	< 14 yrs	Xiao *et al*., 2013
Australia	2012-2013	Dec-May	EV-71	Neurological symptoms, HFMD	< 10 yrs	Zander *et al*., 2014
USA	2011-2012	Nov-Feb	CV-A6	HFMD	< 5 yrs	CDC, 2015
USA	2001	Jan-Aug	E-13	Aseptic meningitis	Unsp.	Noah and Reid *et al*., 2002
Australia	2001	Mar-July	E-30	Aseptic meningitis	14 yrs	Noah and Reid *et al*., 2002
UK Germany France Iceland Netherlands	2000-2001	May-Sept	E-30	Aseptic meningitis	< 5 yrs	Noah and Reid *et al*., 2002
UK France Germany Netherlands	2000	May-Sept	E-13	Aseptic meningitis	< 5 yrs	Noah and Reid *et al*., 2002

*USA= United States of America; UK=United Kingdom; CDC=Centres for Disease Control and Prevention; ECDC=European Centre for Disease Prevention and Control.

The global initialisation of OPV in 1988 lead to PV being on the threshold of eradication, with WPV-2 being eradicated in 1999 and WPV-3 has been undetected since November 2012 (Diop *et al*., 2015). Nigeria was removed from the list of polio

endemic countries in September 2015 as their last AFP case linked to WPV-1 was detected in July 2014 (GPEI, 2016). However, two WPV-1 cases were reported in Nigeria in July 2016 that showed high similarity to a WPV-1 strain from Borno in 2011. This indicates its circulation for more than four years without detection (GPEI, 2016). To date, 34 cases of WPV were reported in the endemic countries, Pakistan (18), Afghanistan (12) and Nigeria (4), with 3 cVDPV cases in Lao People's Democratic Republic (GPEI, 2016). The genetically divergent VDPVs have also become associated with polio outbreaks in areas with low OPV coverage in the form of circulating VDPVs (cVDPVs) (Diop *et al*., 2015). Due to 90% of the VDPV outbreaks being caused by type 2 cVDPVs (cVDPV2), the trivalent OPV has been replaced with the bivalent OPV containing only PV-1 and PV-3 in all OPV-using countries from April 2016 (Morales *et al*., 2016). Countries that have recently been affected by cVDPV outbreaks are Madagascar, Guinea, Ukraine, the Lao People's Democratic Republic, Myanmar and Nigeria (Morales *et al*., 2016).

When analysing the global clinical epidemiology of EV-types, it was determined that E-30, E-6, E-9, CV-B5, E-11, E-4, CV-B3, CV-A16, CV-A9 and E-13 are the top 10 EVs that are associated with hospital cases (Janes *et al*., 2014). Enterovirus epidemiology is highly under-documented in Africa (Opanda *et al*., 2016). A study in Kenya showed that 93.5% (187/200) of the archived isolates collected from the respiratory surveillance program were positive for EV, with the majority of cases being children < 3 years of age (Opanda *et al*., 2016). Enterovirus B species were most commonly isolated, followed by EV-D and EV-A, with no EV-C species detected (Opanda *et al*., 2016).

Environmental distribution

Several countries have applied ES where EVs have been detected in various environmental water matrices, as summarised in Table 1.4. In 2007, an ES was implemented in Singapore and revealed that EVs were detected in 94% of sewage and 89% of secondary effluent (Aw and Gin, 2010). Common EVs that have been identified were most predominantly belonging to the EV-C species (76.5%), followed by EV-B (64.7%) and EV-A (41.2%), with the majority being CV-A1, CV-A11, CV-A17, CV-A22 as well as CV-A24 which has been linked to clinical strains in Singapore

(Aw and Gin, 2010). The presence of CV-A16 in environmental water correlated with an epidemic outbreak of HFMD caused by CV-A16 during time of sampling (Aw and Gin, 2010). According to ES data from the Slovak Republic, CV-Bs (40%) were the most predominant EVs isolated from sewage, followed by Es (37%) and Sabin PVs (21%) in an 11-year surveillance study from 2001-2011 (Klement *et al*., 2013). Similar data were determined in a sewage surveillance study in Italy with a prevalence of CV-B (55.5%), Es (36.3%), CV-A (0.1%) and untypeable EVs (7.5%) (Battistone *et al*., 2014).

Table 1.4. Global distribution of enteroviruses in environmental water sources.

Country	Year	Water Source	Cell Line	Positivity Rate	Main EV types	Reference
China	2008-2012	Sewage	RD L20B HEp-2	99/129 (76.6%)	E-6, CV-B3, E-3, E-12, E-7	Wang *et al*., 2014
Slovak Republic	2001-2011	Sewage	RD L20B	Unsp.	CV-B5, E-3,CV-B2, CV-B3, E-11	Klement *et al*., 2013
Italy	2005-2008	Sewage	RD L20B	680/1392 (48.9%)	CV-B2, CV-B5, E-11, CV-B5, E-6	Battistone *et al*., 2014
	2006-2010	Sewage	RD L20B	256/321 (80%)	E-6, CV-B5, E-11, E-4, CV-B4	Pelligrinelli *et al*., 2013
Tunisia	2009-2010	Sewage	None	52/172 (30.2%)	E-25, CV-B3, E-14	Ibrahim *et al*., 2014
Senegal	2007-2013	Sewage	RD L20B HEp-2	116/271 (42.8%)	CV-A11, CV-A7, E-19, CV-A13, CV-A17	Ndiaye *et al*., 2014
Poland	2011	Sewage	RD L20B Caco-2	127/165 (77%)	E-11, E-3, CV-B, E-6, E-7	Wieczorek *et al*., 2015
Nigeria	2010	Sewage SCW*	RD L20B	9/26 (34.6%)	E-7, E-11, E-13, E-19, E-20	Adeniji and Faleye, 2014
Georgia	2002-2005	Sewage	RD L20B	112/254 (44.1%)	E-6, E-20, E-3, E-7, E-11, CV-B4,	Khetsuriani *et al*., 2010
Singapore	2007	Sewage Effluent	None	17/18 (94%) 16/18 (89%)	CV-A1, C V-A22, CV-A17, CV-A22 CV-A16, CV-A2	Aw and Gin, 2010

*SCW = Sewage contaminated water; CV = Coxsackievirus; E = Echovirus.

An ES applied in Ivory Coast between 2008 and 2009, showed that BGM cell lines (50%) had a higher EV infection sensitivity, compared to the RD (48.5%), HEp-2

(44.1%) and L20B (2.9%) cell lines (Momou *et al*., 2014). When using direct RT-PCR, EVs were detected in 44.1% of the samples positive and no significant difference in the detection rates between direct RT-PCR and RT-PCR after viral amplification was found (Momou *et al*., 2014).

A reduction in the occurrence of VDPVs have been shown in ES studies that were done in conjunction with AFP surveillance after the switch from OPV to IPV, which were replaced by an increase of NPEVs (Klement *et al*., 2013). Environmental surveillance studies have shown that Sabin PV-1 is the least prevalent PV species detected in environmental matrices (Battistone *et al*., 2014; Nakamura *et al*., 2015). It has been suggested that they have reduced replication in the gut or are less stable in the environment indicating lower resistance compared to Sabin PV-2 and PV-3 (Battistone *et al*., 2014). According to an ES in Japan, Sabin PV-2 was the most prevalent (46%) (Nakamura *et al*., 2015). Highly divergent VDPVs have been isolated from several countries, mostly being VDPV-2 (Al-Hello *et al*., 2013, Tao *et al*., 2013; Esteves-Jaramillo *et al*., 2014).

1.7. MOTIVATION FOR INVESTIGATION

There are more than 100 different genotypes that have been identified due to their genetic variability from intratypic and intertypic recombination (Tryfonos *et al*., 2011). Due to the lack of African data on EV epidemiology there is a growing need for environmental surveillance of PVs and NPEVs. The poor infrastructure and treatment at WWTPs facilitates the survival of potentially infectious EVs in water sources and its transmission in the community through potential utilisation of the downstream water sources. By assessing the occurrence of EVs in water sources, especially PVs, there can be an emphasis on the need for proper established guidelines or thresholds for viral indicators and improvement of treatment systems (Hsu *et al*., 2009).

1.8. HYPOTHESIS

It is hypothesised that wastewater treatment systems are not efficiently removing EVs from wastewater, which may be introduced in natural downstream surface water sources after wastewater effluent discharge.

1.9. AIM OF INVESTIGATION

The aim of this project is to detect and characterise EVs with molecular and viral isolation methods, with specific reference to the three Sabin PV strains to determine the viral removal efficiency of selected WWTPs.

1.10. OBJECTIVES

i. To recover EVs from water sources with glass wool adsorption elution or polyethylene glycol/sodium chloride precipitation.

ii. To optimise and apply real-time RT-PCR assays and a conventional nested RT-PCR assay for the detection of EVs in water sources, which include Sabin PV-specific real-time RT-PCR assays.

iii. To isolate viruses in L20B, PLC/PRF/5 and BGM cell lines

iv. To genotype the detected EVs with nucleotide sequence analysis, followed by phylogenetic analysis to examine the genetic relatedness between identified EV strains and recognised EVs strains circulating worldwide.

CHAPTER 2

MATERIALS & METHODS

2.1 SITE SELECTION

Between April 2015 and March 2016, environmental water samples from six WWTPs and three surface water sites were collected on a monthly basis. The water samples provided by a large water utility (n=11) included sewage wastewater (1 L) and wastewater discharge (10 L) samples from five selected WWTPs in the Gauteng and bordering Free State province. An additional downstream surface water (10 L) sample site (VD1), which received wastewater discharge from all five WWTPs, was collected. This surface water site was 100 meter (m) from a dam wall and 200 m from the intake tower for a drinking WTP (Figure 2.1).

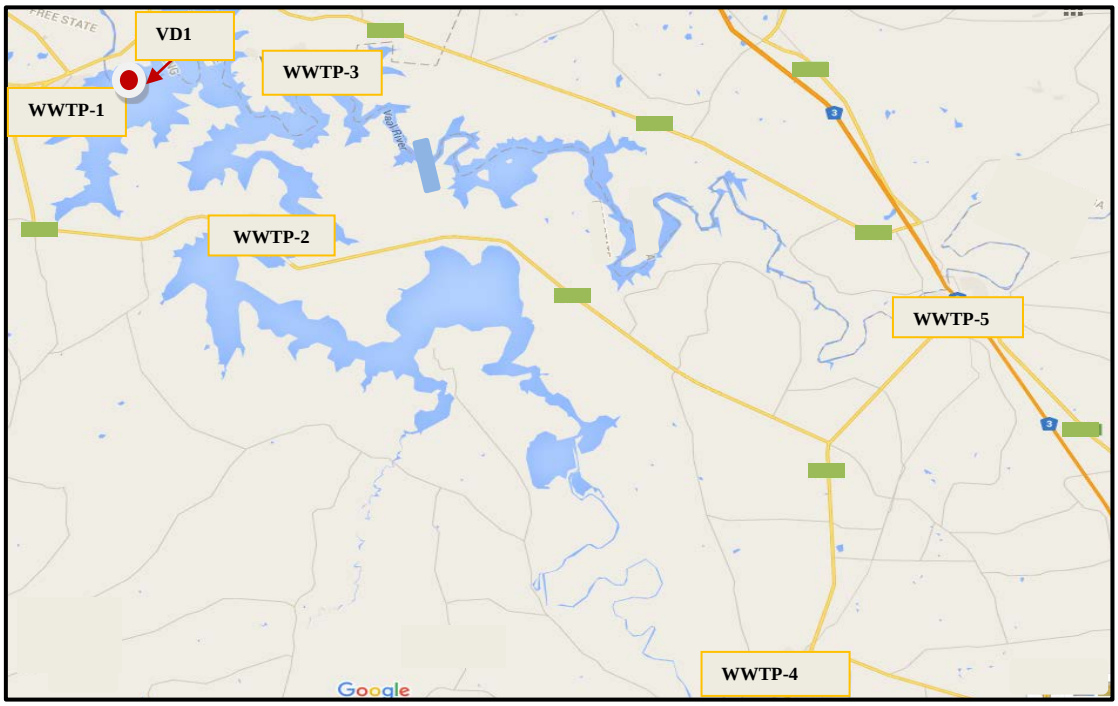


Figure 2.1. Map of the wastewater treatment plant sample sites and downstream surface water sample site in Gauteng and bordering Free State Province provided by a large water utility.

The remaining water samples were provided by a Gauteng municipality in the West Rand (n=12) and included wastewater discharge (1 L) and surface river water 30 m above (1 L) and 20 m below (1 L) the discharge point of the WWTP. The latter water

samples were collected on a weekly basis from April 2015 to March 2016 and each sample type was pooled to a volume of 4 L at the end of each month (Figure 2.2).

Figure 2.2. Map of the sample site of wastewater treatment plant-6 and surface water sample sites provided by a Gauteng municipality.

The WWTP system information of all treatment plants are described in Table 2.1. This study was approved by the Faculty of Health Sciences MSc Committee (Appendix A) as well as the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (Reference no: 164/2015) (Appendix B).

Table 2.1. Technical characteristics of the wastewater treatment plant systems.

Prov.	WWTP	Design capacity (MegaL/d)	Treatment system	Green Drop status	2014 CRR value (%)	2012-2013 EQQ value (%)
FS	1	2.00	Biological trickling filters; solar drying beds	High risk	82.4	57.3
FS	2	0.46	Biological filters and anaerobic ponds; solar drying beds and incineration	High risk	88.2	49.0
GP	3	2.00	Activated sludge and biological nutrient removal; anaerobic digestion	High risk	70.6	80.7
FS	4	2.82	Biological filters and integrated ponds; solar drying beds and composting	Critical	100	53.1
FS	5	1.70	Biological filters and anaerobic ponds; solar drying beds and sludge pond.	Critical	94.1	39.6
GP	6	19.5	Activated sludge and biological nutrient removal	High risk	81.8	37.6

*MegaL/d = Megalitre per day; CRR = Cumulative Risk Rating; EQQ = Effluent Quality Compliance (Department of Water Affairs, 2013).

2.2 INDICATOR ORGANISMS

Escherichia coli was enumerated from ten-fold dilutions of water samples using the membrane filtration technique and m-ColiBlue24®Broth (Hach Company, Loveland, CO). Ten ml of maximum recovery diluent was added to the cellulose nitrate filters (0.45 micrometer [µm]) (Sartorius AG, Göttingen, Germany). One ml of the ten-fold dilutions were filtered through the cellulose nitrate filters (Sartorius AG) using a polytetrafluoroethylene pressure filter holder (Sartorius AG) connected to a Gast vacuum pump (Gast manufacturing Inc., Benton Harbor, MI). Following filtration, the cellulose nitrate filters were placed onto an absorbent pad containing m-ColiBlue24® broth (Hach Company) in a petri dish (9 x 50 mm). The absorbent pad was invertedly incubated at 44.5°C for 24 hours in an Incotherm incubator (Labotec, Midrand, SA). The blue colonies were enumerated as cfu/100 ml (Standard methods for the examination of water and wastewater, 2005; SANS 5221:2006 Ed 4.2).

2.3 VIRAL RECOVERY

2.3.1 Reagent preparation

Glass wool

For glass wool adsorption-elution, three portions of 5 gram (g) of glass wool was teased and compressed into a column, which is a Perspex pipe consisting of open caps on each end that have an extension onto which tubes can attach for inflow and outflow during filtration. Two gauze grids (pore size = 1 mm; 30 mm in diameter) are inserted between the three glass wool sections. The glass wool was soaked with sterile distilled water and pre-treated with 40 ml 1 Molar (M) hydrochloric acid (HCl) (Merck, Darmstadt, Germany), followed by 100 ml sterile distilled water, 40 ml 1 M NaOH (Merck) and 100 ml sterile distilled water to adjust the pH to 7.0 (Vilagenès *et al*., 1993).

Polyethylene glycol$_{8000}$/sodium chloride solution

Polyethylene glycol$_{8000}$/sodium chloride solution (PEG$_{8000}$/NaCl) was prepared for primary concentration of the 1 L raw sewage samples and for secondary precipitation of the 4 L and 10 L wastewater discharge and surface water samples following primary glass wool adsorption-elution. The PEG/NaCl was prepared according to the ISO

standards, 15216-1, 2013 as follows: 250 g PEG_{8000} (Amresco, Solon, OH) and 43.5 g NaCl (Merck) was combined and dissolved in 500 ml distilled H_2O at 50˚C in a Heraeus incubator (Thermo Scientific, Waltham, MA). Once the PEG/NaCl solution was fully dissolved, it was subsequently autoclaved for 15 minutes (min) at 121˚C and stored at room temperature.

2.3.2 Glass wool adsorption-elution

The glass wool column filtration method based on the method of Vilaginès *et al.*, (1993), modified by Venter (2004) was used to recover viruses from the 4 L and 10 L surface water and wastewater discharge samples. These water samples were drawn through positively charged glass wool columns with negative pressure. The negatively charged viruses adsorbed to the glass wool, were eluted twice with 50 ml glycine-beef extract buffer at pH 9.5 (0.05 M glycine [Merck] and 0.5% beef extract [BBL™ Becton Dickinson and Co., Sparks, MD]) with a 15 min holding time. The 100 ml eluate from the column was subsequently adjusted to a pH of 7 with 1 M HCl (Merck).

A final concentration volume of 10 ml in phosphate buffered saline (PBS) (Sigma-Aldrich Co., St. Louis, MO) was attained using the secondary precipitation method, PEG_{8000} (Amresco)/NaCl (Merck) precipitation (ISO 15216-1, 2013). This was achieved by adding 0.25 volumes of PEG_{8000}/NaCl to the supernatant and shaken on ice in a shaking waterbath (GFL, Burgwedel, Germany) for one hour. The supernatant/PEG_{8000}/NaCl mixture was centrifuged at 10 000 x g for 30 min at 4˚C in the Sorvall®SuperT21 centrifuge (DuPont, Wilmington, DE). The supernatant obtained after centrifugation was discarded and the pellet was suspended in 10 ml PBS (pH 7.4) (Sigma-Aldrich Co.). Following the thorough resuspension in PBS, 0.2 volumes of chloroform (Merck) was added to the suspension, shaken thoroughly and centrifuged at 4˚C for 5 min at 10 000 x g in the Sorvall®SuperT21 centrifuge (DuPont). The upper aqueous phase was transferred to a 15 ml sterile tube and stored at -20˚C until nucleic acid extraction.

2.3.3 Polyethylene glycol $_{8000}$/sodium chloride precipitation

The PEG_{8000}/NaCl precipitation method (ISO 15216-1, 2013) was used as a primary concentration method to recover viruses from the 1 L raw wastewater samples. The

samples were centrifuged at 1000 x g for 10 min at 4˚C in 500 ml bottles in the GS-6R centrifuge (Beckman Coulter, CA). The supernatant was transferred to a 2 L sterile bottle and adjusted to a pH of 7.0 with 1 M NaOH (Merck), while the pellets in the 500 ml bottles were kept at 4˚C. The same steps as mentioned above in 2.3.2. were subsequently followed from when the PEG_{8000}/NaCl solution was added to the supernatant. After PBS resuspension the solution was used to dissolve the initial pellets in the 500 ml bottles kept at 4˚C. Subsequently, 0.2 volumes of chloroform (Merck) was added to the suspension, shaken thoroughly and centrifuged at 4˚C for 5 min at 10 000 x g in the Sorvall®SuperT21 centrifuge (DuPont). The upper aqueous phase was transferred to a 15 ml sterile tube and stored at -20˚C until nucleic acid extraction.

2.4. VIRAL ISOLATION

2.4.1. Cell Cultures

Four different cell lines of both human and NHP origin, were used in this investigation. The PLC/PRF/5 human hepatoma cell line (European Collection of Cell Cultures [ECACC] 85061113), was derived from the liver of a chronic carrier of hepatitis B virus who died of primary hepatocellular carcinoma (Alexander *et al* 1976). These cells enhance the detection sensitivity of the majority of EVs, especially CV-B and Es (Rodríguez *et al*., 2008). The BGM cell line (ECACC 90092601) was derived from African green monkey (*Cercopithecus aethiops*) kidney cells and they are susceptible to EVs, more specifically CV-B and PV (Rodriguez *et al*., 2008). L20B cells are mouse L cells that have been genetically modified for PV detection by expressing the gene for the human cellular receptor for PVs (Wood and Hull, 1999). The Vero African Green Monkey cell line (ECACC 84113001), originally isolated from a non-diseased adult African green monkey in Japan, was grown on glass slides and used to monitor the presence of EV-specific inclusion bodies.

2.4.2. Cell culture media and reagents

Antimicrobial and antimycotic pre-treatment

Six millilitre of the recovered viral concentrate was treated with a combination of penicillin-streptomycin-neomycin antibiotics (Sigma-Aldrich Co. GmbH, St.Louis,

MO). The antibiotics consisted of 5 000 units of penicillin, 5 mg streptomycin and 10 mg of neomycin/ml. An antimycotic, Nystatin (Sigma-Aldrich Co.), containing 10 000 units/ml was also added (Vivier *et al*., 2004). Ten microlitre (µl) of the Nystatin and the antibiotic combination was added per ml recovered viral concentrate and is added to prevent cell culture contamination.

Minimum essential media

Each cell line had its own requirements regarding cell culture media. In general, all cells were cultivated in Eagle's minimum essential medium (E-MEM) with Earle's salts, antibiotics mixture (Penicillin-Streptomycin or Penicillin, Streptomycin-Amphotericin B Mixture, Lonza) (GIBCO, Waltham, MA) supplemented with appropriate concentrations of foetal calf serum (FCS, Biochrom GnbH, Germany). The PLC/PRF/5 cell line has an endogenous mycoplasma and 60 µg/ml tylocine (anti-PPLO, Gibco BRL) was added. The L20B cell line required the addition of 0.01 M HEPES (Gibco BRL) to maintain the physiological pH in the cell culture.

2.4.3. Cell culture infection

One ml of viral concentrate was inoculated into duplicate 25 cm^2 culture flasks that contained monolayers of BGM, PLC/PRF/5 and L20B cells, respectively. Prior to infection, the growth media was removed from semi-confluent monolayers and the cells were starved for 60 min at 37°C in serum-free E-MEM. After withdrawal of the serum-free E-MEM, the appropriate volume (one ml) of treated sample was added and allowed to adsorb to the cells for 1 h at 37°C, with gentle swirling every 15 min. Four millilitre of MEM, specific for each cell line, was added to each culture flask and incubated at 37°C in a 5% CO_2 incubator. As a quality control, four ml of MEM was added to a flask of BGM, L20B and PLC/PRF/5 cells only and incubated at 37°C in a 5% CO_2 incubator.

The cultures were monitored daily for the presence of a CPE by light microscopy. If no CPE was observed after seven days, the cells were blind-passaged into a single 25 cm^2 culture flask. This involved preparation of new monolayers of the specific cell lines in 25 cm^2 culture flasks as described above, that were inoculated with 1 ml of the infected cells. It was incubated for a further seven days with daily examination for a CPE. The

flasks containing cells displaying CPE were stored in a 4°C fridge until cell culture harvesting.

2.4.4. Cell culture harvesting

Five hundred microliters of the infected BGM, L20B and PLC/PRF/5 cell cultures were harvested into a 1.5 ml microcentrifuge tube and stored in a -20°C freezer until nucleic acid extraction, including the negative cell culture controls. Following harvesting of all three cell lines, 50 µl of the infected cell cultures were passaged onto cell culture tubes with monolayers of Vero cells on a glass slide and incubated for seven days. The tube cultures were monitored daily for the presence of CPE by light microscopy and the glass slides were stained with haematoxylin and eosin and examined for the presence of EV-specific inclusion bodies as described by Malherbe and Strickland-Cholmley (1980).

2.5 NUCLEIC ACID EXTRACTION

2.5.1 Recovered viral concentrates

One ml of the recovered viral concentrate was seeded with 10 µl of mengovirus (5×10^5 copies) to serve as a process control. Mengovirus is a mutant non-virulent infective strain that is non-enveloped, contains single-stranded RNA, has similar environmental resistance properties to hepatitis A viruses and other single stranded RNA viruses and is not known to occur naturally in tested matrices. Total nucleic acids were extracted using the NucliSENS®EasyMAG® instrument (BioMérieux, Marcy I'Etoile, France) according to the manufacturer's instructions. It is a semi-automated nucleic acid extraction procedure that is based on the principle of magnetic silica particles that capture nucleic acid. Firstly, the sample aliquots were lysed during a 10 min incubation after which 50 µl of the magnetic sillica was added into each well and mixed with a multichannel pipette. The magnet silica particles that captured the nucleic acids were attracted to the NucliSENS®EasyMAG® magnetic device (BioMérieux) that enabled the system to purify the nucleic acid with three different wash buffers. A heating step released the nucleic acid from the magnetic sillica particles and the particles were separated from the eluate by the magnetic device. The extracted nucleic acids were eluted into 100 µl, aliquoted into smaller volumes and stored at -70˚C.

2.5.2 Cell culture suspensions

Five hundred microlitres of the harvested cell culture suspensions, including the negative cell culture controls, were subjected to three cycles of freezing and thawing and centrifuged at 3000 rpm for three min prior to nucleic acid extraction to pellet the cell debris. Nucleic acids were subsequently extracted from 200 µl of the supernatant with the NucliSENS®EasyMAG® instrument (BioMérieux) according to the manufacturer's instructions and as described in 2.5.1. The extracted nucleic acids were eluted into 50 µl, aliquoted into smaller volumes and stored at -70˚C.

2.6 MOLECULAR DETECTION

2.6.1 Mengovirus real-time RT-PCR detection

A one-step rtRT-PCR assay was performed for the detection of seeded mengovirus from the recovered viral suspensions as a nucleic acid extraction process control with the mengo@ceeramTools™kit (Ceerams.a.s, La Chappelle-Sur-Erdre, France) and rtRT-PCR primers and probe described by Pintó *et al*., (2009) (Table 2.2).

Table 2.2. Primers and probe for mengovirus amplification (Pintó *et al*., 2009).

Primer/Probe	Sequence (5'-3')	Orientation
Mengo110-F	GCG GGT CCT GCC GAA AGT	Forward
Mengo209-R	GAA GTA ACA TAT AGA CAG ACG CAC AC	Reverse
Mengo147-probe	FAM-ATC ACA TTA CTG GCC GAA GC-MGB	-

The one-step rtRT-PCR reaction mixture had a total reaction volume of 25 µl, consisting of 1 X mastermix containing mengovirus primers and probes, 1 X RT-PCR enzyme mix and 5 µl of extracted nucleic acids. The one-step rtRT-PCR assay was performed on the LightCycler®2.0 (Roche, Mannheim, Germany) under the following cycling conditions: reverse transcription at 45˚C for 10 min, activation at 95˚C for 10 min and 45 cycles at 95˚C for 15 seconds (s) and 60˚C for 45 s. Acquisition of fluorescence was done after the extension step of each cycle.

2.6.2 Enterovirus real-time RT-PCR detection

A one-step rtRT-PCR assay was performed for the detection of EVs from the recovered viral suspensions as well as the cell culture extracts with the Quantitect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and primers and a hydrolysis probe, described by Führman *et al*, (2005) (Table 2.3).

Table 2.3. Primers and probe for enterovirus real-time RT-PCR amplification (Fuhrman *et al*., 2005).

Primer/Probe	**Sequence (5'-3')**	**Orientation**
EV1	GAT TGT CAC CAT AAG CAG C	Reverse
EV2	CCC CTG AA TGC GGC TAA TC	Forward
EV-probe	FAM-CGG AAC CGA CTA CTT TGG GTG TCC GT-BHQ-Phosphor	-

The PCR reaction mixture consisted of 5 µl of nucleic acid and 15 µl mastermix that included 250 nanomolar (nM) of primer EV1, 250 nM of primer EV2 and 100 nM of the EV-probe. The assay was carried out in a LightCycler®2.0 (Roche Diagnostics) under the following conditions: reverse transcription at 50˚C for 60 min, activation at 95˚C for 5 min and 45 cycles at 94°C for 15 s, 60°C for 1 min and 65°C for 1 min and the fluorescent signal was measured after the extension step.

2.6.3 Sabin-like poliovirus real-time RT-PCR detection

A one-step rtRT-PCR assay was performed for the detection of Sabin PV-1 to 3 from the recovered viral suspensions as well as the cell culture extracts with the Quantitect Probe RT-PCR Kit (Qiagen) with the primers and probes described by Nijst *et al*, (2013) (Table 2.4).

Three individual rtRT-PCR assays for Sabin PV-1, PV-2 and PV-3 were performed and each PCR reaction volume consisted of 5 µl of nucleic acid and 15 µl mastermix that included 250 nM each of forward and reverse primer and 100 nM of probe. The LightCycler®2.0 (Roche Diagnostics) was used for performing the three Sabin PV assays under the following conditions: reverse transcription at 50˚C for 45 min with enzyme activation at 95˚C for 15 min for all three assays.

Table 2.4. Primers and probes for Sabin-like poliovirus amplification (Nijst *et al*., 2013).

Primer/probe	Sequence (5'-3')	Orientation	Specificity
Sabin1-F	TCC CTT TGA CTT AAG TAC AAA	Forward	PV-1 Sabin
Polio1-R	GAT CCT GCC CAG TGT GTG TAG	Reverse	PV-1 Wild/Sabin
Polio1-TM	FAM-AGG GTT CGG TTA AGT GAC AAA CCA CAT AC-BBQ	-	PV-1 Wild/Sabin
Sabin2-F	AAG GAA TTG GTG ACA TGA TTG AGG	Forward	PV-2 Sabin
Sabin2-R	CTC GGC TTT GTG TCA GGC	Reverse	PV-2 Sabin
Sabin2-TM	FAM-TGG AAG TCG GGG GAA CCA ATT GC-BBQ	-	PV-2 Sabin
Sabin3-F	AAT GAC CAG ATT GGT GAT TCC TTG	Forward	PV-3 Sabin
Sabin3-R	GTA AAT GCG GAC TTT GGA GGT TAC T	Reverse	PV-3 Sabin
Sabin3-TM	FAM-TGT GAT CAT TGA CAA CAC GAA CTG CAA-BBQ	-	PV-3 Sabin

The cycling conditions for PV-1 were 35 cycles of 95˚C for 5 s, 55˚C for 1 s, 72˚C for 37 s and 40˚C for 30 s. The ramp rate was adjusted to 2˚C/s in the PV-1 assay between 55˚C to 72˚C and returned to 20˚C/s for cooling at 40˚C for 30 s. For PV-2, the conditions were 35 cycles of 95˚C for 5 s, 58˚C for 15 s, 72˚C for 5 s and cooling at 40˚C for 30 s, while for PV-3 it was 35 cycles of 95˚C for 5 s, 56˚C for 15 s, 72˚C for 5 s and 40˚C for 30 s. For the PV-2 and PV-3 assays the ramp rate was 20˚C/s and acquisition of the fluorescence for all assays were done after the extension step.

2.7 MOLECULAR CHARACTERISATION

2.7.1 Conventional enterovirus RT-nPCR

A conventional RT-nPCR assay was performed to amplify a 375 bp region within the EV VP1 genome region with the first round and nPCR primers described by Nix *et al*., (2006) (Table 2.5).

Table 2.5. Primers for enterovirus VP1 region amplification (Nix *et al*., 2006).

Primers	Sequence (5'-3')	Orientation
EV224	GCI ATG YTI GGI ACI CAY RT	first round PCR, forward
EV222	CIC CIG GIG GIA YRW ACA T	first round PCR, reverse
AN89	CCA GCA CTG ACA GCA GYN GAR AYN GG	nPCR, forward
AN88	TAC TGG ACC ACC TGG NGG NAY RWA CAT	nPCR, reverse

cDNA synthesis

For cDNA synthesis, the Protoscript®II Reverse Transcriptase (New England BioLabs Inc, Ipswich, MA) enzyme was used. Ten µl of RNA was incubated at 95˚C for 2 min on the PTC-100™ thermal cycler (MJ Research Inc., Watertown, MA) and cooled on ice for 2 min. Ten µl of RT reaction mixture was then added to the incubated RNA, which included 0.5 millimolar (mM) deoxynucleotide triphosphates (dNTPs) (New England BioLabs Inc.), 30 µM random hexamers (Roche Diagnostics), 1 X Protoscript II Reverse Transcriptase reaction buffer (New England BioLabs Inc.), 10 mM 1,4-Dithiothreitol (DTT) (New England BioLabs Inc.), 50 Units of Protoscript II Reverse Transcriptase (New England BioLabs Inc.) and 20 U of Protector RNase inhibitor (Thermo Scientific, Langensenbold, Germany). The cDNA was synthesised in the PTC-100™ thermal cycler (MJ Research Inc.) under the following conditions: 25°C for 10 min, 50°C for 60 min and 85°C for 5 min.

First round PCR

The cDNA product was used as a template for the first round PCR that amplifies a 992 bp region of the EV VP1 genome using 1X EmeraldAmp® MAX HS PCR mastermix (Takara Bio Inc., Shiga, Japan), 1 µM EV222 and 1 µM EV224 primers. The total reaction volume was 50 µl, consisting of 10 µl of cDNA template and 25 µl mastermix. The PCR was performed using the BIOER thermal cycler (BIOER Technology Co., Hangzhou, China) with the following cycling conditions: Activation at 95˚C for 3 min, 40 cycles of 95˚C for 30 s, 42˚C for 30 s and 72˚C for 45 s and final extension at 72˚C for 10 min.

Second round PCR

The first round PCR product was used as a template for the nPCR that amplified a 375 bp region of the EV VP1 genome using 1 X EmeraldAmp® MAX HS PCR mastermix (Takara Bio Inc.), 800 nM AN88 and 800 nM AN89 primers. The total reaction volume was 50 µl, consisting of 1 µl first round PCR product and 25 µl mastermix. The PCR was performed using the BIOER thermal cycler (BIOER Technology Co.) with the following cycling conditions: Activation at 95˚C for 3 min, 40 cycles of 95˚C for 30 s, 60˚C for 20 s and 72˚C for 30 s and final extension at 72˚C for 10 min.

Visualisation

Ten microlitre of the first round (992 bp) and nPCR product (375 bp) was analysed by 1.5% agarose gel (FMC Corp., Rockland, USA) electrophoresis and visualised by staining with 5 µl/100 ml gel of ethidium bromide (EtBr) (10 mg/ml) (Promega, Madison, WI) and UV light with the BioRad Gel Dox XR System (Biorad Laboratories Inc., Hercules, CA).

2.7.2 DNA cloning and transformation

TB-agar plate preparation

The broth consisted of 10 g NaCl (Merck), 10 g Bacto™Tryptone (Becton and Dickinson & Co.), 5 g Bacto™Yeast Extract (Becton and Dickinson & Co.) and 15 g of Bacto™Agar (Becton and Dickinson & Co.) per litre. The broth mixture was autoclaved, 1 ml of Ampicillin (100 mg/mL) (Sigma-Aldrich Co.) was added to the TB-agar and an estimated 15-20 ml was poured into each plate.

Cloning

The RT-nPCR products of the correct size (375 bp) were purified using the Zymo DNA Clean and Concentrate Kit (Zymo Research, Irvine, CA) and eluted in 30 µl elution buffer. The NEB®PCR Cloning Kit (New England Biolabs Inc.) was used for cloning the purified PCR products according to the manufacturer's instructions. The ligation reaction consisted of 2.5 ng/µl of linearized pMiniT™ Vector added to a 1-4 µl insert of purified PCR product, with nuclease-free H_2O added up to a volume of 5 µl if

necessary. Four microlitre Cloning Mix 1 and 1 µl Cloning Mix 2 was subsequently added to the reaction mixture to make up a total volume of 10 µl. The ligation reaction was incubated at 25˚C for 5 to 15 min and transferred to ice for 2 min.

Transformation

Transformation was performed using Lucigen *E.cloni* 10 G Duos cells (Lucigen Corp., Middleton, WI) according to the manufacturer's instructions. Four microlitre of ligation reaction was added to 10 to 20 µl of the *E.* cloni cells (Lucigen Corp.) and incubated on ice for 30 min. The cells were heat shocked at 42°C for 45 s in a MJ Minicycler (MJ Research Inc.) after which the samples were returned to ice for 2 min. Following the addition of 400 µl of super optimal broth with catabolite repression (SOC) medium (Lucigen Corp.), the samples were incubated for one hour at 37°C in a shaking incubator (Labnet International Inc. Edison, NJ) at 220 rpm. One hundred microlitres of the cells were plated onto the TB-agar plates and invertedly incubated overnight at 37˚C in an incubator (Labotec, Midrand, SA).

Colony PCR

Colony PCR (cPCR) was performed for the amplification of the cloned fragments from 10 randomly selected colonies using OneTaq® Quick-Load® 1 X NEB Mastermix with standard buffer and 200 nM each of forward and reverse primer (New England Biolabs Inc.) with a total reaction volume of 20 µl. The PCR assay was performed on a BIOER thermal cycler (BIOER Technology Co.) under the following cycling conditions: 30 s at 95°C and 30 cycles of 30 s at 95°C, 30 s at 60°C and 40 s at 68°C, followed by a 5 min delay at 68°C.

Visualisation

Five microlitre of the cPCR product was analysed by 1.5% agarose gel (FMC Corp., Rockland, USA) electrophoresis and visualised by staining with 5 µl/100 ml gel of EtBr (10 mg/ml) (Promega, Madison, WI). The product was illuminated and further visualised with UV light using the BioRad Gel Dox XR System (Biorad Laboratories Inc., Hercules, CA).

2.7.3. Nucleotide sequence analysis

Five direct and two cell culture cPCR products of the correct product size were purified using the Zymo DNA Clean and Concentrate Kit (Zymo Research) according to the manufacturer's instructions. Nucleotide sequence PCR was performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The total reaction volume was 20 µl, consisting of 1 X sequencing buffer, 1 µl terminator mix, 160 nM of the forward or reverse primer and 3 µl of the PCR product. The PCR assay was performed on a BIOER thermal cycler (BIOER Technology Co.) under the following cycling conditions: 94˚C for 3 min and 25 cycles of 94˚C for 30 s, 50˚C for 10 s and 60˚C for 4 min.

The precipitation step was done by the addition of 2 µl of 125 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Co.), 2 µl of 3 M sodium acetate (NaAc) (pH 5.2) (Merck) and 50 µl of absolute EtOH (Merck) after which the sequencing mixtures were incubated at room temperature for 15 min. The reaction mixtures were centrifuged at 14 000 rpm for 20 min at 4˚C in the Heraeus Fresco17 centrifuge (Thermo Scientific, Langensenbold, Germany). The supernatant was removed and 100 µl of 70% EtOH was added. The microcentrifuge tubes were centrifuged at 14 000 rpm for 10 min at 4˚C and the supernatant was removed.

The pellets were dried at 95˚C in a BIOER thermal cycler (BIOER Technology Co.) and subsequently stored in the dark at -20˚C until analysed on an ABI Model 3130XL automated genetic analyser (Applied Biosystems, Foster City, CA).

2.8. PHYLOGENETIC ANALYSIS

The EV sequences from both forward and reverse strands were analysed through the DNA sequence analysis software, Sequencer 4.10.1 to create continuous nucleotide sequences. The continuous nucleotide sequences were further aligned on the Biological Sequence Alignment Editor, BioEdit version 7.0.9.0 (Hall, 1999) and the sequences had to be confirmed with the ability to be translateable protein sequences before further analysis. The aligned sequence genotypes were identified using the BLAST database when compared to known EV sequences in GenBank. In addition, the EV genotype

was confirmed with the Enterovirus Typing Tool V.0.1 (RIVM) (Kroneman *et al*., 2011). The phylogenetic tree was constructed using the neighbour-joining method with the Kimura-2 parameter model of 1000 bootstrap replications on the Molecular Evolutionary Genetic Analysis program (MEGA V.6) (Tamura *et al*., 2013). Selected sequences are in the process to be deposited on Genbank.

2.9. QUALITY CONTROL

In order to reduce the risk of cross-contamination between samples, and samples and DNA amplicons, the sample processing, reagent preparation, reaction preparation, amplification and post-amplification procedures were performed in separate laboratories with its own apparatus, pipettes and reagent tubes. Negative and positive controls were included in each EV detection reaction to monitor for false-negative or false-positive reactions. Negative cell culture controls for BGM, L20B and PLC/PRF/5 were included, as well as negative extraction controls and negative rtRT-PCR and RT-nPCR controls with nuclease-free water. Positive controls were included in each rtRT-PCR and RT-nPCR reaction, which was either the nucleic acid from a characterised cell-culture adapted or wild-type EV.

CHAPTER 3

RESULTS

3.1. SAMPLES RECEIVED

A total of 156 water samples were collected, namely wastewater (n=54), wastewater discharge (n=66), downstream surface dam water (n=12), upstream surface river water (n=12) and downstream surface river water (n=12). No samples were received from WWTP-1 in December 2015 and from WWTP-4 in May, July, October, and November 2015 and February 2016.

3.2. ENTEROVIRUS DETECTION

3.2.1. Virus isolation

The recovered viral suspensions of the 156 water samples were inoculated onto the three different cell lines, with a total of 468 infected cell cultures. Following H & E staining, cytopathogenic EVs were isolated in 26.3% (41/156) of the infected cell cultures when combining the CPE results from all three cell lines for each sample. This consisted of 46.3% (19/41) wastewater, 36.6% (15/41) wastewater discharge and 17.1% (7/41) downstream surface river water-infected cell cultures. No CPE was detected in the cell cultures infected with wastewater discharge from WWTP-3 and 4, downstream surface dam water (VD1) and the upstream surface river water samples.

Of the 468 infected cell cultures, EVs were isolated in 12% (60/468) that included 43.3% (26/60), 40% (24/60) and 16.7% (10/60) of PLC/PRF/5, BGM and L20B cell cultures, respectively. The presence of EV-specific inclusion bodies in the infected cell cultures collected between April 2015 and March 2016 is summarised in Table 3.1. The WWTP with the highest number of infected cell cultures positive for cytopathogenic EVs for each of the three different cell lines was WWTP-5, with 14/60 infected cell cultures. This was followed by WWTP-2 (12/60), 1 (9/60), 4 (5/60) and 3 (4/60).

Table 3.1. Cytopathogenic enteroviruses identified in different cell lines infected with environmental water samples.

WWTP	Sample Type	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
1	Wastewater	-	-	-	BP	BP	BP	-	B		-	B	-
	Discharge	-	-	-	-	-	P	-	-		-	-	-
2	Wastewater	BLP	-	-	-	-	-	BP	BLP	B	-	-	-
	Discharge	B	-	-	-	-	-	-	-	-	P	-	-
3	Wastewater	-	-	-	-	-	BP	P	-	P	-	-	-
	Discharge	-	-	-	-	-	-	-	-	-	-	-	-
4	Wastewater	-		-		-	-			LP	B		BP
	Discharge	-		-		-	-			-	-		-
5	Wastewater	BP	-	P	-	-	-	-	B	BP	-	-	-
	Discharge	-	-	L	L	B	P	-	B	-	B	LP	-
1 TO 5	Downstream	-	-	-	-	-	-	-	-	-	-	-	-
6	Upstream	-	-	-	-	-	-	-	-	-	-	-	-
	Discharge	-	-	P	P	-	-	-	-	BLP	-	LP	B
	Downstream	-	-	P	-	BL	-	BP	L	P	-	P	B

B = BGM cell line; L = L20B cell line; P = PLC/PRF/5 cell line; Downstream = surface water receiving effluent discharge; Upstream = surface water upstream of discharge point. WWTP = Wastewater treatment plant. Areas marked in grey indicate samples not received.

The month with the highest prevalence of EV-specific inclusion bodies determined in the three separate infected cell cultures of each sample was December 2015 with ten infected cell cultures. This was followed by November 2015 (n=7), September 2015 (n=6) and April 2015 (n=6) with no cytopathogenic EVs isolated in May 2015.

Based on the prevalence of EVs represented by the combined result of the three cell lines for each sample, the cell cultures infected with wastewater discharge samples from WWTP-5 still had the highest cytopathogenic EV prevalence. The wastewater discharge-infected cell cultures from WWTP-5 were positive for EVs in 7/12 months. Cytopathogenic EVs were also isolated in 7/12 months from cell cultures infected with downstream surface river water samples. In decreasing order, the wastewater discharge-infected cell cultures from WWTP-6 was second highest in EV prevalence (5/12), followed by WWTP-2 (2/12), and WWTP-1 (1/11). No EVs were isolated from the wastewater discharge samples of WWTP-3 and WWTP-4.

3.2.2. Enterovirus RT-PCR detection

Direct rtRT-PCR

Enteroviruses were detected in 11.5% (18/156) of the recovered viral suspensions with EV one-step rtRT-PCR, of which 88.8% (16/18) and 11.1% (2/18) were from wastewater and wastewater discharge, respectively. No EVs were detected in the surface water samples. The EV-positive recovered viral suspensions peaked in September and October 2015 and again in February and March 2016 (Table 3.2.)

Table 3.2. Recovered viral suspensions of the water samples positive for enterovirus with rtRT-PCR.

WWTP	Sample Type	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
1	Wastewater	-	-	-	-	-	+	+	-		-	+	+
	Discharge	-	-	-	-	-	-	-	-		-	-	-
2	Wastewater	-	-	-	-	-	+	-	-	-	+	+	+
	Discharge	-	+	-	-	-	-	-	-	-	-	-	-
3	Wastewater	-	-	-	-	-	-	-	-	-	-	-	-
	Discharge	-	-	-	-	-	-	-	-	-	-	-	-
4	Wastewater	+		-		+	+			-	+		+
	Discharge	-		-		-	-			-	-		-
5	Wastewater	-	-	+	-	-	+	+	-	-	-	-	-
	Discharge	-	-	-	-	-	-	-	-	-	-	+	-
1 TO 5	Downstream	-	-	-	-	-	-	-	-	-	-	-	-
6	Upstream	-	-	-	-	-	-	-	-	-	-	-	-
	Discharge	-	-	-	-	-	-	-	-	-	-	-	-
	Downstream	-	-	-	-	-	-	-	-	-	-	-	-

*WWTP = Wastewater treatment plant. Areas marked in grey indicate samples not received.

Enteroviruses were detected in the recovered viral suspensions of wastewater samples from WWTP-1 (4/11), 2 (4/12), 4 (5/7) and 5 (3/12), and in the wastewater discharge samples from WWTP-2 (1/12) and 5 (1/12). No EVs were detected in WWTP-3 and 6, as well as the downstream surface dam water and upstream surface river water samples.

Integrated cell culture rtRT-PCR

Enteroviruses were detected in 52.6% (82/156) of the harvested cell culture extracts when representing the combined cell culture results for each sample. This included 85.2% (46/54), 40.9% (27/66) and 25.0% (9/36) of the cell cultures infected with wastewater, wastewater discharge and downstream surface river water samples, respectively. No EVs were detected in the wastewater discharge samples from WWTP-3, the downstream surface dam water samples and the upstream surface river water samples (Table 3.3.).

Table 3.3. Harvested cell culture extracts positive for enteroviruses with integrated cell culture-rtRT-PCR in the respective cell lines.

WWTP	Sample Type	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
1	Wastewater	BP	P	-	BLP	BP	BP	P	BLP		BP	BP	BP
	Discharge	-	-	P	P	P	P	-	-		-	P	-
2	Wastewater	BLP	P	P	BP	P	P	BP	BLP	BP	BP	BP	BP
	Discharge	B	P	-	P	-	-	-	-	-	P	-	-
3	Wastewater	BLP	-	-	-	-	BP	BP	P	BP	-	P	BLP
	Discharge	-	-	-	-	-	-	-	-	-	-	-	-
4	Wastewater	BP		P		BP	BLP			BLP	BP		BLP
	Discharge	P		-		-	P			-	-		-
5	Wastewater	BLP	-	P	P	L	P	-	BP	BP	BLP	BP	BP
	Discharge	-	-	-	L	P	-	B	BP	-	BP	BLP	-
1 TO 5	Downstream	-	-	-	-	-	-	-	-	-	-	-	-
6	Upstream	-	-	-	-	-	-	-	-	-	-	-	-
	Discharge	-	P	P	P	P	BP	-	P	BLP	P	BLP	BP
	Downstream	-	-	P	-	BLP	BP	BP	BP	P	BP	P	BP

B = BGM cell line; L = L20B cell line; P = PLC/PRF/5 cell line; Downstream = surface water receiving effluent discharge; Upstream = surface water upstream of discharge point; WWTP = Wastewater treatment plant. Areas marked in grey indicate samples not received.

When determining the prevalence of EVs in the individual cell lines, 30.5% (143/468) were positive for EVs of which 62.9% (90/143), 25.9% (37/143) and 11.2% (16/143) were harvested cell culture extracts from wastewater, wastewater discharge and downstream surface river water samples, respectively. Enteroviruses were detected in

54.4% (78/143), 33.6% (48/143) and 11.9% (17/143) of the PLC/PRF/5, BGM and L20B harvested cell cultures.

The harvested cell culture extracts of samples from WWTP-6 had the highest EV detection frequency (32/143), followed by WWTP-5 (28/143), WWTP-2 (26/143), WWTP-1 (25/143), WWTP-4 (18/143) and WWTP-3 (14/143). All the wastewater-infected cell cultures from WWTP-2 and 4 were positive for EV with ICC-rtRT-PCR, while their wastewater discharge-infected cell cultures were only positive in 4/12 and 2/7 months, respectively. The cell culture extracts of wastewater samples from WWTP-1 were the third most prevalent with 10/11 months positive for EVs, followed by WWTP-5 with 7/12 months positive for EVs.

When comparing the isolation of cytopathogenic EVs with ICC-rtRT-PCR, cytopathogenic EVs were isolated from 40.2% (33/82) of the cell culture extracts that were positive for EVs with ICC-rtRT-PCR. This included 48.5% (16/33) of harvested cell cultures from wastewater samples, 33.3% (11/33) from wastewater discharge samples and 18.2% (6/33) from downstream surface river water samples.

Conventional RT-nPCR

Only the harvested cell culture extracts that were positive for EV with ICC-rtRT-PCR were selected for EV characterisation with RT-nPCR. The nucleic acid of all the recovered viral suspensions were amplified with EV-specific RT-nPCR, regardless of their EV-specific one-step rtRT-PCR result, and 69.2% (108/156) were positive for EVs. Enteroviruses were detected in 90% (49/54) of the wastewater, 71.2% (47/66) of the wastewater discharge and 33.3% (12/36) of the downstream surface river water samples. No EVs were detected in the upstream surface river water, wastewater discharge WWTP-3 and downstream surface dam water with direct RT-nPCR.

All recovered viral suspensions of the wastewater samples from WWTP-1, 2, 4 and 5 were positive for EV with direct RT-nPCR, except for WWTP-3 with only 7/12 wastewater samples positive for EV. The wastewater discharge samples received from WWTP-5 were positive for EV in the recovered viral suspensions from April 2015 to March 2016, followed by WWTP-2 from May 2015 to March 2016. Enteroviruses were detected in the recovered viral suspensions of 5/7 wastewater discharge samples

received from WWTP-4, and in 7/11 wastewater discharge samples received by WWTP-1 (Table 3.4.)

Table 3.4. Recovered viral suspensions of the water samples positive for enterovirus with RT-nested PCR.

WWTP	Sample Type	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
1	Wastewater	+	+	+	+	+	+	+	+		+	+	+
	Discharge	-	-	+	+	+	-	-	+		+	+	+
2	Wastewater	+	+	+	+	+	+	+	+	+	+	+	+
	Discharge	-	+	+	+	+	+	+	+	+	+	+	+
3	Wastewater	-	+	+	+	+	+	-	-	-	-	+	+
	Discharge	-	-	-	-	-	-	-	-	-	-	-	-
4	Wastewater	+		+		+	+			+	+		+
	Discharge	+		-		+	+			-	+		+
5	Wastewater	+	+	+	+	+	+	+	+	+	+	+	+
	Discharge	+	+	+	+	+	+	+	+	+	+	+	+
1 TO 5	Downstream	-	-	-	-	-	-	-	-	-	-	-	-
6	Upstream	-	-	-	-	-	-	-	-	-	-	-	-
	Discharge	+	+	+	+	+	+	+	+	+	+	+	+
	Downstream	+	+	+	+	+	+	+	+	+	+	+	+

*WWTP = Wastewater treatment plant. Areas marked in grey indicate samples not received.

Both the recovered viral suspensions from the wastewater discharge and downstream surface river water of WWTP-6 were positive for EV with direct RT-nPCR from April 2015 to March 2016. Overall, the highest number of EV-positive recovered viral suspensions were detected in August 2015 and March 2016 with each 11/14 monthly received samples positive for EV with direct RT-nPCR.

With regards to the EV detection difference between the wastewater and wastewater discharge of WWTP-1 to 5, with direct RT-nPCR it was WWTP-5 that had a 100% detection rate in both the wastewater and wastewater discharge. This was followed by WWTP-2 that only had an 8.3% difference (100% to 91.7%), followed by WWTP-4 with a 28.6% difference (100% to 71.4%) and WWTP-1 with a 36.4% difference (100% to 63.6%). When comparing the ICC-rtRT-PCR detection frequency difference for the wastewater and wastewater effluent samples it shows that WWTP-5 only has a 33.3% difference (83.3% to 50%), followed by WWTP-1 with a 45.4% difference (90.9% to

45.5%), WWTP-2 with a 66.7% difference (100% to 33.3%) and WWTP-4 with a 71.4% difference (100% to 28.6%).

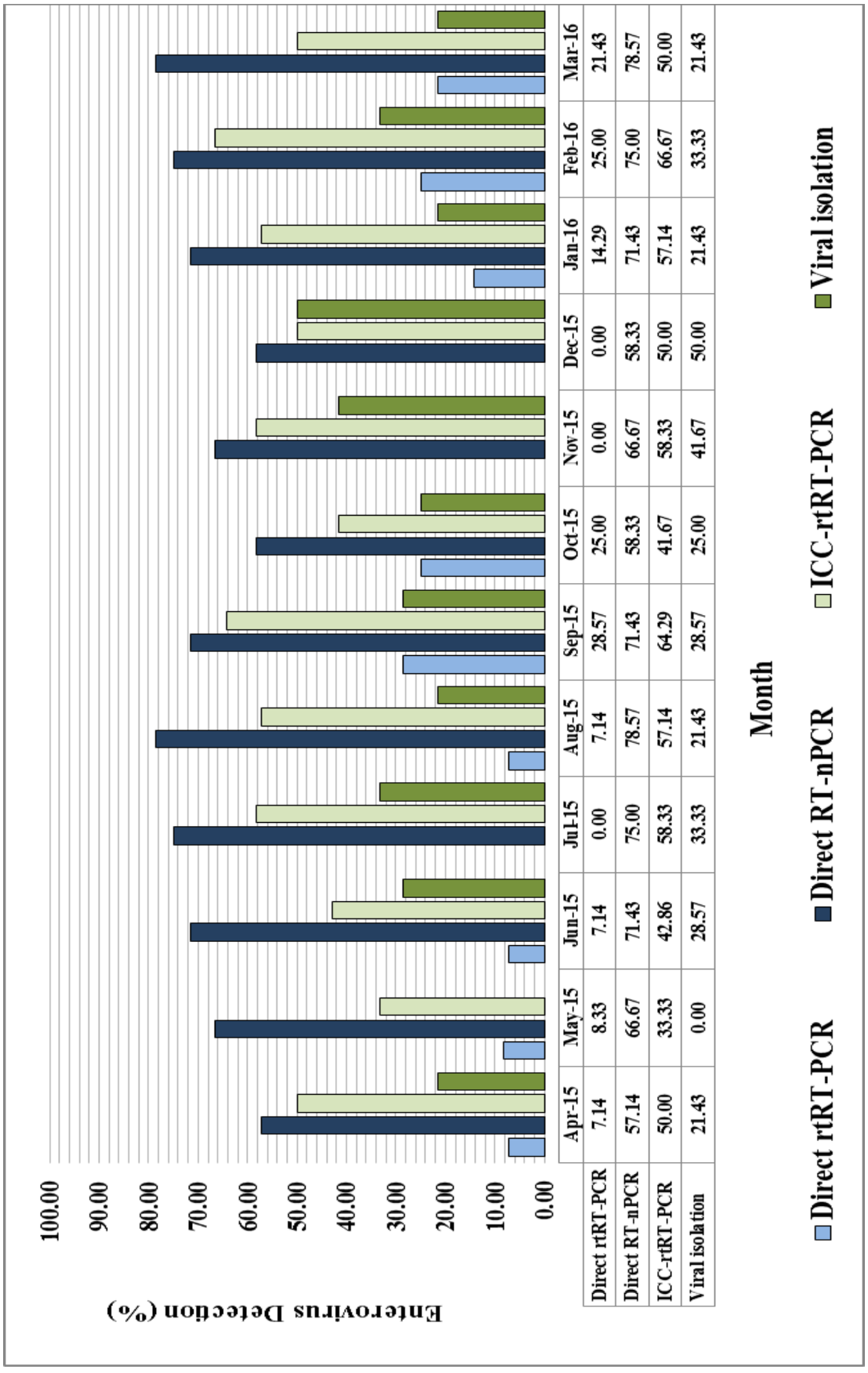
Wastewater treatment plants 1, 2, 4 and 5 had an EV-positivity rate in the wastewater discharge samples of 63.6%, 91.7%, 71.4% and 100% with direct RT-nPCR, respectively. The detection frequency decreased with ICC-rtRT-PCR to 45.5%, 33.3%, 28.6% and 50% of the wastewater discharge samples from WWTP-1, 2, 4 and 5. The viral isolation results showed that 9.1%, 16.7% and 58.3% of the wastewater discharge samples of WWTP-1, 2 and 5 were positive for cytopathogenic EVs, while no cytopathogenic EVs were isolated from WWTP-3 and 4. For WWTP-6, the discharge and downstream surface water samples had a 100% detection frequency with direct RT-nPCR, with an 83.3% and 75% detection rate with ICC-rtRT-PCR, respectively. The frequency of samples positive for cytopathogenic EVs isolated were lower, with a 41.7% and 58.3% detection frequency, respectively

Figure 3.1 displays the monthly prevalence of EVs detected with molecular and viral isolation methods. Figure 3.2 presents the EV detection frequency for each individual WWTP with molecular and viral isolation methods.

Sabin-like poliovirus one-step rtRT-PCR

Three Sabin-like PV one-step rtRT-PCR assays were optimised for each individual serotype. No PVs were detected in the recovered viral suspensions, while 26 harvested cell culture extracts tested positive for Sabin-like PVs, of which 46.2% (12/26), 38.5% (10/26) and 15.4% (4/26) were identified as PV-3, 2 and 1, respectively.

The majority of Sabin-like PVs were detected in harvested cell culture extracts from wastewater (73.1%), followed by the wastewater discharge from WWTP-5 (23.1%) and a single detection in the downstream surface river water sample (3.8%). Sabin-like PV-2 and 3 were detected in all the WWTPs, while Sabin-like PV-1 was limited to wastewater and wastewater discharge from WWTP-1 and 5. The majority of Sabin-like PV detections were in the harvested cell culture extracts of WWTP-4 (7/26) and 5 (7/26).



	Apr-15	May-15	Jun-15	Jul-15	Aug-15	Sep-15	Oct-15	Nov-15	Dec-15	Jan-16	Feb-16	Mar-16
Direct rtRT-PCR	7.14	8.33	7.14	0.00	7.14	28.57	25.00	0.00	0.00	14.29	25.00	21.43
Direct RT-nPCR	57.14	66.67	71.43	75.00	78.57	71.43	58.33	66.67	58.33	71.43	75.00	78.57
ICC-rtRT-PCR	50.00	33.33	42.86	58.33	57.14	64.29	41.67	58.33	50.00	57.14	66.67	50.00
Viral isolation	21.43	0.00	28.57	33.33	21.43	28.57	25.00	41.67	50.00	21.43	33.33	21.43

Figure 3.1. Monthly enterovirus detection frequency in the water samples with molecular and viral isolation methods

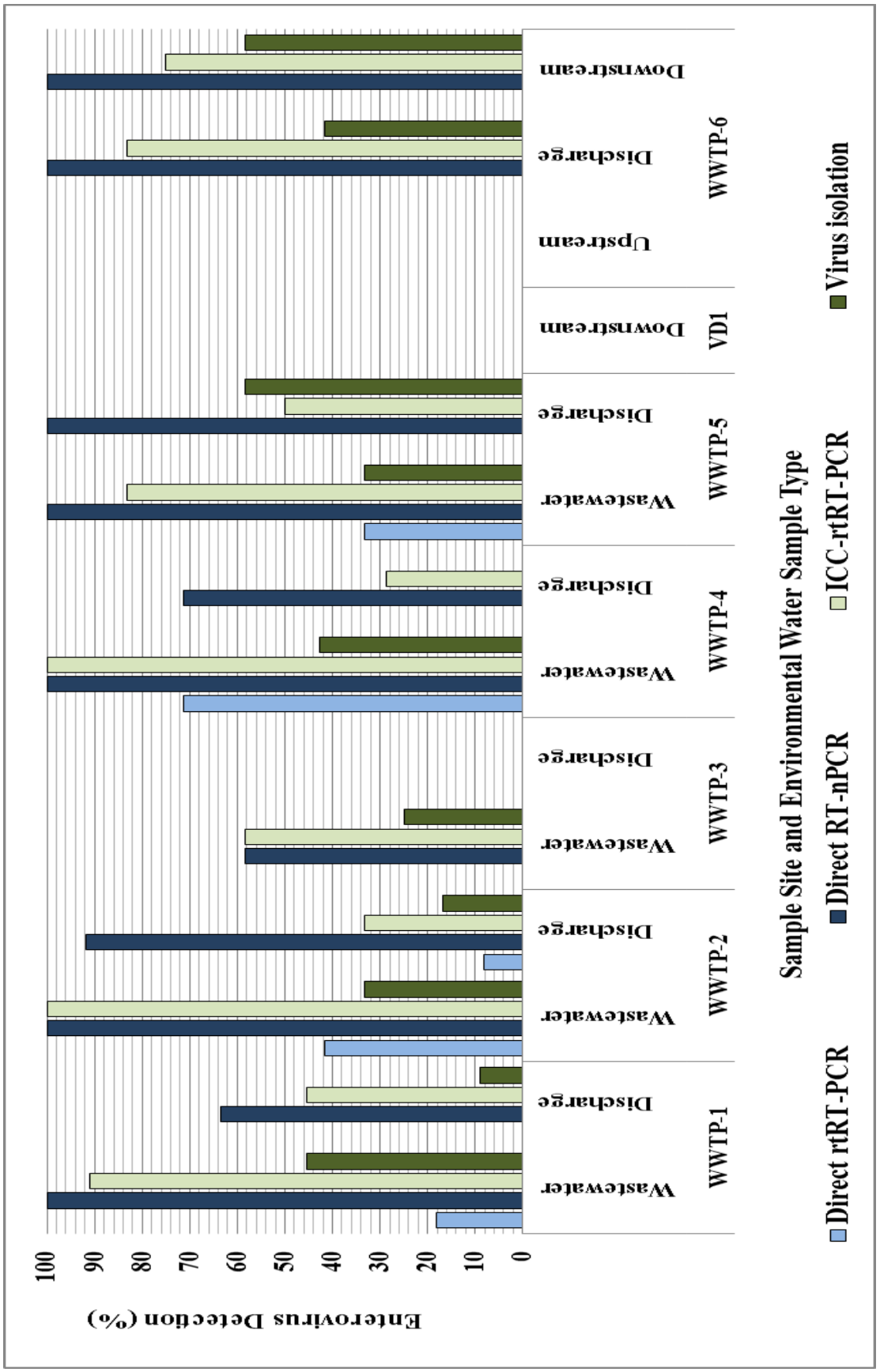


Figure 3.2. Enterovirus detection frequency in the water samples from the selected treatment plants and surface water sites with molecular and viral isolation methods

3.3. NUCLEOTIDE SEQUENCE ANALYSIS

3.3.1. Genotyping from the recovered viral suspensions

In total, 192 EVs were identified in the 108 samples that were EV positive with direct RT-nPCR, of which 45.8%, (88/192), 40.1% (77/192) and 14.1% (27/192) were detected in wastewater, wastewater discharge and downstream surface river water, respectively. Of the 192 EVs, 28 different EV strains were identified, which included eight EV-A species, 14 EV-B species and seven EV-C species. The total number of EVs that were identified within each species was 21.4% (41/192) within EV-A, 24.5% (47/192) within EV-B and 54.2% (104/192) within EV-C species. The 10 most common EV types identified were EV-C99 (21.9%), CV-A22 (10.4%), CV-A20 (8.3%), CV-A13 (7.3%), E-6 (7.3%), CV-A16 (6.8%), CV-A2 (6.3%), EV-C116 (5.2%), EV-A119 (4.7%) and E-14 (3.2%) with CV-A9 (3.2%).

Within each species, CV-A16 (13/41) was the most predominant EV type in the EV-A species, E-6 (14/47) in the EV-B species and EV-C99 (42/104) in the EV-C species. Enterovirus C99 was found to be the most common EV type identified in 27.3% (24/88) of the wastewater and 20.8% (16/77) of the wastewater discharge, while it was E-6 that predominated in 18.5% (5/27) of the downstream surface river water (Figure 3.3 to 3.5).

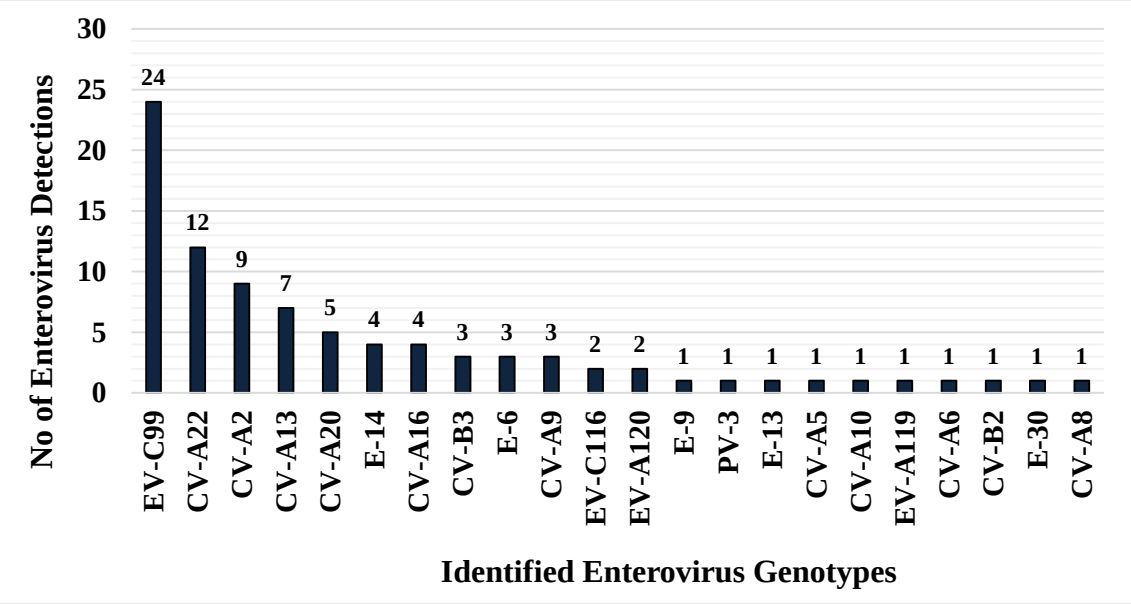


Figure 3.3. Number of enterovirus genotype detections in the wastewater with direct RT-PCR analysis.

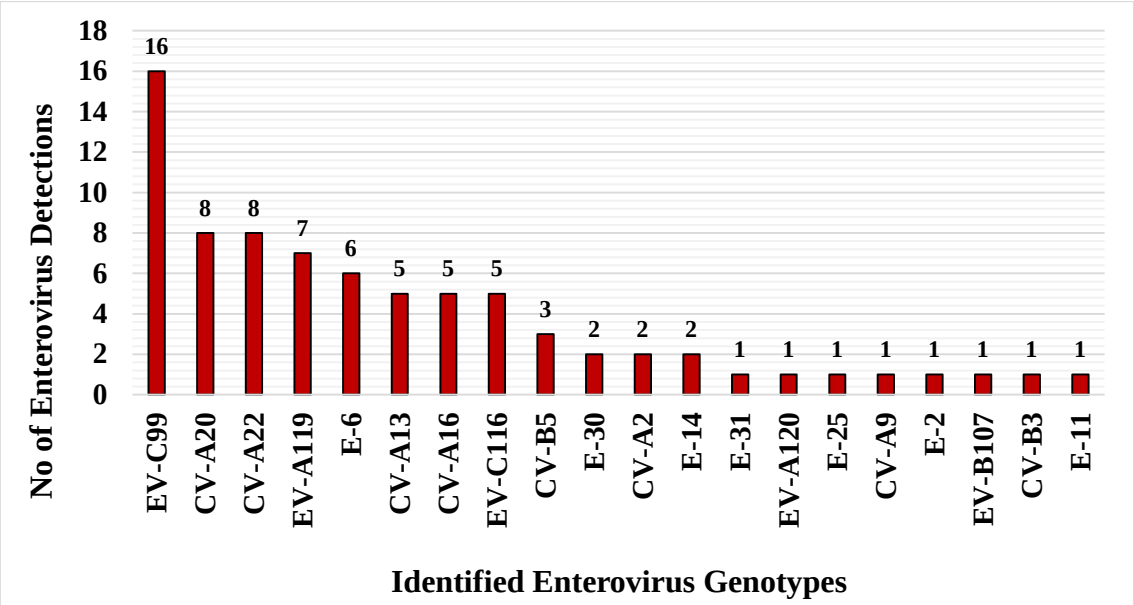


Figure 3.4. Number of enterovirus genotype detections in the wastewater discharge with direct RT-PCR analysis..

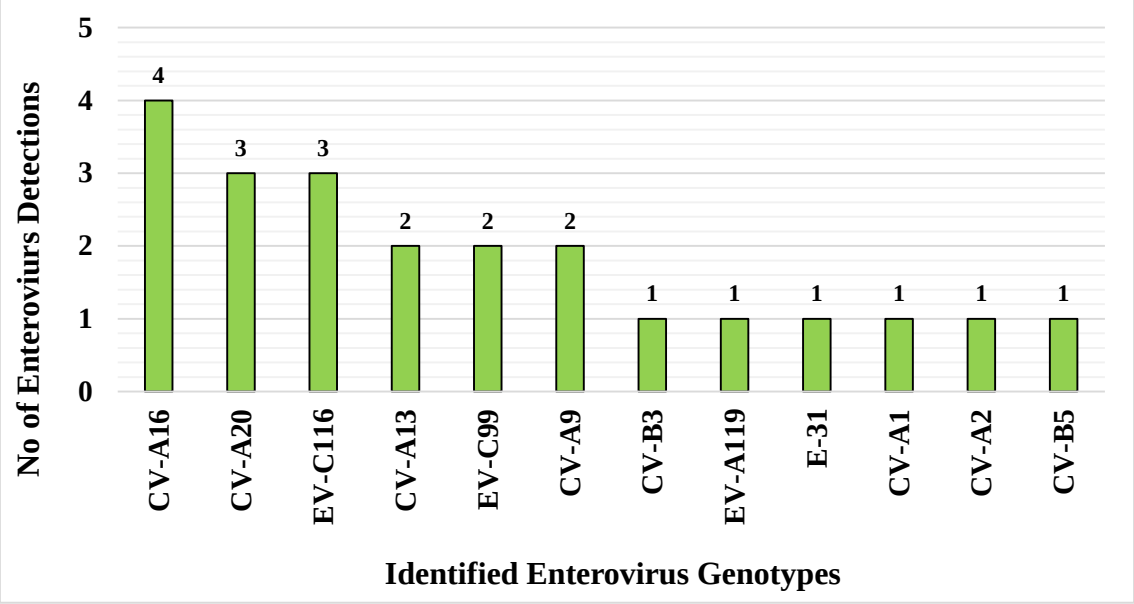


Figure 3.5. Number of enterovirus genotype detections in the downstream surface river water samples with direct RT-PCR analysis.

Ten out of the 28 different EV genotypes were identified in all three water sources, namely wastewater, wastewater discharge and downstream surface river water. These EVs were EV-C99, CV-A20, CV-A13, E-6, CV-A16, CV-A2, EV-C116, EV-A119, CV-A9 and CV-B3 (Figure 3.6).

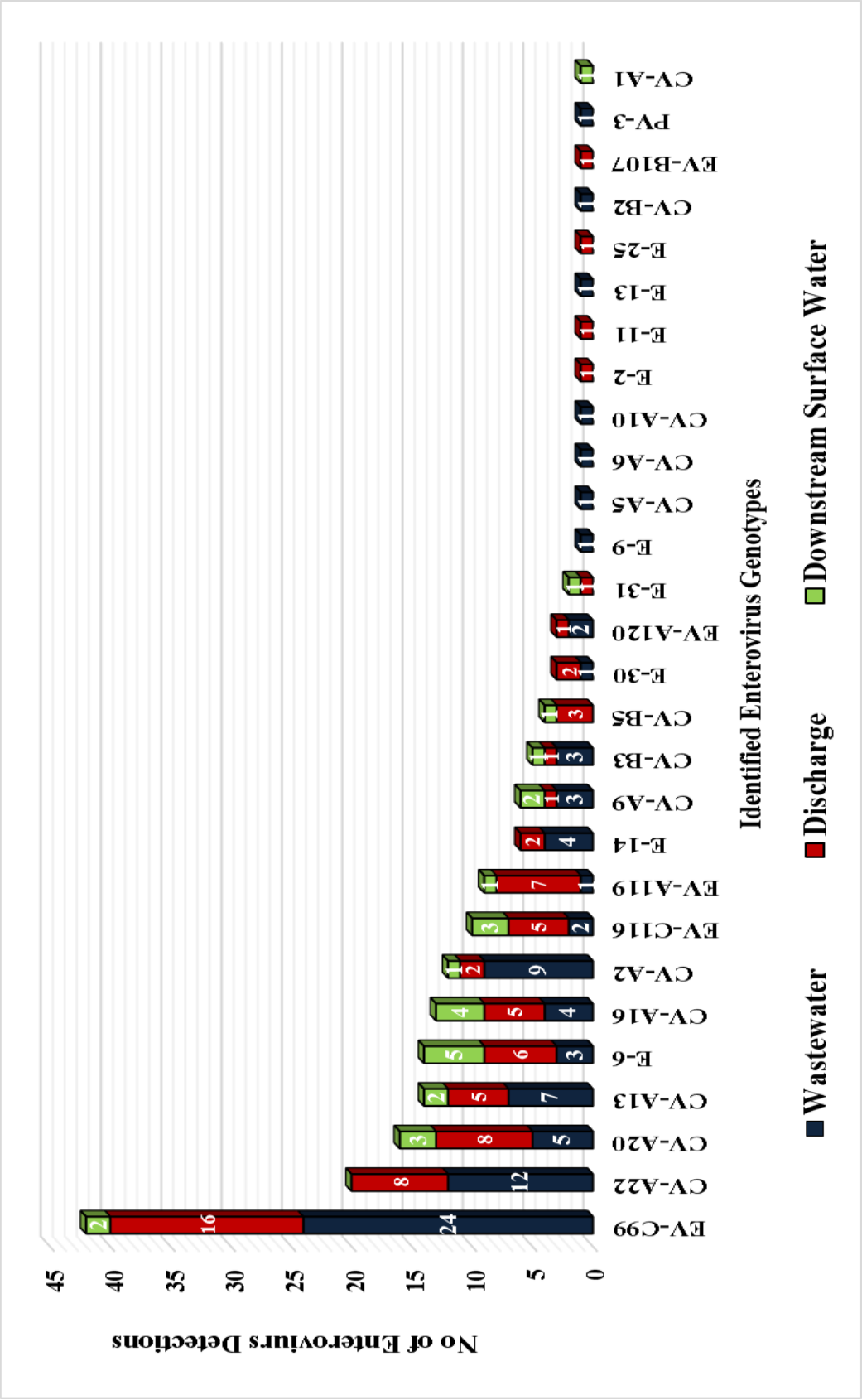


Figure 3.6. Genotypic prevalence of the different enterovirus genotypes identified in the three water types with direct RT-PCR analysis.

The month with the highest percentage of EV genotypes identified was in July 2015 with 12.1% (23/190), followed by January 2016 with 11.6% (22/190). The most diverse EV types were found in January 2016 (13/28), followed by April and July 2015 (12/28).

The widest diversity of EV types were identified in the wastewater samples, namely in WWTP 5 (n=14), followed by WWTP-2 (n=12), WWTP-1 (n=9), WWTP-4 (n=7) and WWTP-3 (n=6). This closely corresponded with the diversity of EV types in the wastewater discharge samples in WWTP-5 (n=10), WWTP-2 (n=9), WWTP-1 (n=6), WWTP-4 (n=5) and WWTP-3 (n=0). Wastewater treatment plant 3 was the only plant where EVs were not detected in the wastewater discharge. Enterovirus-C species were the most prevalent in the wastewater and wastewater discharge in WWTP-1 to 6, namely EV-C99, CV-A22 and EV-C116. Coxsackievirus A2 was the most prevalent in WWTP-5 and EV-A119 in WWTP-6. Echovirus 6 and CV-B5 were most prevalent in WWTP-6 and WWTP-1, respectively. Enterovirus types identified in the wastewater, wastewater discharge and surface water of WWTP 1-6 with direct RT-PCR analysis are summarised in Table 3.5.

Enterovirus C99 was detected in both the wastewater and wastewater discharge of WWTP-1, 2, 4 and 5, as well as the wastewater discharge and downstream surface river water of WWTP-6. Enteroviruses that were detected before and after wastewater treatment in more than one WWTP or in both the discharge and downstream surface water of WWTP-6 was CV-A22 (WWTP-1, 2, 5), CV-A20 (WWTP-1, 2, 6), CV-A2 (WWTP-2, 5), CV-A16 (WWTP-2, 5, 6), EV-A119 (WWTP-5, 6) and E-6 (WWTP-5, 6). The treatment plant with the majority of EV types that were detected before and after wastewater treatment was WWTP-5, namely EV-A119, CV-A2, CV-A16, E-6, E-14, CV-A9, EV-C99 and CV-A22.

This was followed by WWTP-2 with five EV types detected in the wastewater and wastewater discharge, such as CV-A2, CV-A16, EV-C99, CV-A20 and CV-A22. Three EV types were detected before and after treatment in WWTP-1, namely EV-C99, CV-A20 and CV-A22, while EV-C99 was the only EV type in WWTP-4 to be detected before and after treatment.

Table 3.5. Enteroviruses within species A to C identified in the selected wastewater treatment plants with direct RT-PCR analysis.

WWTP	Sample Type	EV-A	EV-B	EV-C
1	**Wastewater**	CV-A2, -A6	CV-B3; E-30	**EV-C99**; CV-A13, -A20, -A22
	Discharge	CV-A16	**CV-B5**	EV-C99, -C116; CV-A20,**-A22**
2	**Wastewater**	CV-A2, -A16;	CV-B2, -B3;CV-A9; E-6, -9, -13	**EV-C99**; CV-A13, -A20, -A22
	Discharge	CV-A2, -A16; EV-A119	E-25, -30;	**EV-C99**; CV-A20, -A22
3	**Wastewater**	CV-A16	E-6, -9, -14	**EV-C99**; **CV-A22**
	Discharge		None	
4	**Wastewater**	CV-A2, -A5, -A16	CV-B3; E-14	**EV-C99**; CV-A13
	Discharge	EV-A119, -A120	E-6;	EV-C99, -C116
5	**Wastewater**	EV-A119, -A120; **CV-A2**, -A8, -A10, -A16	E-6, -14; CV-A9	**EV-C99**, -C116; PV-3; CV-A20, **-A22**
	Discharge	CV-A2, -A16; EV-A119	E-2, -6, -14; CV-A9	**EV-C99**; CV-A13, -A22
1-5	**Downstream**		None	
6	**Upstream**		None	
	Discharge	CV-A16; **EV-A119**	CV-B3; EV-B107; **E-6**, -11, -15, -30, -31	EV-C99, **-C116**; CV-A13, -A20, A22;
	Downstream	CV-A16; EV-A119	CV-B3, -B5; CV-A9; **E-6**	EV-C99, -C116; CV-A1, -A13, -A20;

*WWTP = Wastewater treatment plant; E = Echovirus; CV = Coxsackievirus. Samples labelled in red are the most prevalent EV types identified.

It was mainly EV-C99 that was the most predominant EV type identified in the wastewater from WWTP-1 to 5, along with CV-A22 in WWTP-3 and 5, and CV-A2 in WWTP-5. Coxsackievirus-A22 and CV-B5 were most prevalent in the wastewater discharge of WWTP-1, EV-C99 in WWTP-2 and EV-C99 along with CV-A22 in WWTP-5. One EV type from each EV-A to C species, namely EV-A119, E-6 and EV-C116, were the most prevalent in the wastewater discharge of WWTP-6, while it was only E-6 that predominated in the downstream surface river water. No predominant EV genotype was identified in the discharge of WWTP-4 as there were only single detections of six EV genotypes.

3.3.2. Genotyping of isolates from harvested cell culture extracts

In total, 137 EVs were identified in the 86 samples that were EV positive after ICC-real-time RT-PCR (ICC-rtRT-PCR) analysis, of which 60% (81/137), 27.4% (37/137) and 12.6% (17/137) were detected in wastewater, wastewater discharge and downstream surface river water, respectively. Of the 137 EVs, 21 different EVs were identified, which included two EV-A species, 13 EV-B species and six EV-C species. The total number of EVs that were identified within each species was 2.2% (3/137) in EV-A, 76.6% (105/137) in EV-B and 21.2% (29/137) in EV-C species. The 10 most common EV types identified were CV-B3 (16.8%), E-6 (15.3%), CV-B4 (13.9%), CV-B5 (8.0%), CV-A13 (8.0%), E-13 (7.0%), Sabin-like PV-3 (7.0%), E-1 (4.3%), E-11 (3.6%) and Sabin-like PV-2 (3.6%).

Within each species, CV-A16 (2/3) was the most common EV type in the EV-A species, CV-B3 (23/105) in the EV-B species and CV-A13 (11/29) in the EV-C species. Coxsackievirus B3 predominated in the wastewater with 19.8% (16/81), E-6 in the wastewater discharge with 18.9% (7/37) as well as in the downstream surface river water with 35.3% alongside CV-B4 (Figure 3.7 to 3.9).

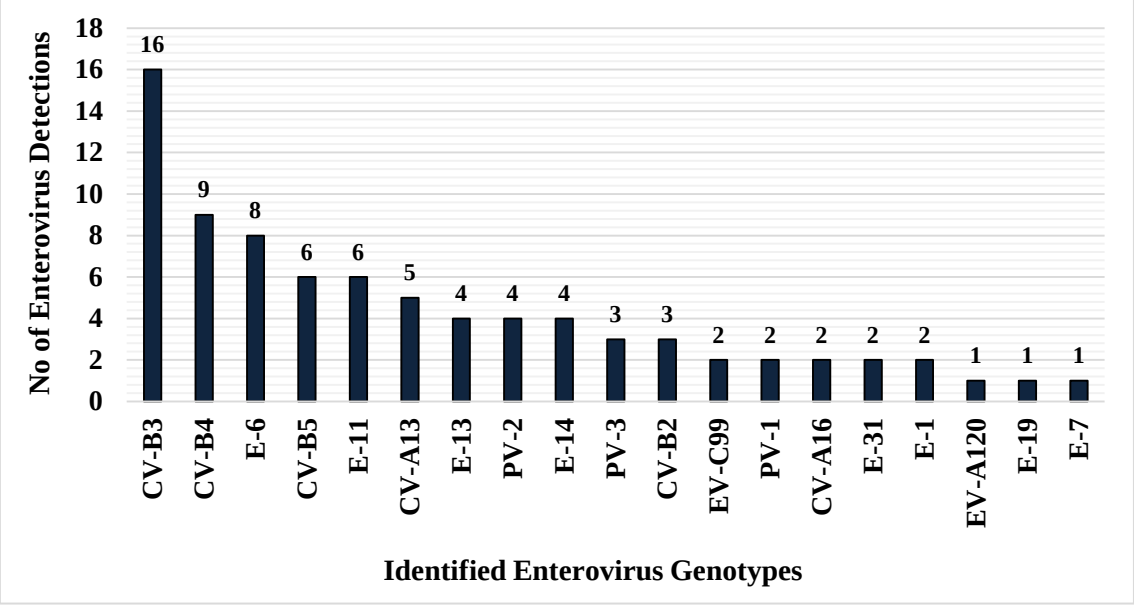


Figure 3.7. Number of enterovirus genotype detections in the wastewater after viral amplification in cell culture.

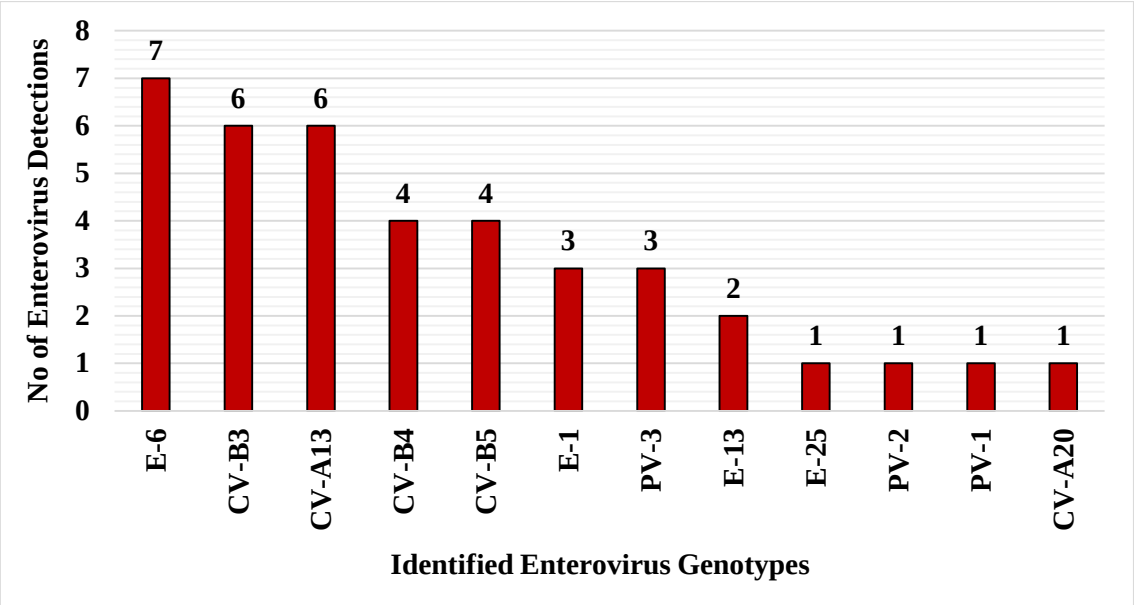


Figure 3.8. Number of enterovirus genotype detections in the wastewater discharge after viral amplification in cell culture.

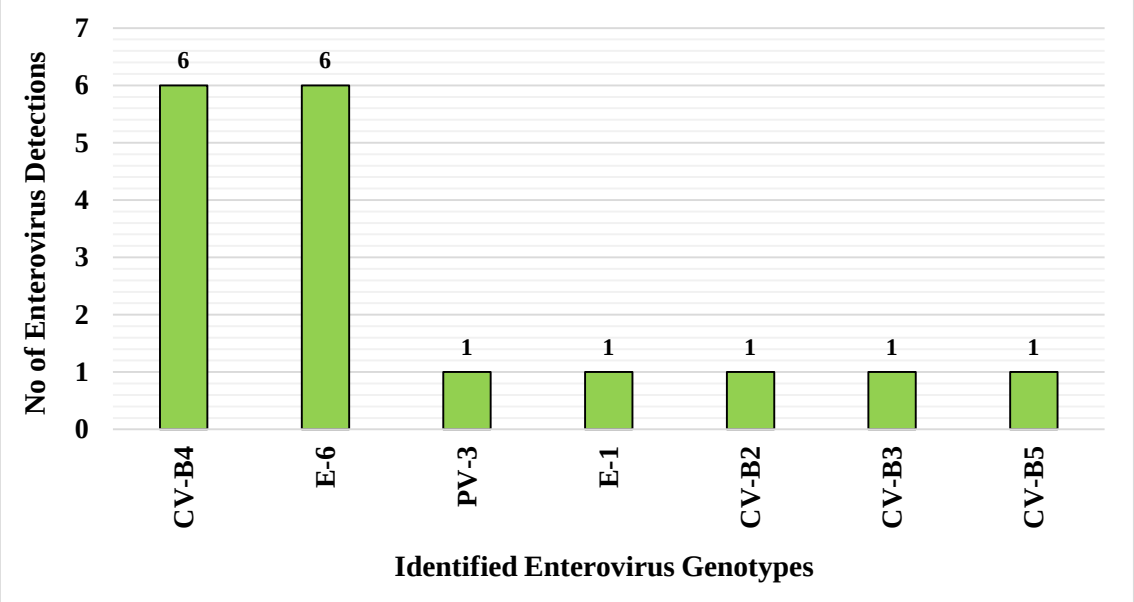


Figure 3.9. Number of enterovirus genotype detections in the downstream surface river water after viral amplification in cell culture.

Six out of the 21 EV genotypes were identified in three different water sources, namely wastewater, wastewater discharge and downstream surface river water. These genotypes included CV-B3, CV-B4, CV-B5, E-1, E-6 and Sabin PV-3 (Figure 3.10).

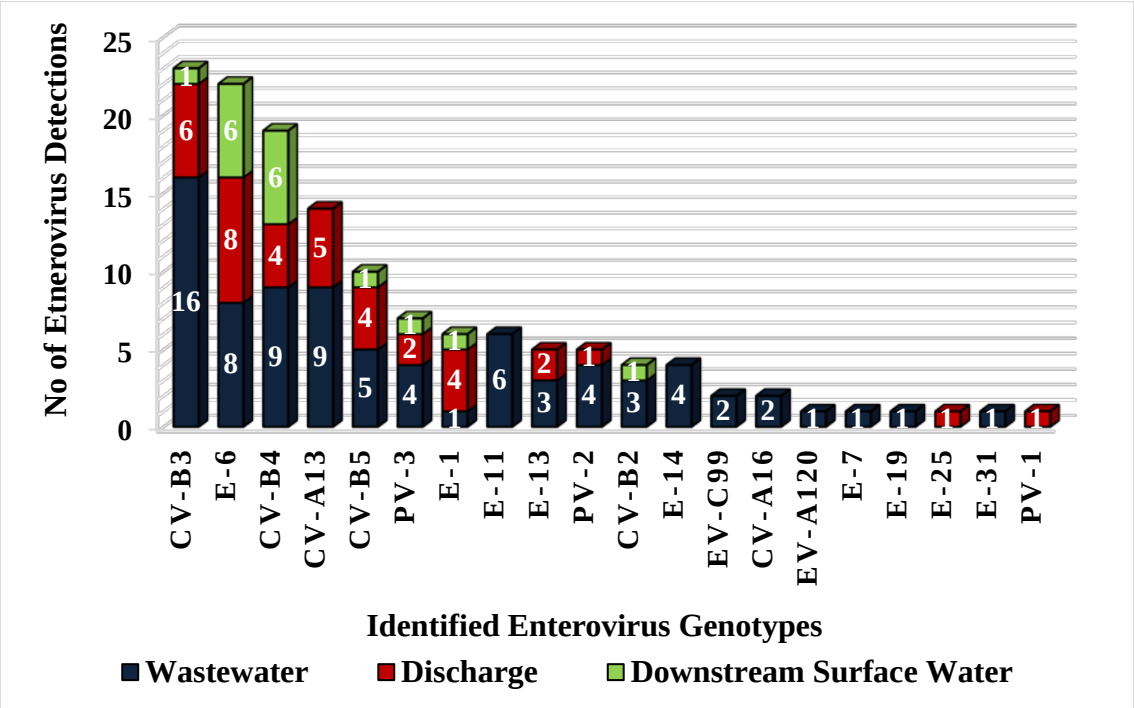


Figure 3.10. Number of enterovirus detections in the three water sample types summarised after viral amplification in cell culture.

The months with the highest percentage of EV genotypes identified were April and September 2015 with each 12.4% (17/137), followed by December 2015 with 10.2% of all EVs (14/137). The EV detection frequency peaked in April, September and December 2015 and February 2016. The widest diversity of EV genotypes were identified in September 2015 (11/21), with the least diversity of EV genotypes identified in May 2015 (3/21). The widest diversity of EV types were identified in the wastewater samples, namely in WWTP 1 (n=12), followed by WWTP-2, WWTP-3 and WWTP-5 (n=10) and WWTP-4 (n=9). The EV diversity in the wastewater discharge was the highest in WWTP-5 (n=9), followed by WWTP-6 (n=8), WWTP-1 (n=4), WWTP-2 (n=4), WWTP-4 (n=1) and WWTP-3 (n=0). Seven different EV types were identified in the downstream surface river water. Wastewater treatment plant 3 was the only plant where EVs were not detected in the wastewater discharge.

Enterovirus B species were most prevalent in the wastewater, wastewater discharge and surface water of WWTP-1 to 6. In the wastewater of WWTP-1 to 3 it was CV-B3 that predominated, while an EV-C species, CV-A13, predominated in WWTP-4 and E-6 in

WWTP-5. Coxsackievirus A13 predominated in the wastewater discharge of WWTP-1, while it was E-6 that predominated in WWTP-5 and 6 (Table 3.6).

Table 3.6. Enteroviruses within species A to C identified in the selected wastewater treatment plants after viral isolation in cell culture.

WWTP	Sample Type	EV-A	EV-B	EV-C
1	**Wastewater**		CV-B2, **-B3**, -B4, -B5; E-11, -13, -14	CV-A13; EV-C99; PV-1, -2
	Discharge		CV-B3, -B5; E-25	**CV-A13**
2	**Wastewater**		CV-B2, **-B3**, -B4, -B5; E-6, -11, -13	EV-C99; PV-2, -3
	Discharge		CV-B3, -B5; E-13	CV-A13
3	**Wastewater**		**CV-B3**, -B4; E-1, -6, -7, -11, -14, -31	PV-2; EV-C116
	Discharge		None	
4	**Wastewater**	CV-A16;	CV-B3-B5; E-11, -31	**CV-A13**; PV-2, -3
	Discharge			CV-A13
5	**Wastewater**	CV-A16; EV-A120	CV-B3-B5; E-1, **-6**, -9, E-14	PV-3
	Discharge		CV-B3-B5; E-1, **-6**	CV-A13; PV-1-3
1-5	**Downstream**		None	
6	**Upstream**		None	
	Discharge		CV-B3-B5; E-1, **-6**, -13	CV-A20; PV-3
	Downstream		CV-B2, -B3, **-B4**, -B5; E-1, **-6**	PV-3

*WWTP = Wastewater treatment plant; E = Echovirus; CV = Coxsackievirus. Samples labelled in red are the most prevalent EV types identified.

No predominant EV types were identified in the discharge of WWTP-2 and 4, since only single detections of each type was identified. The EV types that were detected both before and after treatment in more than one WWTP or in both the discharge and downstream surface water of WWTP-6 were CV-B3 and CV-B5 (WWTP-1, 2, 5, 6), CV-B4 (WWTP-5, 6), CV-A13 (WWTP-1, 4), E-1, E-6 and PV-3 (WWTP-5, 6). The majority of EV types detected both before and after treatment was in WWTP-5 with six EV types, CV-B3-B5, E-1, E-6 and Sabin-like PV-3. The exact same EV types were identified in the wastewater discharge and downstream surface river water of WWTP-6.

The EV-B genotypes, CV-B4 and CV-B3, were the most common EV genotypes that were identified in 10 and eight out of the 12 months, followed by the EV-C genotype, CV-A13 detected in seven out of the 12 months. Sixteen different EV genotypes were identified in the PLC/PRF/5 cell line, compared to 13 and four EV genotypes in the BGM and L20B cell line, respectively (Figure 3.11). As expected, the Sabin-like PV genotypes were propagated in the L20B cell line, besides an EV-A species, EV-A120. No EV-A species were able to be propagated and identified in the BGM cell line, while it was able to propagate 10 EV-B genotypes and three EV-C genotypes (PV-1, -2 and -3). The CV-Bs were more frequently identified in the PLC/PRF/5 cell line with 38 detections, compared to 32 detections in the BGM cell line Echoviruses were also mainly identified in the PLC/PRF/5 cell line with 37 detections, compared to 13 detections in the BGM cell line. Furthermore, CV-A and EV-C99 were only identified in the PLC/PRF/5 cell line.

Enteroviruses were characterised in 71.2% (111/156) of the water samples when combining the nucleotide sequence analysis data from the direct RT-PCR and ICC-RT-PCR, with a total of 329 EVs. This included 13.4% (44/329), 39.5% (130/329), 46.2% (152/329) of EVs within the EV-A, EV-B and EV-C species, respectively.

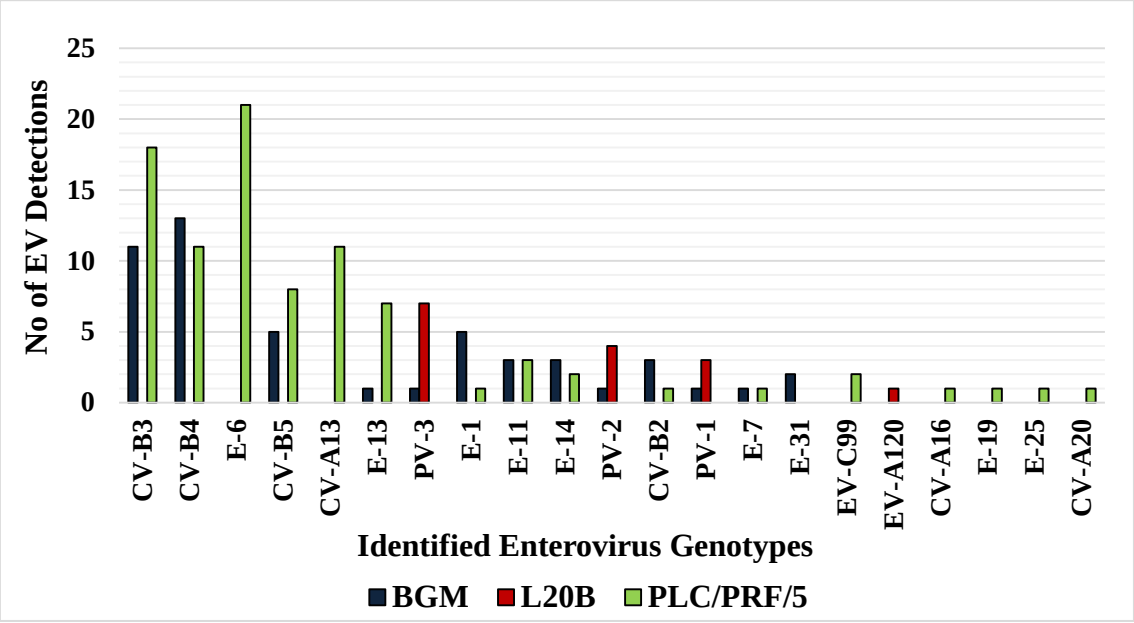


Figure 3.11. Genotypic prevalence of enteroviruses in BGM, L20B and PLC/PRF/5 harvested cell culture extracts.

3.4. PHYLOGENETIC ANALYSIS

3.4.1. Genotypes selected from recovered viral suspensions

The eleven most common EV genotypes, identified in the recovered viral suspensions with direct analysis, were selected for the construction of the Neighbor-Joining phylogenetic tree (Figure 3.12). The EV strains were segregated into three major clusters, EV-A, EV-B and EV-C. Within the EV-A major cluster, three subclusters of CV-A16, EV-A119 and CV-A2 were formed, while in the EV-B major cluster, subclusters of E-6, CV-A9 and CV-B3 were formed. Subclusters of EV-C116, CV-A22, EV-C99 and CV-A, including CV-A13 and CV-A20, were formed in the EV-C major cluster. The strains are labelled according to the WWTP (1-6), sample type [wastewater (R), discharge (E) or downstream river (RD)], year and month of sampling (201510) and the clone number (a,b,c) if multiple clones from one sample are used.

Coxsackievirus A2, E-6, CV-A9, EV-C116 and EV-C99 all had multiple subclusters. For CV-A2, the 5E201506 strain clustered separately from the three other CV-A2 strains, indicating an average of 89.6% nt and 95% aa identity, while both clusters showed similar nt and aa identity towards the CV-A2 reference strains with an average of 92% nt and 97% aa identity. When looking at the EV strains that were identified in the wastewater and wastewater discharge from the same WWTP, it can be seen that two CV-A16 strains identified from WWTP-2 in May 2015 were nearly identical, sharing a nt and aa identity of 99.7% and 99%, respectively. This was also seen with E-6: 5E201602b and 5R201602; CV-B3: 6E201512 and RD201512b; EV-C116: RD201506b and 6E201601b and CV-A20: 2E201603 and 2R201602.

The strains marked in corresponding colours are from the same sample type as well as WWTP but at a different time within the 12-month period. The maroon labelled strains 6E201510 and 6E201601a in the EV-A subgroup are CV-A16 strains identified in discharge samples from WWTP-6 in October 2015 and January 2016 and are shown to be on opposite sides of the cluster. The strains shared a 94.8% nt and 98% aa identity, while 6E201510 shared a higher 98.7% nt and 99% aa identity to the 2R201505 strain from WWTP-2. Similar events were seen with two E-6 strains: RD201504b and RD201603b; two EV-C116 strains: RD201506b and RD201601a and three sets of EV-

C99 strains: 4R, 2E and RD strains. The EV-C99 strains were shown to be the most diverse as the strains had significant where they shared an average nt identity of 82%.

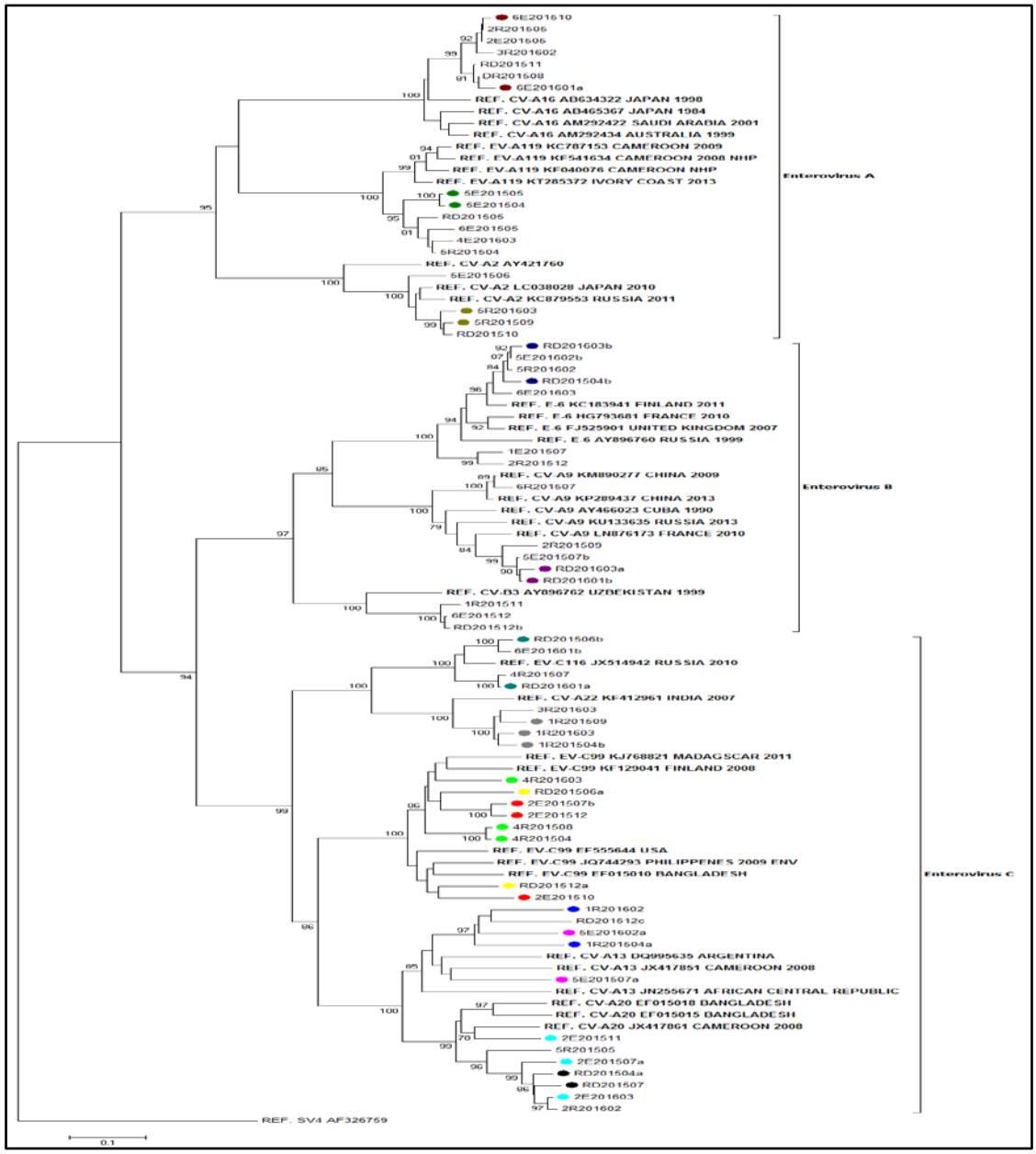


Figure 3.12. Neighbour-Joining phylogenetic tree with a 1000 bootstrap replicates of the enterovirus strains identified in the recovered viral suspensions, including globally recognised reference strains. The evolutionary distances were computed using the Kimura-2-parameter method conducted in MEGA6. The sample ID is the WWTP number and sample type: wastewater (R), discharge (E) or downstream (RD), followed by the year and month of sampling and either an a, b or c if multiple clones from one sample were used. Reference strains are shown in bold and similar EV strains identified from a specific sample over the 12-month period were marked in corresponding colours.

3.4.2. Genotype isolates selected from harvested cell culture extracts

Eight EV genotypes that were identified in the viral isolates were selected for the construction of the Neighbour-Joining phylogenetic tree (Figure 3.13). The EVs were segregated into two major clusters, EV-B and EV-C. Four subclusters were formed within major cluster EV-B: CV-B4, CV-B5, E-1 and E-6. Within EV-C, four subclusters were formed which included CV-A13, Sabin PV-1, PV-2 and PV-3. The strains are labelled with a B (BGM), L (L20B) or P (PLC) depending on the cell line from which they were isolated.

Single subclusters of the specific EV genotypes were formed, which can be seen with CV-B5, E-1, CV-A13, Sabin PV-1, PV-2 and PV-3. Coxsackievirus B4 and E-6 had two subclusters of their EV strains, where the strains were closely identical in terms of their nt and aa identity to the reference strains, but not to each other. The CV-B4 strain, B.RD201603, was in a separate cluster sharing an average nt and aa identity of 89.4% and 89.6% towards the strains in the first subcluster.

Two strains of CV-B5 were identified in the same month from two different sample sites, while showing closer genetic relatedness to each other than to the CV-B5 reference strains, with a 7% nt and 1% aa difference. The three CV-A13 strains that were identified in the wastewater from WWTP-4 in July and September 2015 and January 2016 showed more close genetic relatedness to each other than to the other identified strains from different sites. The Sabin-like PV-1 strains marked in turquoise were identified from wastewater in WWTP-1 in July and November 2015 and shared a 98.1% and 96% nt and aa identity. They clustered together with a closer evolutionary distance from each other compared to their distance from the L.5E201602 strain that was isolated from wastewater discharge from WWTP-5. The Sabin PV strains within the Sabin PV-3 cluster are all genetically grouped together sharing a nt and aa identity ranging between 96-98% and 95-98%, respectively, with the strains furthest from each other showing the lowest identity.

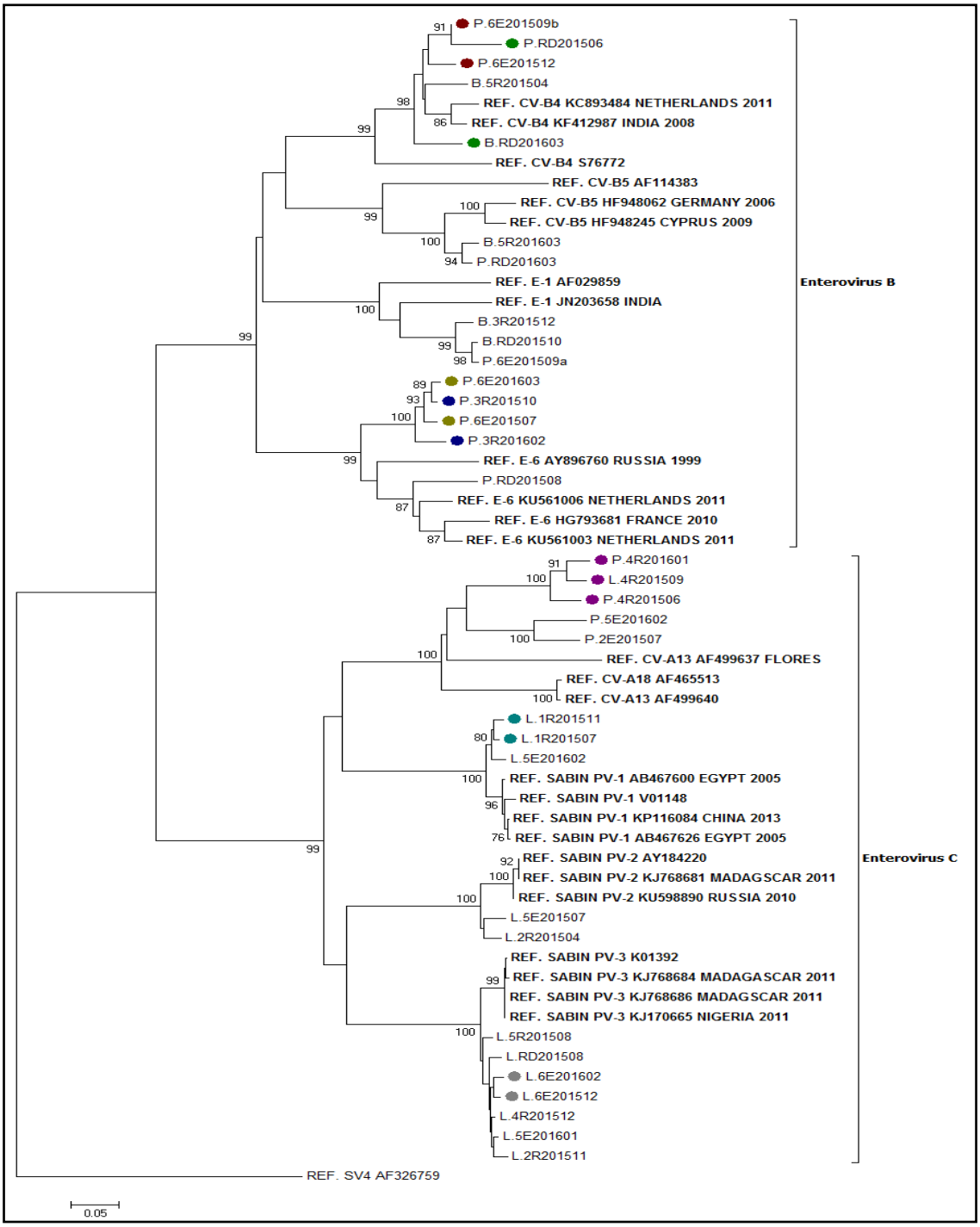


Figure 3.13. Neighbour-Joining phylogenetic tree with a 1000 bootstrap replicates of the enterovirus strains identified in the harvested cell culture extracts, including globally recognised reference strains. The evolutionary distances were computed using the Kimura-2-parameter method conducted in MEGA6. The sample ID is the cell line (BGM, L20B or PLC), WWTP number and sample type: wastewater (R), discharge (E) or downstream (RD), followed by the year and month of sampling and either an a, b or c if multiple clones from one sample were used. Reference strains are shown in bold and similar EV strains identified from a specific sample over the 12-month period were marked in corresponding colours.

3.4.3. Genotypes selected from isolates and recovered viral suspensions

Eleven EV genotypes were selected for the construction of the Neighbor-joining phylogenetic tree which involved comparing the evolutionary distances between direct and isolated EV strains of similar EV genotypes (Figure 3.14). Enterovirus strains were segregated into three major clusters, EV-A, EV-B and EV-C. Within the EV-A major cluster, subclusters of CV-A16 and EV-A120 were formed. Subclusters of CV-B2, E-6, E-25, CV-B3, CV-B5 and E-31 were formed within the EV-B major cluster, while the EV-C major cluster included subclusters of Sabin PV-3, CV-A13 and EV-C99.

When comparing the similar EV genotypes identified from the same sample of a particular month before and after viral amplification in cell culture, the tree displays the formation of separate clusters within their genotype subclusters, as can be seen with EV-A120 (marked in maroon), CV-B3 (marked in red), CV-B5 (marked in grey), CV-A13 (marked in yellow) and EV-C99 (marked in purple). From the EV-A120 cluster, it can be seen that L.5R201504 and 5R201504 are more closely related to each other than to 5R201506 where the strains from April 2015 have an 8% nt difference from the June 2015 strain of EV-A120. When comparing the wastewater and wastewater discharge strains, E-6 strains from WWTP-5 shared a bootstrap value of 95.

Two separate clusters of E-6 were formed, where one cluster only consists of E-6 strains identified from recovered viral suspensions with direct analysis. The second cluster consists of a combination of direct and isolated E-6 strains with the majority being isolated from harvested cell culture extracts. Echovirus 6 strains, 5E201602b and P.5E201602, have been identified in the exact same sample before and after viral amplification in separate clusters sharing an nt identity of 86.4%. Within the two separate clusters, the various strains all shared an nt identity ranging between 94-99% with each other. The EV-C99 strain, 2R201508, is not as closely clustered with the other EV-C99 strains sharing an 82.7% nt identity to the same EV-C99 strain, P.2R201508, after viral amplification in cell culture. The P.2R201508 strain was shown to be more closely related to the other two EV-C99 strains with an nt identity of 88.7%. The same event can be seen with the Sabin PV-3 strains, 5R201505 and L.5R201508 which are strains identified three months apart, where it showed closer genetic relatedness to the reference strains than to each other.

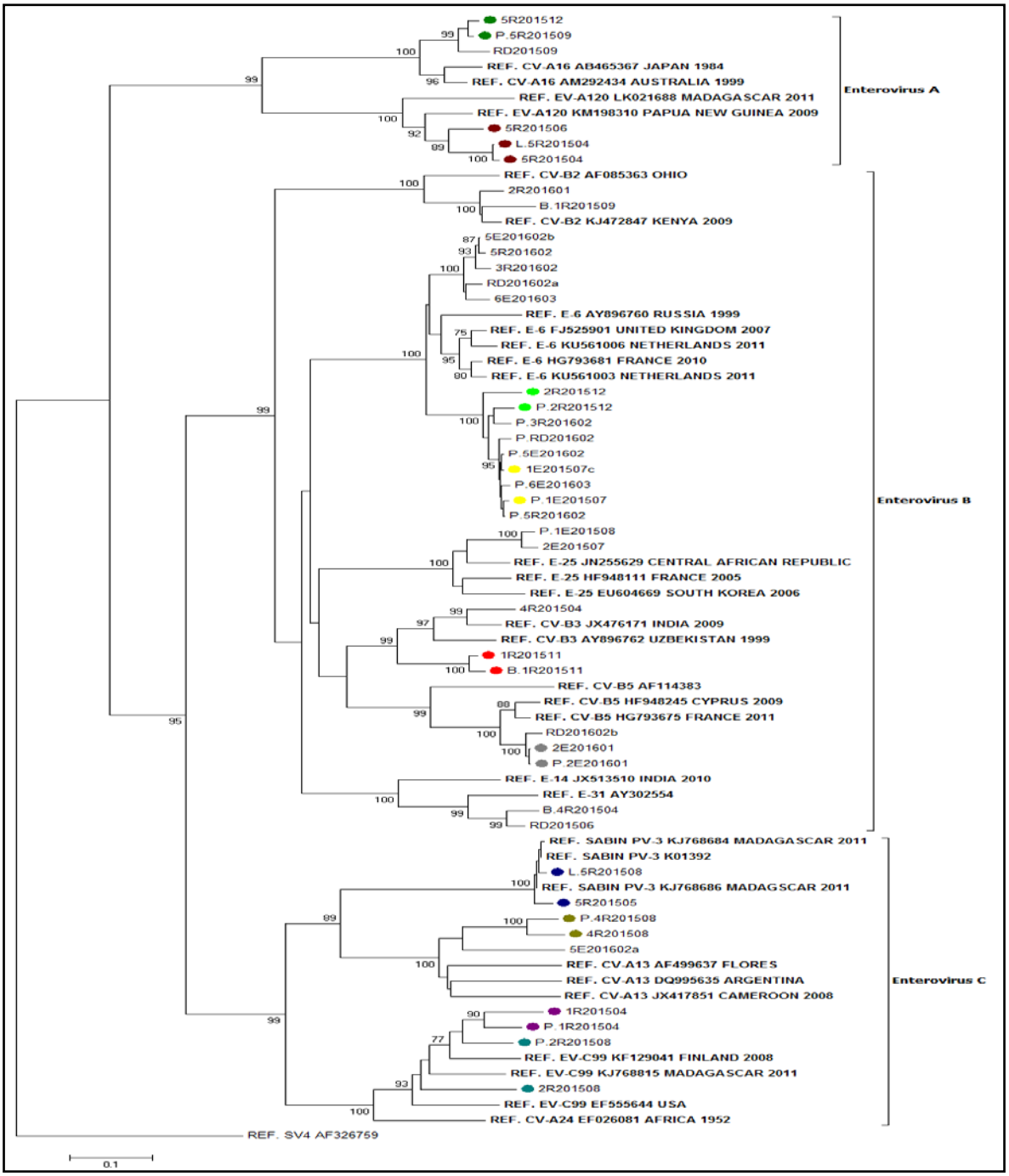


Figure 3.14. Neighbour-Joining phylogenetic tree with a 1000 bootstrap replicates of isolated enterovirus strains and from recovered viral suspensions, including globally recognised reference strains. The evolutionary distances were computed using the Kimura-2-parameter method conducted in MEGA6. The evolutionary distances were computed using the Kimura-2-parameter method conducted in MEGA6. The sample ID is the WWTP number and sample type: wastewater (R), discharge (E) or downstream (RD), followed by the year and month of sampling and either an a, b or c if multiple clones from one sample were used. Reference strains are shown in bold and similar EV strains identified from a specific sample over the 12-month period were marked in corresponding colours.

3.5. INDICATOR ORGANISMS

The total cfu/100 ml for *E.coli* was determined in 120 samples that included samples from WWTP-1 to 5 and VD1. The average *E.coli* count for the wastewater samples were 3.86 x 10^6 cfu/100 ml, 3.16 x 10^5 cfu/100 ml for the discharge samples and 71 cfu/100 ml for VD1. The discharge samples from WWTP-3 had the lowest *E.coli* count enumerated at 25.8 cfu/100 ml, followed by WWTP-1 (255 cfu/100 ml), WWTP-4 (817 cfu/100 ml), WWTP-5 (1.06 x 10^5 cfu/100 ml) and WWTP-2 (1.19 x 10^6 cfu/100 ml) (Table 3.6)

Table 3.7. A summary of the *E.coli* counts (cfu/100 ml) as well as the enterovirus detection results in both direct and ICC-RT-PCR for the 120 samples.

MONTH	DETECTION	WWTP-1		WWTP-2		WWTP-3		WWTP-4		WWTP-5		VD1
		Wastewater (1L)	Effluent (10L)	Wastewater (1L)	Effluent (10L)	Wastewater (1L)	Effluent (10L)	Wastewater (1L)	Effluent (10L)	Wastewater (1L)	Effluent (10L)	Downstream (10 L)
APR-15	*E.coli* cfu/100 ml	6.30 X 10^6	0	9.50 X 10^6	1.21 X 10^5	1.13 X 10^5	0	7.00 X 10^6	4 800	6.30E X 10^6	1 100	0
	EV Direct RT-PCR	EV (+)	(-)	EV (+)	(-)	(-)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
MAY-15	*E.coli* cfu/100 ml	2.0 X 10^7	0	7.00 X 10^6	1.00 X 10^7	7.00 X 10^6	0	SAMPLES NOT RECEIVED		3.00 X 10^6	3.10 X 10^4	0
	EV Direct RT-PCR	EV (+)	(-)	EV (+)	EV (+)	EV (+)	(-)			EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	EV (+)	(-)	(-)			(-)	(-)	(-)
JUN-15	*E.coli* cfu/100 ml	1.70 X 10^5	0	2.00 X 10^6	9.30 X 10^5	0	0	0	0	1.60 X 10^6	8.90 X 10^5	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	(-)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	(-)	EV (+)	EV (+)	(-)	(-)	(-)	EV (+)	(-)	EV (+)	(-)	(-)
JUL-15	*E.coli* cfu/100 ml	1.30 X 10^6	500	8.00 X 10^6	300	1.15 X 10^6	0	SAMPLES NOT RECEIVED		8.50 X 10^5	0	800
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)			EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	(-)	(-)			EV (+)	EV (+)	(-)
AUG-15	*E.coli* cfu/100 ml	1.85 X 10^6	0	1.00 X 10^6	0	7.00 X 10^5	10	1.30 X 10^6	0	1.00 X 10^6	0	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	EV (+)	EV (+)	(-)	(-)	(-)	EV (+)	(-)	EV (+)	EV (+)	(-)
SEP-15	*E.coli* cfu/100 ml	1.60 X 10^6	400	2.80 X 10^6	1000	1.30 X 10^6	0	2.30 X 10^6	0	1.30 X 10^6	700	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	EV (+)	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	EV (+)	EV (+)	(-)
OCT-15	*E.coli* cfu/100 ml	1.20 X 10^6	100	9.50X10^3	400	1.05 X 10^6	0	SAMPLES NOT RECEIVED		1.40 X 10^6	400	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)			EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)			EV (+)	EV (+)	(-)
NOV-15	*E.coli* cfu/100 ml	5.40 X 10^6	900	9.80 X 10^6	3.10 X 10^6	1.20 X 10^6	0	SAMPLES NOT RECEIVED		3.15 X 10^6	1.50 X 10^5	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	(-)	(-)			EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)			EV (+)	EV (+)	(-)
DEC-15	*E.coli* cfu/100 ml	SAMPLES NOT RECEIVED		0	300	1.15 X 10^6	0	1.30 X 10^6	500	8.50 X 10^5	0	0
	EV Direct RT-PCR			EV (+)	EV (+)	EV (+)	(-)	EV (+)	(-)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR			EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	(-)
JAN-16	*E.coli* cfu/100 ml	4.6 X 10^6	100	3.00 X 10^6	1 200	1.54 X 10^6	300	9.20 X 10^6	300	3.08 X 10^6	3 500	20
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	EV (+)	(-)	(-)	EV (+)	(-)	EV (+)	EV (+)	(-)
FEB-16	*E.coli* cfu/100 ml	5.00 X 10^6	700	2.00 X 10^7	1.20 X 10^5	3.00 X 10^6	0	SAMPLES NOT OBTAINED		1.50 X 10^7	2.00 X 10^5	40
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)			EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	EV (+)	EV (+)	(-)	EV (+)	(-)			EV (+)	EV (+)	(-)
MAR-16	*E.coli* cfu/100 ml	4.00 X 10^6	100	7.00 X 10^5	3 500	2.00 X 10^6	0	2.30 X 10^6	120	4.00 X 10^6	120	0
	EV Direct RT-PCR	EV (+)	EV (+)	EV (+)	EV (+)	EV (+)	(-)	EV (+)	EV (+)	EV (+)	EV (+)	(-)
	EV ICC-RT-PCR	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	EV (+)	(-)	(-)
TOTAL AVERAGE cfu/100 ml		4.67 X 10^6	255	5.80 X 10^6	1.19 X 10^6	1.68 X 10^6	25.8	3.34 X 10^6	817	3.46 X 10^6	1.06 X 10^5	71.7

*Red-labelled samples are EV positive and have an *E.coli* count below 1000 cfu/100 ml.

It was determined that 74.1% (40/54) of the wastewater effluent samples had an *E.coli* count below 1000 cfu/100 ml, of which 67.5% (27/40) were positive for EVs with direct and ICC-RT-PCR combined. Nevertheless, potentially infectious EVs were detected in only 25% (10/40) of the wastewater effluent samples that had an *E.coli* count below 1000 cfu/100 ml.

CHAPTER 4

DISCUSSION

Enteroviruses were detected in wastewater, wastewater discharge and downstream surface river water before and after viral amplification in cell culture with both molecular and viral isolation methods. This indicates the wide prevalence of EVs in various water sources and its persistence to wastewater treatment, posing a public health concern when discharged into natural water sources utilized for domestic, agricultural or recreational purposes. Enteroviruses were most prevalent between August and September 2015 and February and March 2016. The highest prevalence of EVs belonged to the EV-C species and the widest diversity of EVs belonged to the EV-B species, with EV-A species being rarely detected with both direct and ICC-RT-nPCR characterisation. According to phylogenetic analysis, the EV-C99 strains showed the most divergence and were also the most prevalent EV in all water types. The majority of strains showed significant divergence from globally recognised reference strains, indicating South African specific EV strains.

When evaluating the different EV detection outcomes between the four detection methods, it was found that direct RT-nPCR (69.2%) provided the highest EV detection frequency, followed by ICC-rtRT-PCR (52.6%), virus isolation (26.3%) and lastly direct rtRT-PCR (11.5%). This correlates with global ES studies that show the direct detection of EV genomes is significantly higher than infectious EV isolation (Gantzer *et al*., 1999). The conventional RT-nPCR assay currently used has previously been shown to have a higher sensitivity for the detection of EVs directly from water samples than rtRT-PCR (personal communication: Dr WB van Zyl, 2016). Even though the risk of contamination is higher with RT-nPCR, the two different primer sets used potentially increases the chance of amplifying the target sequence. Since direct RT-nPCR detects EVs regardless of whether it is infectious or inactivated it further contributed to the high detection frequency of EVs in the water samples. Not all EV types, especially EV-A species and CV-As of the EV-C species, grow well in cell culture or are cytopathogenic, and different EVs need numerous cell passages to propagate (Khetsuriani *et al*., 2010;

Pelligrinelli *et al*., 2013). It is therefore not surprising that viral isolation and ICC-rtRT-PCR exhibited a lower detection frequency, compared to direct RT-nPCR. Nevertheless, since non-cytopathogenic EVs can be detected with ICC-rtRT-PCR it explains the greater EV prevalence in the harvested cell culture extracts compared to only virus isolation of cytopathogenic EVs (Hot *et al*., 2003). Fulmic and humic acids, metal ions and polyphenol are not properly removed during viral recovery and can inhibit PCR reactions even when present at low concentrations (Schrader *et al*., 2012), which may explain the low detection frequency with direct rtRT-PCR. The Sabin PVs were isolated from both the L20B and BGM cell cultures, but the L20B proved more suitable. This cell line is PV-specific and is therefore part of the WHO guidelines for ES of PV (Faleye *et al*., 2016). Since Sabin-like PVs were detected in the harvested cell culture extracts and not in the recovered viral suspensions with the three PV one-step rtRT-PCR assays it indicates its increased sensitivity after viral amplification in cell culture. Inhibition of PCR assays can furthermore be overcome when the sample is diluted by cell culture media and the infectious virus concentration increased (Reynolds *et al*., 1996), which explains why Sabin-like PVs were detected in the cell culture extracts and not in the recovered viral suspensions of the water samples. Sabin PV-3 was the most predominant PV type detected in the water samples and correlates with previous South African data (Grabow, 1999), with Sabin PV-1 being the least prevalently detected PV type.

Enteroviruses were most prevalent and diverse in the recovered viral suspensions and harvested cell culture extracts of the wastewater samples, as faecally shed EVs from infected individuals are introduced to the wastewater treatment inflow. The wastewater discharge and downstream surface river water samples were the second and third most EV prevalent. No EVs were detected in the wastewater discharge of WWTP-3, the surface water upstream from WWTP-6, and in the surface dam water (VD1) downstream from WWTP-1 to 5. The direct rtRT-PCR detection frequency was too low to be used in the evaluation of removal efficiency of the WWTPs, and is therefore based on direct RT-nPCR, ICC-rtRT-PCR and viral isolation.

Since no EVs were detected in the wastewater discharge of WWTP-3, it is evident that there is either a low circulation of EVs in this region since only 60% of the wastewater

samples were positive for EVs or the treatment system is effective in the removal of EVs, especially pathogenic EVs. The treatment system that is implemented by WWTP-3 is an activated sludge and biological nutrient removal as well as anaerobic digestion. It has a cumulative risk rating of 70.6%, indicating that the treatment plant has reached 70.6% of its capacity and an effluent quality compliance of 80.7%, based on chemical, biological and physical compliance (Department of Water Affairs, 2013).

According to the EV detection methods, it was WWTP-5 that presented as the treatment plant with the poorest removal efficiency of EVs, especially potentially infectious EVs. Wastewater treatment plant 5 had a 100% detection rate in the wastewater and wastewater discharge with direct RT-nPCR, cytopathogenic EVs were isolated in 60% of the wastewater discharge samples and 50% of these discharge samples were positive for EVs with ICC-rtRT-PCR. This indicates a high survival rate of potentially infectious EVs after treatment in WWTP-5, which according to its Green Drop Status has a cumulative risk rating of 94.1% and an effluent quality compliance of 39.6% (Department of Water Affairs, 2013). While WWTP-2 had the second highest EV detection frequency with direct RT-nPCR, it was WWTP-1 that followed after WWTP-5 with the second highest number of potentially infectious EVs in almost 50% of its wastewater discharge samples. This indicates that a high prevalence of EVs were present in WWTP-2 after treatment, but were either already inactivated or were unable to efficiently propagate in cell culture due to viral damage or the EV types present were not suitable for growth on the selected cell lines. The wastewater discharge of WWTP-6 and downstream surface river water showed similar detection results, with a 100% detection rate with direct RT-nPCR and between 75-85% with ICC-rtRT-PCR. This treatment plant applies activated sludge and biological nutrient removal and has a cumulative risk rating of 81.8% and an effluent quality compliance of 37.6% (Department of Water Affairs, 2013). By detecting a high prevalence of EVs in WWTP-6 and the downstream surface river water into which it is discharged, indicates viral pollution of natural water sources and the EV resistibility to treatment and its survival in downstream surface water.

As EVs, especially potentially infectious EVs were detected in the wastewater discharge of WWTP-1, 2, 4, 5 and 6 it indicates the either inefficient removal of EVs from

wastewater and/or the high EV resistibility to wastewater treatment, while remaining viable within water sources, such as the downstream surface river water associated with WWTP-6. It is evident that there is a larger detection frequency difference between the wastewater and wastewater discharge after viral amplification in cell culture, compared to the direct RT-nPCR results. This may indicate that the presence of potentially infectious EVs decrease after wastewater treatment and that the majority of inactivated EVs remain and are subsequently detected and identified with direct RT-nPCR. There were, however, no associations found between the wastewater discharge of WWTP-1 to 5 and the downstream surface dam water, VD1, as no EVs were detected in VD1. This observation was consistent with global data reporting the presence of EVs in wastewater discharge (Irving and Smith., 1981; Ehlers *et al*., 2005; Sedmak *et al*., 2005; La Rosa *et al*., 2010; Battistone *et al*., 2014). The detection of potentially infectious EVs in downstream surface river water points to wastewater contamination and the capability of EVs to survive in natural water sources after discharge, with EVs being detected in river water in both global and local ES studies (Cho *et al*., 2000; Hot *et al*., 2003; Lee *et al*., 2004; Ehlers *et al*., 2005; Shieh *et al*., 2008; Chigor *et al*., 2014; Connell *et al*., 2012). Considering the low infectious dose of EVs, with an estimated daily risk of infection from recreational water ranging between 1.32×10^4 to 5.70×10^2 and the presence of potentially infectious EVs in receiving water bodies raises a public health concern (Battistone *et al*., 2014; Chigor *et al*., 2014). Undetected EVs at the VD1 sample site can be explained by the extended distance between the discharge points of WWTP-1 to 5 and VD1, ranging between 10 – 30 km. This would decrease the probability of EVs discharged by the WWTPs to be detected at VD1, which would also be diluted once it is distributed into the dam. The upstream surface river water sample site is before the discharge point of WWTP-6 and could explain why no EVs were detected in these samples. Enteroviruses can furthermore survive for prolonged periods of time in the sediment of natural water sources and can therefore fail to be detected in the surface water (Bosch, 1998).

The National Water Act (36/1998) states that wastewater discharge released into natural water sources is allowed a target water quality range (TWQR) of 1000 cfu/100 ml for faecal coliforms. When evaluating the relation between wastewater discharge samples within the TWQR and the presence of EVs, it would seem that *E.coli* is not a proper

indicator of viral contamination of wastewater discharge. This correlates with previously described observations where EVs were detected in water samples having an *E.coli* count within the TWQR (Pusch *et al*., 2005). However, potentially infectious EVs were not as prevalent in wastewater discharge samples that were within the TWQR for *E.coli* that indicated that it might have presented as a partially suitable indicator of potentially viable EVs that were circulating in the water sources.

The monthly distribution in Figure 3.1 demonstrates that EV prevalence slightly decreased at the start of winter in May 2015 and gradually increased until it reached a peak during the early spring months of August to September 2015. It subsequently drops at the end of spring and early summer between October and December 2015, after which it reached a second peak in late summer and early autumn in February and March 2016. This seasonal pattern has been previously observed, specifically in Cape Town during the EV outbreaks between 1981 and 1989 that peaked in the late winter and spring months (McIntyre and Keen, 1993). The high detection frequency during the early spring season may also indicate its clinical predominance for the following summer (Sedmak *et al*., 2004). Since EVs were detected in both dry and wet seasons throughout the year it was generally difficult to establish a definite seasonal pattern. This correlates with ES data from African and other subtropical countries where EVs were detected during all seasons (Urashima *et al*., 2003; Ehlers *et al*., 2005; Kiulia *et al*., 2010). The summer and autumn EV seasonality generally occurs in temperate climates (Fisman, 2012; Battistone *et al*., 2014).

Of the 111 samples in which EVs were characterised with nucleotide sequence analysis, 22 contained a single EV genotype, 27 contained two different genotypes, 28 contained three, 27 contained four, six contained five and one contained six different EV genotypes. This emphasized on the wide diversity of EV types that were characterised in a single sample when combining the direct RT-nPCR and ICC-RT-nPCR nucleotide sequence analysis, which was less varied when doing the one or the other on its own. The samples, in which EVs were only detected in the recovered viral suspensions and not in the harvested cell culture extracts, showed that these EVs were either EV-A or EV-C species. The prevalence of EVs within each species differed meaningfully between direct RT-nPCR and ICC-RT-nPCR nucleotide sequence analysis. The

prevalence of EV-A and EV-C species characterised with direct RT-nPCR decreased drastically after ICC-RT-nPCR characterisation from 21.4% to 2.2% and 54.2% to 21.2%, respectively. The EV-B prevalence, however, increased from 24.5% with direct RT-nPCR characterisation to 76.6% with ICC-RT-nPCR characterisation. A higher prevalence as well as a wider diversity of different EV genotypes were detected and characterised in the recovered viral suspensions compared to the harvested cell culture extracts. This could have been due to five clones per sample being sequenced after direct RT-nPCR, while only two clones were sequenced after ICC-RT-nPCR. Five clones were selected to increase the characterization of various different EV types within a single sample, while after viral amplification in cell culture it was one EV type that predominated over the other potentially present EV types and therefore only two were sequenced. As previously mentioned, it was suggested that EV-A and EV-C species, specifically CV-As, have a limited ability to propagate in cell culture (Khetsuriani *et al*., 2010; Pelligrinelli *et al*., 2013). This explains the low detection rate of EV-A species (three compared to 41 EVs identified directly from the recovered viral suspensions) and EV-C species (29 compared to 104 EVs identified directly from the recovered viral suspensions) with ICC-RT-PCR nucleotide sequence analysis.

By detecting mainly EV-B species with virus isolation in cell culture creates a biased concept that EV-B species are the most prevalent and diverse EVs or the most evolutionary successful, while it is more likely cell-line specific related (Faleye *et al*., 2016). This raises concern with regards to the true epidemiological significance of EVs in water with viral isolation in cell culture only, which clearly holds preference to EV-Bs. It is clear from the direct RT-nPCR characterization analysis that EV-A and EV-C species are also widely prevalent, but its viability is broadly undetermined as its growth is limited in cells. Nevertheless, when relying only on molecular identification without viral isolation in cell culture it also skews the diversity landscape as EVs that were not detected directly were able to be identified after being subjected to cell culture (Faleye *et al*., 2016). By combining both direct RT-nPCR and ICC-RT-nPCR nucleotide sequence analysis it broadens the EV landscape, providing more significance for ES, especially newly emerging EV strains. It is evident that the BGM and PLC/PRF/5 cell lines are more specific for the propagation of EV-B species, than EV-A and EV-C species. The harvested PLC/PRF/5 cell cultures proved to be more reliable in isolating

EVs-A, B and C species as it was the only cell line to isolate CV-As (n=14) and EV-C99 (n=2), while the three Sabin PVs were isolated from the BGM cell line. The WHO recommends the L20B and RD cell line for ES of PV and NPEVs and studies have shown that the RD cell line is more specific for EV-Bs, while other cell lines such as MCF and LLC-MK2 are more reliable for the isolation of EV-C species (Adeniji and Faleye, 2014). It is therefore important to determine specifically what the ES study is aiming to accomplish when deciding on the type of cell line to use for EV isolation, since a combination of different cell lines will allow a broader perspective of EV epidemiology. This is especially significant since there is a lack of data on the genetic diversity and prevalence of EVs in sub-Saharan Africa (Sadeuh-Mba *et al*., 2013).

In overall, the 10 most common EVs identified were EV-C99, E-6, CV-B3, CV-A13, CV-A22, CV-B4, CV-A20, CV-A16, CV-B5 and CV-A2. Compared to previous ES data from South Africa, the CV-Bs, predominantly CV-B3 and CV-B5, isolated from BGM and PLC/PRF/5 cell lines were the most prevalent in treated and untreated water sources (Ehlers *et al*., 2005). The low CPE-positive samples correlated with the low number of EV-specific inclusion bodies isolated from these previous studies, indicating that EVs were capable of infecting and replicating in the susceptible host cells, while lacking the ability to produce a CPE (Vivier *et al*., 2004).

Enterovirus C99 was the most common EV identified in both the wastewater and wastewater discharge after direct RT-PCR nucleotide sequence analysis, but only detected twice after viral amplification in cell culture and detected in a total of 44/156 samples. It is a novel EV type that has been identified in the Republic of Congo (Mombo *et al*., 2015), Mozambique (Bero *et al*., 2015), Cameroon (Sadeuh-Mba *et al*., 2013), Ivory Coast (Cristianziano *et al*., 2015), China (Tao *et al*., 2013) and Finland (Smura *et al*., 2014). According to the phylogenetic analysis of the partial VP1 region (375 bp) of the EV sequences it showed a high genetic diversity with multiple clusters, of which a similar pattern was observed for E-6, CV-A2, CV-A9, EV-C116, EV-C99 (Figure 3.12), CV-B4 (Figure 3.13), CV-B2, E-6, CV-B3 and EV-C99 (Figure 3.14). The one EV-C99 cluster grouped with strains from Madagascar and Finland, while the second cluster grouped with strains from Bangladesh and the Philippines. Different EV-C99 strains are circulating, such as the strains from WWTP-2, 2E201512 and

2E201507b sharing a nt identity of 96.7%, that only share a 79% nt identity with strains from WWTP-4, 4R201504 and 4R201508 (sharing a nt identity of 99.6%). This is also true for strains from the same region but identified in July and December 2015, RD201506a and RD201512a, sharing a nt identity of 78.8% and grouped into different clusters. Since 1% of nt substitutions accumulate in EVs, especially EV-Cs, a 20% nt distance may correspond to divergence from a common ancestor around 10 years ago (Mombo *et al*., 2015). The phylogenetic analysis shown in Figure 3.12 to 3.14 furthermore indicates the wide concurrent circulation of similar strains in more than one region that shared a closer nt identity towards each other than to globally recognized strains from Asia, Africa and Europe.

The high prevalence of E-6 correlates with the global EV epidemiology, where E-6 has been classified as the most predominant EV worldwide (Belguith *et al*., 2007; Bailly *et al*., 2011; Smura *et al*., 2013) and associated with seasonal outbreaks of aseptic meningitis (Chomel *et al*., 2003; Luchs *et al*., 2008). Echovirus 30 was only identified in three samples, while it has been suggested that E-6 and E-30 predominantly co-circulate (Bailly *et al*., 2011). The high prevalence of CV-A13 correlates with data from other African EV surveillance studies, where CV-A13 is the most predominant EV type circulating in Cameroon, Gabon and Chad (Sadeuh-Mba *et al*., 2013) as well as Nigeria (Adeniji and Faleye, 2015). Coxsackievirus-A13, CV-A20 and EV-C99 are the three most common EV-C species and the most genetically diverse EVs identified according to sub Saharan Africa data (Sadeuh-Mba *et al*., 2015; Bero *et al*., 2015). The high prevalence of CV-A13 and CV-A20 does, however, raise a concern with regards to its close similarity to PVs in the capsid coding region, which makes them more likely to recombine with PVs that may lead to the emergence of pathogenic cVDPVs (Sadeuh-Mba *et al*., 2015). Rarely described EVs, such as EV-A119, appeared in April and May 2015 and again in March 2016, suggesting a seasonal prevalence of this type of newly emerging EV. This EV type has previously been detected in environmental water sources from 2014 – 2015 (Muilwijk, 2014). This novel EV type was first recorded in a chimpanzee in 2008 and subsequently isolated from a healthy child in Cameroon in 2009 (Ayukekbong *et al*., 2013; Adeniji *et al*., 2016). Since then it has only been detected in a healthy child in Ivory Coast in 2013 and in an AFP patient in Nigeria in 2016 (Cristanziano *et al*., 2015; Adeniji *et al*., 2016). This is the first reporting of EV-

A119 in South Africa and it is clear that is a strain that is only circulating in Africa. Cross-species transmission of EV-A119 and CV-A13 from human to NHP has been documented in Cameroon (Sadeuh-Mba *et al*., 2014) and the Democratic Republic of Congo (Harvala *et al*., 2014), respectively.

The wastewater and wastewater discharge of WWTP-5 had the widest diversity of EV types identified in the recovered viral suspensions and harvested cell culture extracts, compared to WWTP-1 to 4. This indicates that the population is shedding various EV types, while not necessarily being detected that often, since the majority of EV types are either detected only once or twice within the 12-month study period. Seventeen different EV genotypes were identified in the wastewater discharge of WWTP-5 of which nine were detected after viral amplification in cell culture, namely E-6, CV-A13 and CV-B3. The Sabin-like PVs and two CV-Bs were only detected once in the wastewater discharge. Since E-6 appeared prevalent in the discharge of WWTP-5 in both the direct recovered viral suspensions and harvested cell culture extracts and is the most common EV type circulating worldwide it may indicate that this type is more evolved and resistant to treatment compared to other EV types. Only a single detection of six EV types were identified in the wastewater discharge of WWTP-4 with only CV-A13 being detected after viral amplification in cell culture. Since there were a meaningful number of viable and diverse EVs, mainly CV-A13 and EV-C99, detected in the wastewater of WWTP-4, it may indicate the efficient removal or inactivation of EVs. The EV diversity can furthermore be low due to only having received seven out of the 12 months of expected samples. While yet a wide diversity of EV types were identified in the wastewater of WWTP-3, of which 10 out of the 14 EV types were potentially viable EVs, no EVs were detected in the wastewater discharge. This implies that the wastewater treatment system may be efficient in the removal of EVs from wastewater, but since the population size of this region is only 701 it may suggest a low circulation of EVs, as the majority of EV types were only detected once. In the wastewater from WWTP-2, 36 EVs were identified, with EV-C99 predominating in the direct and CV-B3 in the ICC-RT-nPCR analysis. In the wastewater effluent, however, there were yet 23 EVs of which EV-C99 and CV-A20 predominated. The majority of EVs in the wastewater discharge were detected in the recovered viral suspensions, with only four EVs detected after viral amplification in cell culture. The detection of

potentially infectious EVs decreases meaningfully after wastewater treatment, as it is evident in the number of detections in the discharge of WWTP-1, 2 and 4 compared to detections in the wastewater. The EV diversity and prevalence in the wastewater discharge and downstream surface river water were closely similar, as 37 and 39 EVs were detected, respectively, where E-6 predominated in both. This indicates the poor removal efficiency of EVs from WWTP-6 and its ability to survive in natural water sources after discharge that similar prevalence and EV types were found in these two sample sites.

CHAPTER 5

CONCLUSION

Pollution and scarcity of water sources has become a major global health concern. According to the National Water Act (36/1998) wastewater effluent that is discharged into water resources is allowed a TWQR of 1000 cfu/100 ml faecal coliforms. Poor infrastructure and inadequate treatment at wastewater treatment plants contribute to the emergence of waterborne infectious diseases through ingestion of contaminated water in both industrialised and developing countries worldwide (Ashbolt, 2004). Poor water quality and lack of access to safe water and sanitation contribute to an estimated global 1.7 million deaths per year (Ashbolt, 2004). The stability of EVs in water after wastewater treatment makes them detectable in wastewater discharge and downstream water sources, indicating poor EV removal efficiency. It is therefore important to determine the removal efficiency of EVs from wastewater, as it raises a public health concern when these downstream water sources are utilized by the public. There is furthermore a lack of data on the presence of EVs, specifically potentially infectious EVs, in water sources as well as the current epidemiology on EV circulation in South Africa.

The first objective was to recover EVs from 60 wastewater samples with primary PEG8000/NaCl precipitation and from 72 wastewater effluent and 36 surface water samples with primary glass wool adsorption-elution and secondary PEG8000/NaCl precipitation. The second objective was to optimise and perform EV and Sabin PV rtRT-PCR assays, followed by characterisation with conventional RT-nPCR. The Sabin PV assay was optimised into three individual assays for specifically Sabin-like PV-1, PV-2 and PV-3. This method proved to be more sensitive for the detection of PVs after viral amplification in cell culture compared to direct analysis. The same observation was made with the sensitivity for the detection of EVs with rtRT-PCR after viral amplification in cell culture compared to direct analysis. The third objective was to isolate EVs in the BGM, L20B and PLC/PRF/5 cell lines. It was determined that the PLC/PRF/5 cell line was the most efficient cell line in isolating the widest diversity of

EVs from the EV-A, EV-B and EV-C species. The fourth objective was to genotype the detected EVs with nucleotide sequence analysis and assess the genetic relatedness between identified strains and globally recognised strains with phylogenetic analysis. In total, more than 300 EVs were identified that belonged to 35 different EV types with the majority belonging to the EV-B species, which correlated with the current African epidemiological landscape (Sadeuh-Mba et al., 2013; Cristianziano et al., 2015). This therefore shows that similar EVs are predominating worldwide, while the phylogenetic analysis indicated that the EV strains still varied significantly from its reference strains originating from other continents. The high genetic similarities between EV strains that have been identified concurrently in more than one WWTP shows the wide distribution of similar EV strains in different demographic regions. There is a lack of data on the current risk of EV infection from natural water sources in South Africa and the data obtained in this study may motivate that it is of significance to implement risk assessment analysis studies. Since there is limited data on South African EV epidemiology this study will therefore contribute to identifying major EV strains that may now add to the African and global epidemiological perspective on EV circulation.

To conclude, based on the EV removal efficiency of the selected WWTPs the EV detection prevalence in wastewater discharge was evidently the highest in WWTP-5 and WWTP-6. The infected cell cultures from the wastewater discharge and downstream surface river water had the highest EV isolation frequency, indicating the survival of potentially viable EVs that are resistant to wastewater treatment and are detectable in downstream water sources. Only WWTP-3 showed a promising EV removal efficiency since no EVs were detected in its wastewater discharge. The detection of potentially infectious EVs after wastewater treatment proves the hypothesis that EVs are not effectively removed from wastewater at the selected WWTPs and are subsequently discharged into natural water sources. This might rise as a public health concern since an estimated 7 million people in South Africa do not have access to safe water, where natural water sources are utilised for domestic and agricultural purposes (Pallansch et al., 2013).

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APPENDIX A

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

2/06/2016

Approval Certificate
New Application

Ethics Reference No.: 164/2015

Title: The assessment of sewage treatment efficacy through the detection and characterisation of human enteroviruses

Dear Miss Maxime Muilwijk

The **New Application** as supported by documents specified in your cover letter dated 14/04/2015 for your research received on the 14/04/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 24/06/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**164/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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APPENDIX B

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

MSc Committee

3 June 2016

Dr W van Zyl
Department of Medical Virology
Faculty of Health Sciences

Dear Dr van Zyl,

Ms M Muilwijk, Student no 14327903

Please receive the following comments regarding the MSc submission of the abovementioned student:

Student name	M Muilwijk	**Student number**	14327903
Name of study leader	Dr Walda van Zyl		
Department	Medical Virology		
Title of MSc	The assessment of sewage treatment efficacy through the detection and characterisation of enteroviruses.		
Date of first submission	April 2015		
June 2016	• Thank you for submitting ethics approval • Thank you submitting the permission letter from Rand Water		
Decision	This protocol has been approved Ethics approval obtained The internal and external examiners were approved		

Yours sincerely

Prof Riana Cockeran
Chair: MSc Committee

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