

**Characterisation of Non-Polio Enteroviruses Identified in Disease Biomes in
South Africa**

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A thesis submitted to the Faculty of Health Sciences, University of Witwatersrand, in
fulfilment of the requirements for the degree of Master of Science in Medicine in
Virology

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Declaration

I, Wayne Howard, declare that this thesis is my own work. It is being submitted for the degree of Master of Science in Medicine in Virology in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

A handwritten signature in black ink, appearing to read 'Wayne Howard', written in a cursive style.

16th day of August , 2016.

Publications and presentations arising from this study

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Abstract

Human enteroviruses (family *Picornaviridae*) consist of 106 serotypes and are divided into four species: *Human enterovirus* (HEV)–A, B, C, and D. Enteroviruses cause a variety of clinical symptoms from severe (e.g. acute flaccid paralysis) to less severe (e.g. hand-foot-and-mouth disease). Whilst there is currently no antiviral treatment, viral genotyping allows for: identification of increased virulence, identification of new enteroviruses, correlation of virus types with immunity, epidemiological investigations and provides information on viral inter-relationships. A comprehensive study is underway to determine the prevalence and type of circulating non-polio enteroviruses in South Africa, specifically for those involved in recent outbreaks. This study investigated the prevalence of non-polio enteroviruses circulating in South African between 2010 and 2012 using samples obtained from 2 national surveillance programs conducted at the National Institute for Communicable Diseases: Acute Flaccid Paralysis (AFP) and Rotavirus. Typing was performed using a Real-Time PCR (RT-PCR) assay, followed by Sanger sequencing. 832 samples were tested to date (562 from the Rotavirus and 270 from the AFP surveillance programs, respectively). 446 positive enterovirus samples were detected from which 308 samples were successfully sequenced. Specimens from the AFP program yielded mostly HEV-B serotypes (90.40%), whereas samples typed directly from the Rotavirus program stools yielded mostly HEV-C serotypes (47.20%). 92.8% of typed samples were from patients under 5 years. Despite most detections being HEV-B (56.55%), the most commonly detected virus was Enterovirus 99 (8.63%) from the HEV-C species. RT-PCR and sequencing, whilst more expensive, have proven more accurate than cell culture and neutralization assays for typing enteroviruses. In South Africa, HEV-B viruses were predominant, and in comparison to studies from other countries, a larger proportion of HEV-C viruses were detected. Detecting EV directly from stool yielded a larger diversity of the viruses, and while disease associated viruses were detected, they did not contribute significantly to the associated disease burden.

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Nomenclature

aa – amino acid

AFP – Acute Flaccid Paralysis

BLAST – Basic Local Alignment Search Tool

cDNA – copy DNA

CNS – Central Nervous System

CPE – Cytopathic Effect

CSF – Cerebrospinal Fluid

CVA – Coxsackievirus A

CVB – Coxsackievirus B

DNA – Deoxyribonucleic Acid

DTT - Dithiothreitol

E - Echovirus

EV - Enterovirus

HEV – Human Enterovirus

IPV – Inactivated Polio Vaccine

IRES – Internal Ribosome Entry Site

mRNA – Messenger RNA

µl - Micro litre

NCBI – National Centre for Biotechnology Information

ng - Nanogram

NICD – National Institute for Communicable Diseases

NPENT – Non-Polio Enterovirus

nt - Nucleotide

OPV – Oral Polio Vaccine

ORF – Open Reading Frame

PCR – Polymerase Chain Reaction

Pol - Polymerase

PV - Poliovirus

PVR – Poliovirus Receptor

QCMD – Quality Control for Molecular Diagnostics

RIVM – National Institute for Public Health and the Environment

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcriptase PCR

SARI – Severe Acute Respiratory Infection

siRNA – Small Interfering RNA

STAR – Stool Transport And Recovery

UTR – Untranslated Region

VP – Viral Protein

VPg – Viral Protein Genome associated

WHO – World Health Organisation

1.Introduction

1.1 Human Enterovirus

1.1.1 General information and history

Human Enteroviruses are part of the *Picornaviridae* family, in the Enterovirus genus (www.ictvonline.org). They are separated into four species (Human Enterovirus (HEV)-A, B, C and D; Appendix A, Tables 1.1-1.4) that contain the different serotypes. HEV-A contains Coxsackie A viruses and some numbered Enteroviruses. HEV-B is a large species group with 1 Coxsackie A virus, Coxsackie B viruses 1-6, all the Echoviruses as well as numbered Enteroviruses. HEV-C contains the three Polioviruses (PV), a number of Coxsackie A viruses and numbered Enteroviruses. HEV-D is the smallest species group currently containing only 5 numbered Enteroviruses. Infections are characteristically in the summer and autumn months, though infections can be detected all year round (Pons-Salort et al., 2015). Most infections are asymptomatic, though there are estimates that large numbers of symptomatic infections occur every year (Pons-Salort et al., 2015). In the United States, this number could be as many as 5 to 10 million infections per year (Strikas et al., 1986). Most of these infections are not serious, but some infections can lead to serious disease especially in infants (Nasri et al., 2007). Enteroviruses (EV) are also the leading cause of viral aseptic meningitis (Tapparel et al., 2013), and have been implicated in a wide range of acute and chronic infections. These include conjunctivitis, gastroenteritis, hand-foot-and-mouth disease (acute) and dermatomyositis, polymyositis, dilated cardiomyopathy, and diabetes mellitus (chronic) (Tapparel et al., 2013, Patil et al., 2015).

Most EVs are transmitted through the oral route, though some viruses have different routes of transmission, usually associated with specific disease phenotypes. CVA21, which is a major cause of respiratory disease, is transmitted through contaminated respiratory secretions, and EV70 (a cause of acute haemorrhagic conjunctivitis) through ocular and respiratory secretions, or indirectly through contaminated items (Fields, 2007). Infections are considered acute, though there have been instances of persistent virus shedding, noted especially in immunodeficient individuals (Li et al., 2014).

Treatment for EV infections is symptomatic and there is no currently available drug in clinical use, though several potential therapies exist. For example: interferon and antiviral antibodies have been studied as possible therapies (Langford et al., 1988, Abzug et al., 1995), although problems with these therapies include delivery of the agent to infected cells, as well as ensuring the correct dosage of the antiviral therapy reaches the infected cell. As with many RNA viruses, resistance to treatment may also arise through natural selection and evolution of the viruses. Small interfering RNAs (siRNA) have also been investigated as a possible treatment for EV infections, and *In vitro* infections have been inhibited in some studies (Ahn et al., 2005, Gitlin et al., 2005), though further studies in animals and humans are needed, as well as effective delivery systems.

EV history has been dominated by studies on the three polioviruses (PV), and many landmarks in virology have also been based on PV. Poliomyelitis is believed to be an ancient disease as Egyptian hieroglyphs depict a young man with an atrophied leg that is believed to be a result of poliomyelitis, which dates from the second millennium BC (Fields, 2007). In the 1800s there was much progress made in studying the disease, but the 1900s is when the beginning of understanding the nature of the infectiveness of the disease was started. The communicable nature, enteric involvement and the infectious nature of the virus were all demonstrated in early studies (Trask JD, 1938, Landsteiner K, 1908, Wickman, 1907). Despite this progress there were a few misconceptions about poliomyelitis that confused scientists and misdirected efforts for control. By the mid-1900s, studies had begun to correct these misconceptions (Bodian D, 1955, Enders et al., 1949, Aykock, 1928, Bodian et al., 1949), which led to the framework for vaccine development. While a variety of vaccines were developed, 2 vaccines became the most well-known, and were introduced and used to control Poliovirus infections: the Salk Inactivated Polio Vaccine (IPV) which was delivered intramuscularly and the Sabin live attenuated vaccine which was delivered orally (Oral Polio Vaccine (OPV)).

1.1.2 Virus structure

Enteroviruses are spherical viruses with a single-stranded Ribonucleic Acid (RNA) genome of positive polarity. They are about 30nm in diameter and are simple, consisting of a protein capsid without a lipid membrane surrounding the naked RNA genome (Dales et al., 1965). As enteroviruses pass through the stomach to access

the intestines, they are also acid stable (Fields, 2007). Virus stability is temperature dependent, though serotype also seems to play a role (Lo et al., 1976).

The capsid consists of 4 viral proteins (VP1-4) and these form an icosahedron, which is a 20-sided solid structure with 12 vertices. The 20 sides are triangular and have VP1-3 proteins on the surface, with VP4 binding to the inner face of the triangle. None of the viral proteins have a sequence homology, yet they all form the same topology: an eight-stranded, antiparallel β -barrel. This forms a wedge that facilitates the packing of the structural units into a dense, rigid protein shell (Acharya et al., 1989, Page et al., 1988).

The surface of the virion is corrugated, with a mesa showing a fivefold symmetry surrounded by a deep canyon. Around the canyon are protrusions with threefold symmetry and the canyon has been proved to be the receptor binding site for Polioviruses. Neutralising and antigenic sites on the virus are on the surface, formed by the connecting loops and C-termini of the capsid proteins. These also are the viral serotype determinants. Mutations in the canyons can alter the affinity of binding to receptors and antibodies (Colston and Racaniello, 1994, Colston and Racaniello, 1995).

The viral genome is infectious since it is translated as soon as it is introduced to the cytoplasm of a cell and produces all the proteins needed for viral replication (Henry and Youngner, 1963). This is due to the single stranded nature, and the positive

polarity that makes it very similar to mRNA. The RNA is also covalently linked to a protein called *VPg* (virion protein, genome linked) at the 5' end, though *VPg* isn't required for infectivity. Nucleotide sequence analysis of Human Enteroviruses, show a common organizational pattern (Figure 1.1) with the genome length consisting of approximately 7500 nucleotides (nt). The 5' non-coding regions are long (624 nt to 1199 nt), highly structured and contain sequences that control replication and translation. It contains the Internal Ribosome Entry Site (IRES) that directs translation of the viral messenger RNA (mRNA). The 3' non-coding region is shorter, ranging from 14 bases to 125 bases, has secondary structure and has been implicated in controlling RNA synthesis, though if the 3' non-coding region is removed, the RNA is still infectious. Downstream of the 3' non-coding region is a poly-A tail. If this poly-A tail is removed, the resulting RNA is non-infectious.

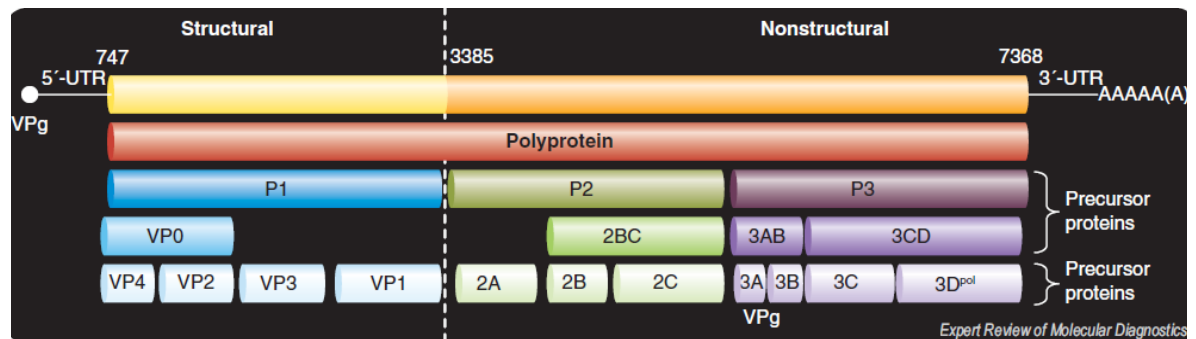


Figure 1.1: Schematic illustration of the genomic organization of enteroviruses and the successive cleavages of the polyprotein. Nucleotide positions correspond to the numbering of the sequence of poliovirus type 2, Lansing strain. Pol: Polymerase; UTR: Untranslated Region; VPg: Genomic Viral Protein (Nasri et al., 2007).

Between the two non-coding regions is the open-reading frame (ORF) that encodes a polyprotein. The translated polyprotein is processed to form the individual proteins. The full-length polyprotein is not observed in natural synthesis as it is cleaved during the synthesising process. The polyprotein is divided up into 3 main regions: P1, which encodes the capsid proteins; P2 and P3, which encode the proteins involved in protein processing (2A^{pro}, 3C^{pro}, 3CD^{pro}) and genome replication (2B, 2C, 3AB, 3B^{VPg}, 3CD^{pro}, 3D^{pol}).

1.2 Diversity in Enteroviruses

Enteroviruses are dependent on an RNA-dependent RNA polymerase for genome synthesis and, like with other RNA viruses, this enzyme is error prone due its lack of proof-reading capabilities. This results in quasispecies arising within a single infection as error rates can be as high as one misincorporation per 10^3 to 10^4 nucleotides (Domingo and Holland, 1997). Enteroviruses have been suggested to exist on the threshold of 'error catastrophe'. Either an increase (Crotty et al., 2000, Gu et al., 2006) or a decrease (Pfeiffer and Kirkegaard, 2005) in the mutation rate of enteroviruses, reduces the survival rate of these strains in comparison to their wild type strains.

This evolutionary process has given rise to a large number of readily distinguishable members within the EV group that have been categorized antigenically as different serotypes. Each of the serotypes correlates with the immunologic response of the

human host, protection from disease, receptor usage, and to a lesser extent, the spectrum of clinical disease (Fields, 2007). Human enteroviruses had previously been classified as Polioviruses, Coxsackie A or B viruses, and Echoviruses based on biological activity and disease. This included human CNS disease with flaccid paralysis (poliomyelitis); flaccid paralysis in new-born mice, human CNS disease, and herpangia (Coxsackie A viruses); spastic paralysis in new-born mice and human CNS and cardiac disease (Coxsackie B viruses); and absence of disease in mice and originally absence of disease in humans (Echoviruses). This original classification became problematic with the identification of viruses serologically identical to Echoviruses that were found to cause disease in mice and humans. Other inconsistencies in the classification were found to occur and led to a numbering of new EV serotypes starting at EV68. This classification also found that some viruses that were previously thought to be HEV should be classified in different family groups: echovirus 10 is reovirus 1, enterovirus 72 is human hepatitis A virus, and Echoviruses 22 and 23 are parechoviruses 1 and 2 respectively. Other enterovirus serotypes were found to be the same as previously identified serotypes: coxsackievirus A15 is the same as CAV11, CAV18 is the same as CAV13 and CAV23 is the same as echovirus 9 (<http://www.ictvonline.org/>). The antigenic groups also became more complicated as more viruses were discovered, and isolates were discovered that were weakly antigenically related to known serotypes. As the serotype is still the single most important physical and immunologic property that distinguishes different EVs, it is still used currently, though the advent of molecular technology has resulted in the older methods of classification becoming obsolete.

Current methods for classification utilise the genome organisation and sequence similarity, as well as the biological properties of the viruses. The 4 species of human enteroviruses (HEV-A, B, C and D) are classified by (Fauquet, 2005):

- a) Sharing greater than 70% amino acid (aa) identity in P1
- b) Sharing greater than 70% aa identity in the non-structural protein 2C+3CD
- c) Sharing a limited range of host cell receptors
- d) Sharing a limited natural host range
- e) Having a genome base composition (G+C) which varies by no more than 2.5%
- f) Sharing a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination

The P1 coding region for the capsid proteins provides a reliable correlation between sequence relatedness and the previous definition of serotype, except for VP4, so this sequence is less reliable for serotype identification. The VP1 capsid protein has many of the antigenic sites involved in virus-host interactions, and the sequence can be used as a surrogate for antigenic typing by means of neutralization tests in order to differentiate EV serotypes. VP1 sequence homology of at least 75% (85% aa identity) between an isolate and a serotype prototype strain suggests that the isolate is serotypically identical to the prototype (assuming that the next highest identity with other prototype strains is <70%) (Oberste et al., 2004b, Oberste et al., 2005). Using the VP1 sequences has supported and clarified early serologic data and also led to proposals regarding the classification of isolates into the new EV serotypes. Studies of the 5' untranslated region (UTR) of enteroviruses show that there are two clusters

formed by utilising this region for classification. HEV-C and HEV-D form one cluster and HEV-A and HEV-B, the other (Hyypia et al., 1997, Brown et al., 2003). These factors, with additional information from sequencing studies, indicate that the current revised method of classification has some shortcomings, and that there will need to be a continual refinement of the classification and criteria for the EV genus.

1.3 Recombination

Recombination, an exchange of nucleotide sequences among different RNA genome molecules, has been found to occur in Enteroviruses. This process results in a genome that consists of sequences from the parent virus as well as sequences from a co-infecting virus. Recombination can occur in up to 1% of a growth cycle, and it has been noted in vaccinees that sequences from all three serotypes of the Sabin polio vaccine strains have been observed in one virus genome (Cammack et al., 1988). The significance of recombination is unknown, but it has been suggested that recombinants are selected for their improved ability to replicate in the human gut over their parental viruses. Recombination is not limited to polioviruses, and has been demonstrated in non-polio enteroviruses (Simmonds and Welch, 2006). The process of recombination has been demonstrated to occur during negative strand synthesis as part of the RNA genome synthesis process, due to the fact that the concentration of positive-strand acceptors for the template switching process is 30 to 70 times greater than that of negative-strand acceptors (Kirkegaard and Baltimore, 1986, Tang et al., 1997) (Figure 1.2). Recombination between viruses has been seen to be more common in the same serotype than between different serotypes (King, 1988).

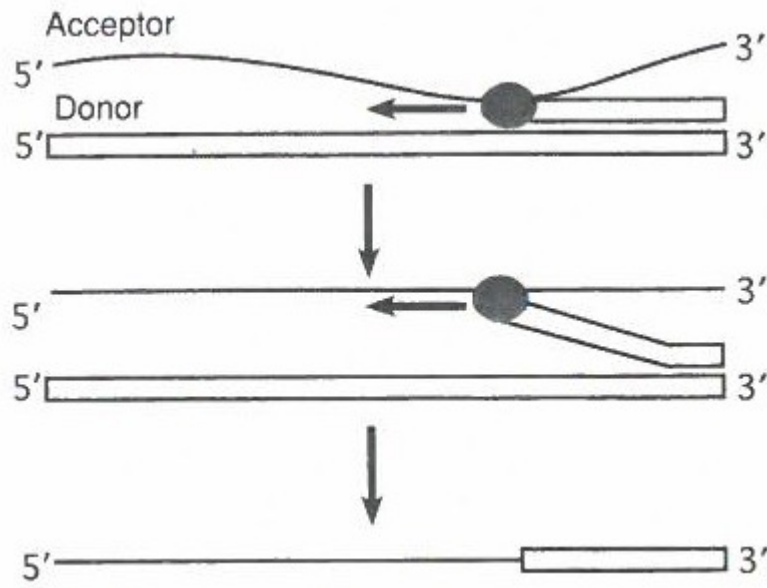


Figure 1.2: RNA recombination. Schematic diagram of RNA recombination in enterovirus-infected cells by template switching (copy choice). Two parental genomes, the acceptor and the donor, are shown as a line and box respectively. The RNA polymerase (black oval) is shown copying the 3' end of donor RNA and switching to the acceptor genome (middle). As a result of this template switch, the recombinant RNA shown is formed (bottom)(Fields, 2007).

As demonstrated in Figure 1.2, studies have shown that between members of HEV-C and poliovirus, there are incongruities between the capsid region and noncapsid regions (Brown et al., 2003). They also suggest that viruses with a PV capsid may recombine with HEV-C non-structural protein sequences and vice versa (Liu et al., 2000). This shuffling of the different genomic regions may lead to serotypes with selective advantages that become dominant. The frequency of recombination in the noncapsid region supports the proposal that Enterovirus serotypes are defined by the

capsid region and that limited correlations exist between the serotype of isolates and other phenotypic characteristics not associated with the capsid proteins. This also changes the phylogenetic relationship between the viruses depending on which region of the genome is compared.

Members of HEV-A and HEV-B have also been seen to recombine within their species groups (Oberste et al., 2004b, Oberste et al., 2004a). It was noted that between different species groups, the recombination was seen in the non-structural regions and not the capsid regions, probably due to structural constraints on the serotype, while capsid recombination was seen within 1 species group (Oberste et al., 2004c).

1.4 Epidemiology

EV infections have a wide variety of clinical presentations, and infect individuals in all age groups. Despite this variation, there are factors that influence disease and produce consistent characteristics. Gender, socioeconomic status, and most importantly, age, have largely predictable effects (Tapparel et al., 2013).

Different age groups have different susceptibilities to infection, severity of disease, and clinical manifestations. Generalisations are possible to be made, though understanding these age-effects are complicated by the prior history of EV infection and resulting immunity (Froeschle et al., 1966, Fields, 2007).

Most infections are during childhood, due to the high number of infections in the population; therefore the children are the most important vectors for transmission, especially in households. The greater exposure makes them more likely to be affected by serious disease than adults, e.g. aseptic meningitis (Gondo et al., 1995, Irvine et al., 1967).

Historically, polio incidence was found to be low in ages 0-6 months due to protection from maternal antibodies (in countries where polio wasn't under control through vaccination). In these countries, increased incidence was seen of paralytic disease in children over 6 months old. In areas with improved hygiene, the incidence declined in this age group, but resulted in a susceptible population of older individuals which, in an outbreak, developed severe paralytic symptoms (Prevots et al., 1998). As of the beginning of 2015, wild Poliovirus is endemic in only two countries: Pakistan and Afghanistan. Active vaccination programs and surveillance systems have eradicated Polioviruses from the rest of the countries, though Vaccine Derived Polio Viruses (VDPVs) can arise when vaccine coverage of the population is too low to prevent the transmission of the Sabin vaccine. Cases occur regularly in third world and developing countries, with cases in 2015 occurring in Lao, Ukraine, and Madagascar, among others (<http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx>). The last wild type poliomyelitis case in South Africa was in 1989 (Chezzi et al., 1997). In adults, polio infection is more likely to cause paralytic poliomyelitis, rather than the acute, non-paralytic CNS symptoms like aseptic meningitis, or asymptomatic infections seen

in younger children (Horstmann, 1955). The discrepancy seen in the different age groups is also seen in other EVs, such as EV71 (Komatsu et al., 1999, Lum et al., 1998) and CVB (Dery et al., 1974). Poor socioeconomic status increases the likelihood of infection (Honig et al., 1956, Otatume and Addy, 1975), although, as mentioned above with polio, the better the socioeconomic status, the less likely the infection, but the more severe the symptoms or complications of an EV infection (Melnick, 1984).

With the elimination of Polioviruses from the majority of the planet, there is a possibility of the emergence of other serotypes into the void created by the absence of Polioviruses. In addition AFP cases are still being diagnosed that are not caused by PV, and may be caused by other EV serotypes (Mehrabi et al., 2011). While many studies have tried to determine the cause of these AFP cases, many different EV have been typed, and no clear association can be made between a particular serotype and the AFP cases still detected. South African surveillance studies on non-polio EV have been limited to single outbreaks and in very localised geographic locations, and none of the studies focused on AFP (McIntyre and Keen, 1993, Schoub et al., 1985, Yeats et al., 2005), however these studies may give an initial insight into what symptoms some other serotypes cause.

Incidence data about particular EV-caused disease can be obtained from prospective longitudinal studies, although these are very difficult and expensive (Cooney et al., 1972, Kogon et al., 1969, Strikas et al., 1986). Recent studies in China show that CVA6, CVA16 and E71 (associated with hand-foot-and-mouth disease) have infected

almost 90% of the adolescent population, and at least 50% of the population has been infected by more than one of the viruses (Ang et al., 2015). These studies have the advantages of avoiding the problem of passive surveillance, and allow for analysis of infections and disease incidence, despite the costs. Passive case finding is cheaper, but the information yielded is less useful. The surveillance might miss a case as it will only detect samples from individuals with an easily identified symptom, and diagnosed by someone who decides to report it. Yet, the information from this type of surveillance may detect trends in infections (Bell and McCartney, 1984, Strikas et al., 1986). Surveillance data collected from notifiable disease networks (e.g. Polio) is the most accessible data, but least representative of all the systems.

Incidence is difficult to determine for EV as its transmission in populations can be endemic, sporadic or as an epidemic and activity in children is often subclinical or asymptomatic. Large populations often have multiple serotypes circulating concurrently and in no particular pattern. Outbreaks can occur in varying degrees and they can be characterised by serotype, time, location, and disease (Moore, 1982, McIntyre and Keen, 1993, Rossouw et al., 1991, Yeats et al., 2005, Kumar et al., Bal et al., 2015).

Studies on PV have greatly influenced the field of molecular virology. PV was the first animal virus completely cloned and sequenced (Kitamura et al., 1981, Racaniello and Baltimore, 1981b), the first RNA animal virus for which an infected clone was constructed (Racaniello and Baltimore, 1981a), and the first human virus that had its three-dimensional structure solved by x-ray crystallography (Hogle et al., 1985). The

PV receptor, after its discovery in 1989 (Mendelsohn et al., 1989), was then generated in mice carrying the CD155 as a transgene (Koike et al., 1991, Ren et al., 1990). Molecular epidemiology has helped refine EV studies, and improve our understanding of the viruses by the following: identifying unequivocal strains, providing insights into taxonomy and classification, clarifying origins of outbreaks, and allowing identification of strains transmitted between outbreaks (Fields, 2007). Older studies were conducted using monoclonal antibodies and oligonucleotide fingerprinting, but the most accurate and useful assay for EV classification is nucleic acid sequencing, which is able to detect even small differences in closely related strains (Rico-Hesse et al., 1987). Various studies on EV have been conducted using molecular sequencing techniques, including those analysing CVB1, CVB5, Echovirus (E) 5 and EV71 (Brown et al., 1999, Drebot et al., 1999, Kopecka et al., 1995, Oberste et al., 1999a, Zoll et al., 1994), which have improved understandings on circulation, outbreaks and different genotypes of the viruses. The Oberste study in 1999 studied E30 and its evolution over decades (Oberste et al., 1999a). E30 was significant for the study as it was, and still is, an important virus causing aseptic meningitis. They used the full VP1 gene to monitor its molecular evolution over time. They found that E30 had periods where it was prevalent in the population, though the virus circulating in the late 1990s had evolved from the viruses circulating decades before. Also the virus lineages were traceable and distinct, changing through each temporal prevalence spike.

More recent studies identify non-polio enteroviruses that are isolated in cell culture from the acute flaccid paralysis surveillance programs operating in the respective

countries (Dhole et al., 2009), though a small number of studies extract RNA from stool samples directly and then amplify and sequence the genetic material present in the samples (Mehrabi et al., 2011). Enterovirus specific primers are used to amplify only viral RNA and then type the Enterovirus positive samples (Mehrabi et al., 2011).

The common trend found between these studies is that Enteroviruses are found endemically in healthy and diseased patients. There will be a difference in the predominant types when comparing regions, and comparing time periods within a region. A study in Cameroon, using 3 cell lines to isolate enteroviruses showed great diversity in HEV-B and –C, though no HEV-A viruses were typed. HEV-C viruses constituted the bulk (63.1%) of all the typed viruses. In this particular study Coxsackievirus A13 was the predominant subtype found over 2 years (Sadeuh-Mba et al., 2012). In comparison, an Indian study utilising the CPE positive, Polio negative viral isolates from their AFP Surveillance program, found that most of the typed viruses over two years were from HEV-B. HEV-A and –C viruses were also detected. In contrast to the Cameroon study, an HEV-A virus, Enterovirus 76, was the predominant type in the 1st year of the study, and 2 viruses from HEV-B were predominant in the second year: Echovirus 7 and Echovirus 20 (Laxmivandana et al., 2013). A study conducted over a period of 10 years using various specimen types, and many enterovirus sensitive cell lines, display the variety of enteroviruses circulating in the population, and the change in distribution over time (Trallero et al., 2010). The study also demonstrates how difficult it would be to diagnose a patient without molecular typing, as a number of the different types were found distributed between different symptoms, and specimen types.

1.5 Diagnosis and Surveillance tools

Clinical diagnosis is difficult considering the factors of EV infection already described. Symptoms vary widely, and many are similar to other pathogenic and non-pathogenic diseases. Also, as many EV infections are asymptomatic, the detection of the virus in a sample may not prove disease causation. If there is an outbreak or epidemic, clinical diagnoses are made easier, as the causative agent is much more likely to be an EV. The most accurate way to detect the viruses when looking for a causative disease is by taking a sample from the infected area, for example: cerebro-spinal fluid (CSF) for aseptic meningitis or cardiac tissue for myocarditis. The sample with the highest sensitivity for detecting EV is a stool sample, regardless of clinical presentation (Mintz and Drew, 1980) though this may be complicated by the intermittent viral shedding during disease, and concurrent infections by more than one enterovirus.

Many of the procedures for the detection of EV using virus isolation have been described (Grandien M, 1989), but these techniques are long and labour intensive. If the appropriate cell lines are used, isolation of EV from these cells can take 2 to 3 days and remains a sensitive method for detecting these viruses. Preferred samples are stool samples, but throat swabs and washings, and spinal fluid can also be used to inoculate the cells. Unfortunately, no single cell line can be used to culture all HEV, therefore a combination of several cell lines is commonly used to detect EV (Chonmaitree et al., 1988). Despite using these cell line combinations, several CVA serotypes are difficult to grow in culture (Nsaibia et al., 2007) and suckling mice are

then used to propagate these serotypes. Transformed cells, such as the L20B cells containing the CD155 receptor for PV attachment and entry are also used to select for certain serotypes, even in the presence of other serotypes (Hovi and Stenvik, 1994). Confirmatory testing is performed using these cells as there are a few non-polio serotypes that grow in these murine cells.

After detecting cytopathic effect (CPE) in these cells, identification is typically done by neutralization with type-specific antisera prepared in horses and available from the World Health Organisation (WHO) (Melnick and Hampil, 1973); though currently supplies are becoming limited. When these antisera were being produced there were at least 66 known serotypes. Individual typing was impractical so antisera were combined in intersecting pools in such a way that antibody to any one type was present in only a limited number of pools (Melnick et al., 1973). Even using these pools, untypable EV were still detected due to mixed infections, aggregates of viruses, extreme antigenic drift, or undiscovered types. In theory, it was possible to type these viruses, but as it is seldom critical to type non-polio EV for clinical management of a routine case, the labour intensity and time it took to conduct these assays rarely justified the result. Thus neutralisation assays are more useful for epidemiology, than for clinical diagnosis.

Molecular techniques have greatly improved EV diagnostics. Since these methods are much quicker than the conventional methods of EV detection (viral culture and antibody tests), one such molecular technique, polymerase chain reaction (PCR) has become widespread in use. The most common use of PCR for EV diagnosis is the

direct detection of EV from clinical specimens (Kessler et al., 1997, Olive et al., 1990, Rotbart et al., 1994). These PCRs may have slight differences, but the main characteristic that is common, is that they target the 5' UTR (the most conserved region) of the EV genome. PCR is able to detect small amounts of EV RNA from samples such as spinal fluid, as well as being able to detect EV serotypes that do not grow well in cell culture. One of the disadvantages however, is that the sensitivity of PCR to detect EV varies in different specimen types. For example, some studies have not been able to gain any advantage over the more traditional methods of EV detection in specimen types such as stool (Ahmed et al., 1997, Tanel et al., 1996).

By changing the genome target sites for the PCR, the specificity of the reaction can be modified to detect specific EV serotypes only rather than detecting a number of similar serotypes (Kilpatrick et al., 1998). To accomplish this, despite the number of synonymous nucleotide mutations, is the use of inosine in primer synthesis. This method has been used extensively to target the VP1 gene to classify different PV through PCR (Kilpatrick et al., 1996).

Genetic sequencing has also had an impact on EV typing. A combination of PCR and genetic sequencing can be used to assign an unknown virus to a specific serotype, even though the genetic locations to many viral functions are unknown (Oberste et al., 1999b). Current serotyping assays are based on sequencing the 3' end (to characterise a virus into its species group) or the entire VP1 gene (to characterise a virus by its serotype) of Enteroviruses. The sequences are then used to characterise the virus based on the criteria listed above in section *1.2. Diversity in Enteroviruses*.

There are two different sequencing methods that are commonly used in current molecular laboratories. The traditional, older method is Sanger sequencing (Sanger et al., 1977). This method uses the structure of the deoxyribose sugar to produce different lengths of oligonucleotides. These oligonucleotides are terminated by nucleotides with a 3' carbon that is missing an extra oxygen molecule, which would then not allow another nucleotide to attach to it. Modern equipment allows for these terminator nucleotides to be detected by a laser as the terminators are also labelled by a fluorescent tag, with a different colour tag for each nucleotide: A, C, T, and G. The equipment detects the different lengthed oligonucleotides, each with their own tagged terminator, and an attached computer aligns the tags into a sequence that is then able to be analysed. Sanger sequencing is precise and accurate as primers are used to detect a targeted sequence. If non-specific sequences are needed to be sequenced, then more recent methods are available for this: Next-Generation Sequencing.

The more recent developments in molecular sequencing allow for more rapid assays that are able to cover a large amount of sequence information (either very long sequences, or many different sequences in a single sample), or resolve sequences to a point where single nucleotide mutations can be detected between quasispecies in a sample (Hert et al., 2008). The next-generation sequencing methods are varied though costly. Since the introduction of these methods, costs have been steadily declining, and the availability of the methods for studies has been more widespread.

The information mined using these techniques decreases the costs of sequencing large amounts of genetic information.

EVs have genomes approximately 7500 bases in length, therefore Sanger sequencing of the entire genome will be expensive and time consuming. The new techniques decrease the costs of sequencing whole genomes, as well as allowing for studies involving viral recombination, or small differences in viruses circulating in the population such as quasispecies studies and the effects of single nucleotide polymorphisms, on virus infectivity, symptoms caused, and severity of disease.

1.6 Aim of the study

The majority of studies on Enteroviruses in South Africa have been focused on Poliovirus or outbreaks of other enteroviruses. Outbreak studies have been short term studies that only looked at one particular virus causing disease (Yeats et al., 2005, Schoub et al., 1985) and did not follow the endemic disease-causing viruses in the population. The last known study describing the prevalence of non-polio enteroviruses in South Africa was a retrospective study performed in 1993 (McIntyre and Keen, 1993) that focused only on Cape Town. It was found that Coxsackie B viruses were endemic, though the meningitis outbreaks (that were in the summer months) were caused by a number of HEV-B viruses: Echoviruses 4 and 9, and Coxsackievirus A9. While it was stated that enteroviruses were endemic and were the largest cause of meningitis in the population, the study only focused on meningitis. Since PV has been

eliminated from South Africa, what is of particular importance is that acute flaccid paralysis cases are still being diagnosed in the country. These cases may be caused by other non-polio enteroviruses and hence the continued surveillance for other enteroviruses should be considered (Dhole et al., 2009, Mehrabi et al., 2011). Some EV cause more than one symptom of disease and may cause serious complications from an asymptomatic or subclinical infection and the predominant serotype causing a symptom may also change over time (Pons-Salort et al., 2015). The circulation and changes in predominance in serotypes is complex and varied, however a surveillance program would be able to track and identify these problem serotypes and the diseases they are associated with.

The objectives of the study are:

- To develop a multiplex real-time PCR assay capable of differentiating between the 4 enterovirus species: HEV-A, -B, -C, and -D.
- To develop a Sanger-based sequencing assay able to identify, and differentiate between all non-polio enterovirus subtypes.
- To develop a novel assay to identify non-polio enterovirus subtypes/mixed infections using deep level sequencing (Next Generation sequencing). This can then possibly be used as a basis to develop an assay to investigate into enterovirus recombination and its patterns.
- To compare neutralization assays used currently with the real-time PCR multiplex assay, the Sanger sequencing assay, and the deep level sequencing assay results, with regards to turn-around-time, cost-effectiveness, workflow, sensitivity and accuracy. This is to determine whether sequencing is a more efficient method than

neutralization assays in a diagnostic setting, and will determine which type of sequencing assay is most suitable.

- To determine the epidemiology of non-polio enteroviruses circulating in the South African population from 2010 to 2012, and to determine the importance of non-polio enteroviruses in causing acute flaccid paralysis.

2. Materials and Methods

2.1 Sample selection

Two different surveillance programs running at the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa, sourced eight hundred and thirty two (832) samples for this study from the period 2010-2012: the Acute Flaccid Paralysis (AFP) Surveillance Program and the Rotavirus Vaccine Surveillance Program. The AFP Surveillance Program collects stool samples with the aim of detecting any Polioviruses that cause acute flaccid paralysis in children under the age of 15, as per the World Health Organisation guidelines. The Rotavirus Vaccine Surveillance Program obtains stool samples from young children (under the age of 5 with diarrhoea severe enough to be hospitalised) (Msimang et al., 2013).

These samples constituted 3 different specimen types: Enterovirus positive culture specimens from the AFP program that are not Poliovirus, stool specimens from the AFP program that did not yield a positive Poliovirus result or positive Non-Polio

Enterovirus result, and a random selection of stools from the Rotavirus program where the presence of Enteroviruses present in the samples is unknown.

The AFP Surveillance Program supplied viral cultures that were Poliovirus negative by PCR, yet had cytopathic effect (CPE) indicative of enterovirus infection in the RD and/or L20B cell lines (Wood and Hull, 1999, WHO, 2004). This specific combination of CPE and PCR result in these cell lines indicates the presence of a Non-Polio Enterovirus. 175 culture samples that resulted in a Non-Polio Enterovirus (NPENT) were selected from the three years. The NPENT samples are used as an indicator (at least 10 NPENTs per 100000 individuals) for a sensitive surveillance system in the program. The AFP program also supplied ninety five (95) culture negative stool samples (one from each of the nine provinces in the country per month of 2012, with some provinces not sending at least one sample every month). Culture negative samples were only obtained from 2012 as the culture negative stools are discarded 1 year after receipt, so samples from the years 2010 and 2011 were unavailable. These negative stool samples were treated with chloroform and phosphate buffered saline to retrieve a supernatant that potentially contained Enteroviruses (WHO, 2004). The stool samples from the AFP program were used to detect any Enteroviruses not grown on RD and L20B cell lines.

The Rotavirus Vaccine Surveillance Program supplied five hundred and sixty two (562) stool samples. There were 4 provinces covered by this program: Gauteng, Kwa-Zulu Natal, Western Cape and Mpumalanga, covering a mixture of rural, peri-urban and urban populations (Msimang et al., 2013). These samples were selected by

obtaining the first four samples arriving at the NICD from each site per month for the years 2010-2012. Sample size per year was calculated using the calculation $n = [Z^2 * p * (1-p)] / c^2$ (Confidence level of 95%, $Z = 1.96$) whereas 'p' is as per Table 2.1:

Table 2.1: Estimated Sample Size for non-AFP Enterovirus Frequency in a Population

Hypothesized % frequency of outcome (p):	15
Confidence Interval % of :	95
SAMPLE SIZE	196

The hypothesized percentage frequency was based on the number of non-polio enteroviruses detected in the AFP surveillance network for South African patients per year. The site in the Western Cape only started collecting samples in May 2010, resulting in a lower number of samples for that year (175 samples). In order not to bias one site over another it was decided not to distribute the collection of the outstanding samples over the other sites and to keep the sample size for 2010 at 175.

The Severe Acute Respiratory Infection (SARI) Surveillance Program conducted their own enterovirus genotyping, and typing results for 37 detected enteroviruses present in respiratory samples for the years 2010 and 2011 were obtained. These results have not been published by this group, and were included in this study as an

indication of the Enteroviruses that are associated with respiratory infections that are able to be detected in respiratory specimens. Approximately 30% of the enterovirus positive samples were typed from 2010 to 2011, and permission to type these remaining positive specimens was not granted.

2.2 Control specimens

Control specimens used to validate the sequencing assays were obtained from QCMD (Quality Control for Molecular Diagnostics, Glasgow, Scotland). The panel samples viruses from all four Human Enterovirus species: Coxsackievirus A16, Enterovirus 71 (HEV-A); Echoviruses 11, 16, 25, 30, Coxsackievirus A9 (HEV-B); Coxsackieviruses A21, A24 (HEV-C); and Enterovirus 68 (HEV-D). In addition, Coxsackievirus B1-6 and Poliovirus 1-3 SABIN strain virus cultures were obtained from the virus isolation laboratory in the AFP Surveillance Program as part of the methods validation.

A positive control obtained from Coxsackievirus B2 cultures, and a negative water control was included from the initial extraction step through to conclusion of the assay, thus controlling for all steps performed.

2.3 Nucleic Acid Extraction

Extractions were conducted on automated as well as manual systems. The automated system used was the Maxwell 16 (Promega, Madison, WI, USA). Manual extractions were conducted using the Qiagen Qiamp Viral Mini Kit (Qiagen, Venlo, Netherlands). The manual and automated extractions on stool samples (not cultures) were preceded by a treatment of the samples with a Stool Transport and Recovery (STAR) buffer (Roche, Mannheim, Germany). This buffer stabilises any pathogens, neutralises nucleases, as well as binding PCR inhibitors present in the sample.

Automated extractions were performed using 100 μ l of initial stool or culture sample. The stool samples were processed as supplied from the surveillance programs after they had completed routine testing. The STAR buffer treated stool samples were centrifuged at 1000 rpm in a microcentrifuge for 1 minute to sediment the solids and to allow the supernatant to be aliquoted for extraction. The Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) was used to extract the viral RNA.

The initial screening PCR was conducted on the extracted material and sequencing was attempted on these extracts. Those samples that failed to yield a useable sequence were then extracted manually and rerun on the sequencing assay to obtain more results.

The Qiagen Qiampr Viral Mini Kit (Qiagen, Venlo, Netherlands) was used for manual extractions of the viral RNA, as per the manufacturer's instructions (Schuurman et al., 2007). The same STAR buffer treated stool sample used in the automated extraction was used for the manual extraction.

2.4 Enterovirus Screening Real-Time PCR

2.4.1 PCR assay for Enterovirus screening

The (Nijhuis et al., 2002) PCR protocol (Table 2.2) was used to screen samples for the presence of Enteroviruses before moving on to sequencing. This protocol had been used diagnostically within the NICD for the routine AFP surveillance, and had been shown to be specific and sensitive to Human Enteroviruses. This assay screened all the specimens in this study.

2.4.2 RNA dependent DNA polymerase used in the screening assay

A comparison was conducted between two cDNA synthesis kits. The kit used in the diagnostic enterovirus assay was the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Mannheim, Germany). This was compared with the Transcriptor First

Strand cDNA Synthesis kit (Roche, Mannheim, Germany). The Transcriptor Kit was used for the final assay.

2.5 Enterovirus Sequencing by the Sanger Method

2.5.1 PCR and sequencing primers

Nix et al (2006) used semi-nested PCR primers and sequencing primers that would detect all Human Enteroviruses, but were non-specific with many 'wobble' sites to ensure that all viruses were detected (Figure 2.1, Table 2.2). Using this protocol, the primers were tested and validated on QCMD control samples as well as on the culture controls obtained.

An agarose gel was run using the PCR product to separate the Enterovirus amplicon from the non-specific amplicons. This was performed using a 30cm gel of 1.5% agarose concentration for a period of 3 hours at 130 volts in a Tris-Borate-EDTA buffer. The full 50 μ l of PCR product was gel electrophoresed and excised before the clean-up procedure.

2.5.2 PCR product excision and clean up

The agarose gel was visualised under UV light and the molecular marker and positive control used to confirm the presence of an enterovirus amplicon. Excision was done using a scalpel to remove the band from the gel. The Wizard SV Gel and PCR Clean Up System (Promega, Madison, WI, USA) was used as per the manufacturer's instructions to clean the DNA band from the agarose gel as a preparation for the sequencing PCR.

2.5.3 Sanger sequencing

Sanger Sequencing was then conducted as per the BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) using the primers described by Nix et. al. (2006). A schematic of the PCR reactions performed in the Nix sequencing protocol performed on the GeneAmp 9700 (Life Technologies, Carlsbad, CA, USA) can be found in Figure 2.1. Primers and probes used in the screening and sequencing PCR reactions are described in Table 2.2. The sequences were analysed on the 3130 analyser (Life Technologies, Carlsbad, CA, USA).

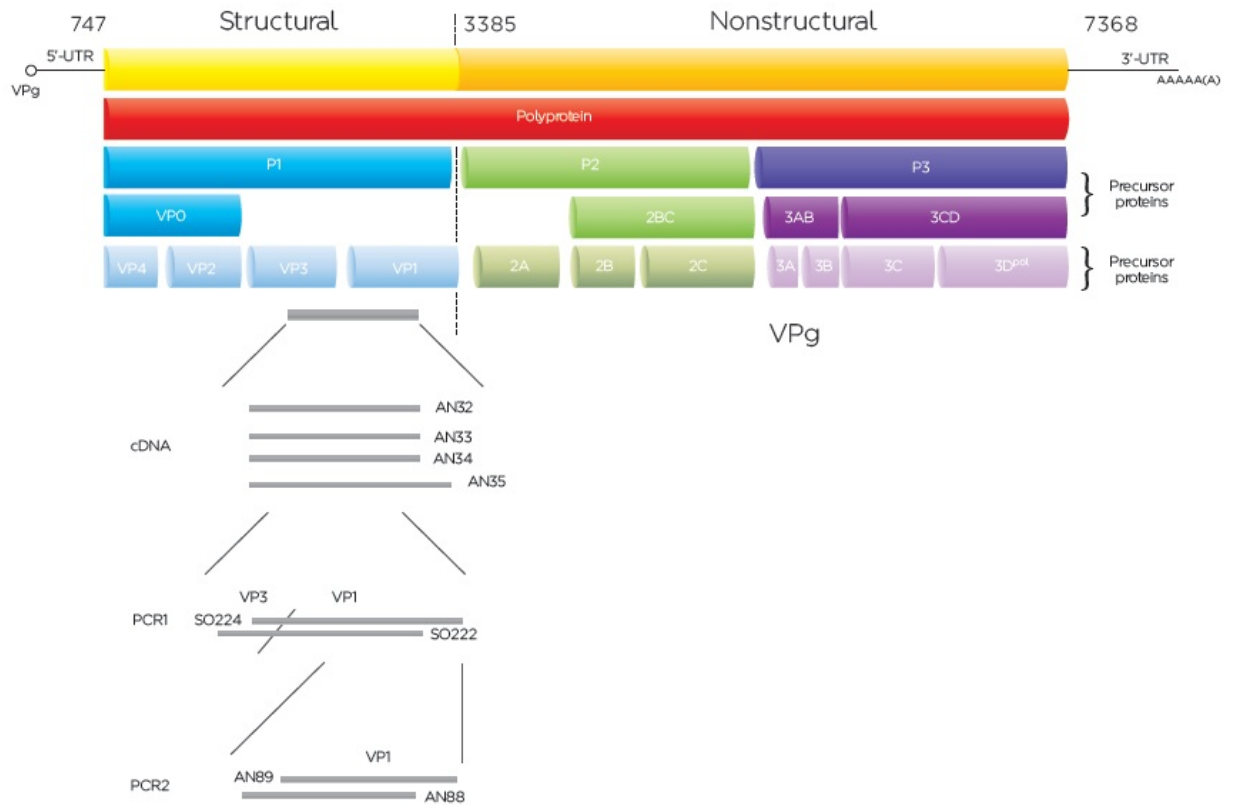


Figure 2.1: Schematic illustration of the genomic organization of enteroviruses and the successive cleavages of the polyprotein, as well as the PCR reactions of the sequencing PCR (Nix et al., 2006). Nucleotide positions correspond to the numbering of the sequence of poliovirus type 2, Lansing strain. Pol: Polymerase; UTR: Untranslated Region; VPg: Genomic Viral Protein

Table 2.2: Primers and probes used in the screening and sequencing PCR reactions.

A= Adenine; T= Thymine; C= Cytosine; G= Guanine; I= Deoxyinosine; Y= C or T; R= A or G; W= A or T; N= G, A, T or C.

PCR reaction	Primer/Probe	Sequence	Reference
Screening cDNA	Random	Hexamer	Roche Transcriptor cDNA

			Synthesis Package Insert
Screening PCR	Forward primer	5' – TCCTCCGGCCCCTGA – 3'	(Nijhuis et al., 2002)
	Reverse primer 1	5' – AATTGTCACCATAAGCAGCCA – 3'	
	Reverse primer 2	5' – GATTGTCACCATAAGCAGCCA – 3'	
	Probe 1	5' – CGGAACCGACTACTTTGGGTGTCCGT	
	Probe 2	5' – CGGAACCGACTACTTTGGGTGACCGT	
Sequencing cDNA	AN32	5' – GTYTGCCA – 3'	(Nix et al., 2006)
	AN33	5' – GAYTGCCA – 3'	
	AN34	5' – CCRTCRTA – 3'	
	AN35	5' – RCTTYGCCA – 3'	
1 st round PCR	SO224	5' - GCIATGYTIGGIACICAYRT – 3'	
	SO222	5' – CICCIGGIGGIAYRWACAT – 3'	
Semi-nested PCR, and sequencing primers	AN89	5' – CCAGCACTGACAGCAGYNGARAYNGG – 3'	
	AN88	5' – TACTGGACCACCTGGNGGNAYRWACAT – 3'	
Alternative sequencing primers	AN232	5' – CCAGCACTGACAGCA – 3'	
	AN233	5' – TACTGGACCACCTGG – 3'	

2.6 Next Generation Sequencing

Culture positive samples from the AFP network were selected for Next Generation Sequencing according to their species. HEV-C viruses were selected based on their increased likelihood to have recombined with the Polio Sabin vaccine strains. Primers were sourced from a study by (Boot et al., 2004) which covered the entire EV genome and were specific to HEV-C. cDNA was synthesised using three combinations: 1) random primers only, 2) random primers and anchored primers, 3) and anchored

primers only. PCR was then conducted with the sourced primers and the Expand Long Template PCR System (Roche, Mannheim, Germany) as per the manufacturer's instructions. This kit contained a Taq polymerase enzyme able to synthesize long strands of DNA. Sanger sequencing on the ABI 3130 analyser (Life Technologies, Carlsbad, CA, USA) and next generation sequencing on the GS Junior (Roche, Mannheim, Germany) were conducted and the results compared.

A second method was attempted to create double stranded cDNA. The cDNA Synthesis System (Roche, Mannheim, Germany) was utilized for this assay as per the manufacturer's instructions. This kit contains all the reagents needed to create cDNA and double stranded cDNA. Combinations of anchored, random and specific primers can be used with this kit and was tested as with the Long Template PCR System (Roche, Mannheim, Germany). Variations of magnesium chloride concentrations, RNA concentrations, and combinations of the different primers were attempted to obtain usable cDNA for sequencing

2.7 Enterovirus Serotyping by Genotyping

From the Sanger sequencing assays, all specimen controls were serotyped correctly by genotyping as per Oberste's criteria for typing enteroviruses (i.e. greater than 75% sequence homology to the published sequences (Oberste et al., 2006). The National Centre for Biotechnology Information (NCBI) database was used as a source to

BLAST (Basic Local Alignment Search Tool) the enterovirus sequences obtained in the study.

The National Institute for Public Health and the Environment (RIVM), Netherlands, developed an Enterovirus Typing Tool based on the NCBI BLAST tool (Kroneman et al., 2011). This tool was similar to the BLAST tool, but was found to be more user-friendly.

2.8 Sequence Analysis

Initial chromatogram analysis and visual checking was conducted using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Consensus sequences were exported into a text file that was used in the BLAST programs mentioned in section 2.7 *Enterovirus Serotyping by Genotyping*.

2.9 Data mining

Microsoft Excel was used to sort data and create tables and graphs for total samples tested, serotypes detected and comparisons. Epi Info (CDC, Atlanta, USA) was used to create the country distribution image in Section 3: *Results*.

2.10 Ethics

Ethics was obtained from the University of Witwatersrand Ethics Committee under the study numbers: M120467, M119034, and M111145.

3. Results

3.1 Stool Sample treatment before extraction

The STAR buffer (Roche, Mannheim, Germany) improved the yield of sequences from the stool samples. Before treatment the yield was 30% for 2010, 26.27% for 2011, and 37.89% for 2012 in the Rotavirus Vaccine Surveillance samples. After treatment, the yield improved to 58.12% for 2010, 55.88% for 2011 and 60.00% for 2012. The AFP Surveillance programs stool samples improved from 62.50% sequences obtained to 68.75% (26.62% improvement overall for all samples). A paired t-test with a Confidence Interval of 95% yielded a p value of less than 0.0001, showing a statistically significant difference in the yield of sequences after treating the samples with STAR buffer before extraction. After the comparison was taken into account, and the treatment of stools with the STAR buffer was tested, the recommendation was for manual extractions to be done at all times, and automated extractions only done if there are large numbers of samples that make manual extractions unfeasible.

3.2 Comparison between AMV enzyme and Transcriptor Enzyme

The Nijhuis et al (2002) protocol was in use for diagnosing the presence of Enteroviruses in routine patient samples at NICD, using the AMV reverse transcriptase. A newer enzyme available on the market, the Transcriptor enzyme, was made available and was tested and compared to the AMV enzyme as per Table 3.1.

Table 3.1: Comparison of the First Strand cDNA Synthesis Kit for RT-PCR (AMV), and the Transcriptor First Strand cDNA Synthesis Kit. CSF – Cerebrospinal Fluid

Sample number	Sample type	Qualitative result	Real-Time PCR Crossing point (AMV Kit)	Real-Time PCR Crossing point (Transcriptor Kit)
Neg		Neg		
1	CSF	Pos	36.02	0
2	CSF	Neg		
3	Blood (plasma)	Pos	24.67	20.68
4	CSF	Pos	31.05	27.97
5	Stool (clarified)	Pos	38.19	35.25
6	CSF	Neg		
7	CSF	Pos	33.8	32.1
8	CSF	Neg		

The Transcriptor Kit showed an improvement in detection of the EV genome, in the limited available diagnostic samples, as the crossing points of the real-time PCR curves were earlier in all specimens except one. This one sample was a CSF sample, and had undergone a freeze-thaw before being used for this comparison. This may have degraded the viral RNA to the point where it could not be detected. The use of blood plasma and clarified stool was to indicate that other sample types could be

used, and would yield similar results. The Transcriptor Kit was then implemented in the screening assay to improve sensitivity.

3.3 Real Time PCR

Initial investigations to detect enterovirus nucleic acids from the extracted samples used primers targeting the 3' Untranslated region of Enteroviruses with the aim of distinguishing the samples by species, as this region is conserved within a species, but differs with viruses of another species. A method described by (Oberste et al., 2006) described these primers. The results from their PCR were then used to determine species specific primers that gave sequences able to be used for typing. Using the protocol described, no product was detected from the extracted samples using the Roche LightCycler FastStart DNA Master SYBR Green I Kit (Mannheim, Germany). Extracted RNA of an enterovirus control provided a constant starting nucleic acid concentration, and attempts were made to improve the protocol by varying the primer concentrations, increasing the number of PCR cycles, using a G-C rich buffer, and varying the annealing temperatures during the PCR (using a gradient PCR, ranging from 36.0°C to 46.0°C in 0.5°C increments). No amplification was seen throughout these trials, though non-specific amplification was seen in the tests where the cycle numbers were increased. This is most likely primer-dimer formation, and it was not observed in any other aspect of the trials.

Conventional PCR was also attempted with these primers, though they had to be loaded onto a gel without loading dye as the products are approximately the same size at which the dye runs on the agarose gels, which might mask the bands, but no positive results were obtained. The molecular weight marker was loaded with dye as a reference. For each of the controls used (1 for each species), resultant cDNA concentrations were measured using a mass spectrophotometer, and approximately 10ng/ μ l were produced from the PCR. In order to confirm that the control specimens did indeed contain virus, an experiment was performed using the routine Enterovirus diagnostic Real-Time PCR used within the NICD (Nijhuis et al., 2002) and a clear presence of the virus was shown in the controls.

A protocol by (Nix et al., 2006) differed from the Oberste protocol in that it only contained primers targeting the most conserved area of the 5' untranslated area of the EV genome and that these would be able to detect all the HEV serotypes but not be able to differentiate between them (Figure 2.1).

3.4 Comparison between the Qiagen Gel extraction kit and the Promega Wizard SV PCR and Gel Clean Up System

During the troubleshooting of the samples that did not yield clean sequences that were able to be typed on the BLAST programs, a comparison was done to try improve the PCR product obtained from the clean-up of the amplicon band excised from the

agarose gel. 1 sample was amplified in separate tubes, and 3 tubes were tested by each kit.

Table 3.2: Comparison of two post PCR product clean up kits from Promega and Qiagen

Promega Wizard SV Gel and PCR Clean up System				QIAquick Gel Extraction Kit			
Sample	ng/ μ l	260:280	260:230	Sample	ng/ μ l	260:280	260:230
Promega 1	19.44	2.09	1.34	Qiagen 1	15.85	2.3	0.03
Promega 2	13.44	2.27	1.17	Qiagen 2	17.1	2.28	0.03
Promega 3	15.67	2	1.5	Qiagen 3	13.35	2.31	0.04
Average	16.18	2.12	1.34		15.43	2.30	0.03

The Promega kit did not yield larger quantities of DNA from the gel bands than the Qiagen kit when measured on the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) (Table 3.2). The Promega kit had a slightly better yield (16.18>15.43 ng/ μ l), a better DNA:RNA purity (2.12<2.3; a 260 λ :280 λ ratio of between 1.8 and 2.0 is ideal), and less contaminants (1.34>0.03; a 260 λ :230 λ ratio of 2.0-2.2 is ideal). In particular, the Qiagen kit gave a 260 λ :230 λ ratio of 0.03 on the spectrophotometer showing that there were large amounts of chemical contaminants inhibiting the absorbance of light by DNA at the 260 wavelength. The Promega kit was also more user friendly, and required less time to extract the PCR product than when using the Qiagen Kit.

3.5 Sanger Sequencing

3.5.1 Sequencing Primers

Due to the failure of the Oberste et al (2006) screening protocol, the sequencing primers from that study were not used, as the species type was required for them to be selected. The Nix et al (2006) protocol used primers that detected all Enteroviruses, but these primers also detected other related viruses within the *Picornaviridae* genus, such as Rhinoviruses. The screening Real-Time PCR (Nijhuis et. al., 2002) ensured that samples screened were positive for Enteroviruses only, and this reduced the possibility of non-specificity being a factor in the sequencing PCR. Mixed infections with two or more Enteroviruses cannot be typed though, as the Nix primers would amplify and select for all enteroviruses, and then unusable sequence information would be obtained from the genetic analyser.

3.5.2 cDNA Production

Dithiothreitol (DTT), though included in the Roche PCR enzymes used (Transcriptor Reverse Transcriptase package insert), was found to improve PCR yields if added in extra volumes, and so an extra 1 μ l was added during the cDNA synthesis for each sample as suggested by the Nix et al (2006) protocol.

3.5.3 Gel Electrophoresis

Initially 10 μ l of sequencing PCR product was run on the gel and the remainder was cleaned up with the QIAquick Gel Extraction Kit from Qiagen (Qiagen, Venlo, Netherlands). Subsequently, it was found that using this cleaned product for sequencing resulted in many results with mixed sequences due to non-specific amplification, even though the EV positive band was the strongest band present on the gel. The full 50 μ l was gel electrophoresed and excised from the gel before the clean-up procedure using the Promega kit as mentioned in *3.4 Comparison between the Qiagen Gel extraction kit and the Promega Wizard SV PCR and Gel Clean Up System*. This eliminated most of the mixed results, though the remaining mixed results indicated that there are still products being amplified very close to the enterovirus product size, making band separation difficult; or mixed infections which Sanger sequencing cannot separate.

3.6 Next generation sequencing

Successful generation of cDNA was unfortunately not obtained by either of the two systems used. Using a spectrophotometer, RNA presence was confirmed on the eluent from the sample extraction, however, gel bands were not present after various steps in both systems. Troubleshooting of the kits has not yielded any positive results thus far.

3 samples were obtained from cell culture and in HEV-C: a CA22 from 2010, a CA21 from year 2011, and an EV99 from 2012. The Expand Long Template Kit (Roche, Mannheim, Germany) was used with three combinations of primers to create cDNA. Anchored primers did not yield any bands on the agarose gel used to try detecting the HEV-C samples that were typed. Non-specific smudges were seen on the gel after nested PCR reactions were conducted using the Boot study (Boot et al., 2004). Concentration variations of deoxynucleotides were then attempted. 300 μM , 500 μM , 700 μM and 1000 μM concentrations were attempted in the 2 PCR reactions that constituted the nested reactions. A smudge was detected on the agarose gel using a 300 μM concentration in the first round PCR and 500 μM in the second round PCR. This smudge was excised from the gel and cleaned up as described. The DNA was then sequenced using Sanger sequencing, and on the GS Junior (Roche, Mannheim, Germany), and next generation sequencer. On both instruments only Poly-T sequence was obtained.

Attempts using the cDNA Synthesis System Kit (Roche, Mannheim, Germany) were done using the kit instructions. When no band was obtained in the control RNA reaction, concentrations of starting RNA, Mg^{2+} , and different combinations of anchored, random and specific primers were attempted. Still no control band was obtained, and consultations with the manufacturer yielded no further progress. Budget constraints then halted these investigations. Further studies will need to be conducted to find a method that is able to amplify the full genome of EV, and is able to amplify all

the viruses, to ensure ease of use as well as the ability to detect mixed infections and recombined viral genomes.

3.7 BLAST Analysis

Both the NCBI BLAST tool and the Dutch BLAST tool from RVIM were used to type results. For the control specimens it was found that the identification had a 100% correlation between the two tools. However, with patient samples, slight typing differences were observed between the 2 tools (e.g. viruses that are more closely related such as Coxsackie A24 and Enterovirus 99). Using both tools concurrently gave the most accurate serotyping results.

3.8 Tested samples

A total of 832 samples were tested from the AFP Surveillance Program, and the Rotavirus Surveillance Program from 2010 to 2012. From the AFP program, 175 samples were screened after being classified as a non-polio enterovirus (NPENT) by the NICD AFP surveillance laboratory. In addition, ninety five samples were obtained from the culture negative stools from 2012. The Rotavirus Surveillance Program yielded 562 samples to be screened (Table 3.3).

Table 3.3: Number of samples tested from the AFP surveillance network and the Rotavirus surveillance network.

Surveillance source	Number of samples	Total Samples		Detection rate	Sequenced	Final result	Failure rate
		Negative	Positive				
AFP Culture positives	175	29	137	78.29	137	125	8.76
AFP culture negatives	95	63	32	33.68	32	22	31.25
ROTA surveillance	562	285	277	49.29	277	161	41.88
Average (%)				53.61			30.94
Totals	832	377	446		446	308	

Four hundred and forty six (446) (53.61%) samples were positive for EV presence using the real-time PCR screen and 308 (69.04%) of the positive samples yielded sequences able to be compared to the NCBI database and typed. An additional 37 sequences were obtained from the Severe Acute Respiratory Infection (SARI) Surveillance Program, conducted at the NICD (unpublished data).

Male patients constituted 55.51% of the EV positive samples, and the majority of the samples came from children under the age of 5 years old. 41.53% of patients were under 1 year old, 51.27% between the ages of 1 year and 5 years, and the remainder (7.2%) over the age of 5. Patient demographics for these EV positive samples (including the SARI data) are indicated in Table 3.4. Samples originated from all provinces in the country, though the majority were from the sites where the Rotavirus

surveillance network was in operation (Figure 3.1): Gauteng, Western Cape, Kwa-Zulu Natal, and the 2 hospitals in Mpumalanga.

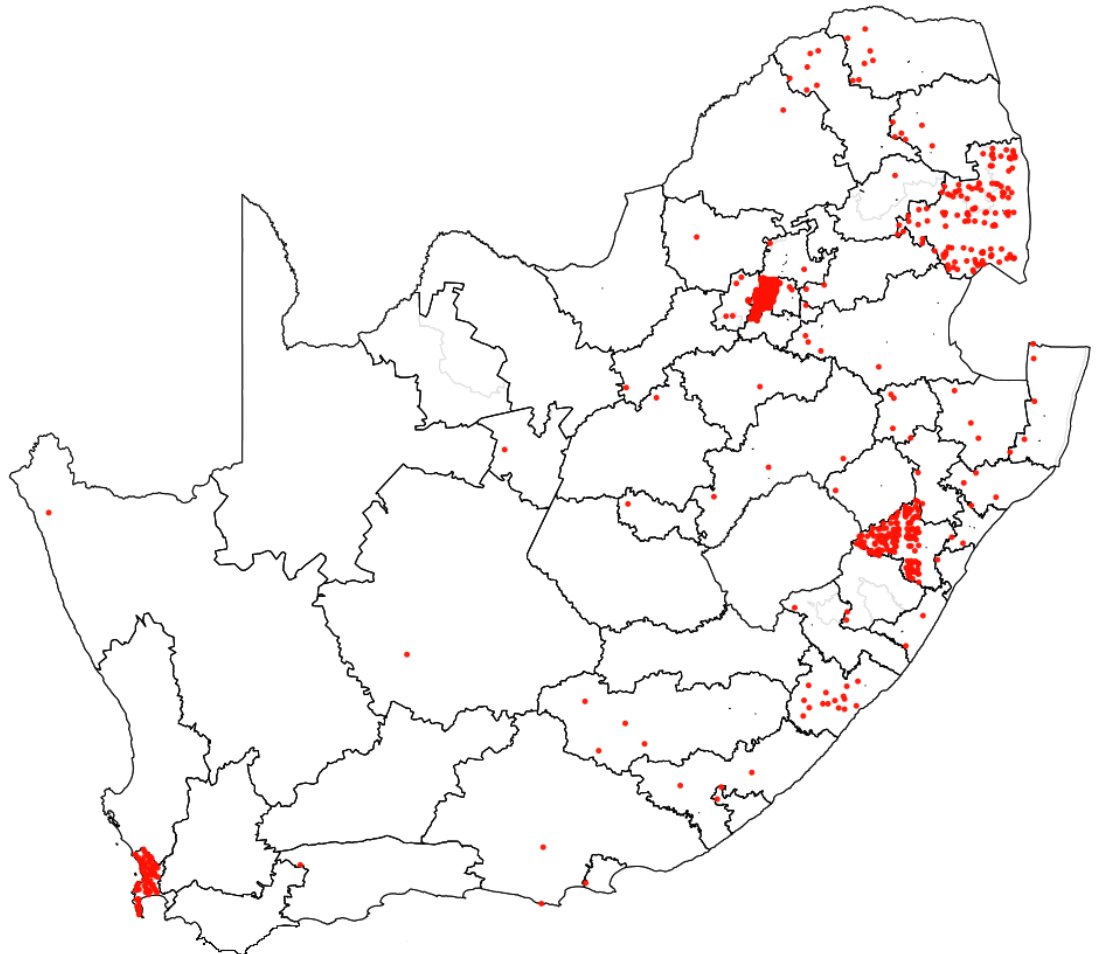


Figure 3.1: Dot-plot image of the health districts in South Africa where the positive samples from the Acute Flaccid Paralysis and Rotavirus surveillance specimens originated

Table 3.4: Gender and age of patients with Enterovirus positive samples

	Percentage (%)	
<u>Gender</u>	55.51 (Male); 44.49 (Female)	
<u>Patients <1 Year old</u>	41.53	(92.8 % under the age of 5 years old)
<u>Patients >1 year and <5 years old</u>	51.27	
<u>Patients >5 years old</u>	7.2	

Sixty four (64) serotypes were detected from all four species groups, HEV-A to –D (Table 3.5). 32 serotypes were from HEV-A, 177 from HEV-B, 95 from HEV-C, and 9 from HEV-D. EV99, from HEV-C, was the serotype most frequently detected. The diversity through HEV-A to -C is high and there is no dominant serotype in HEV-A and HEV-B. Only 1 HEV-D serotype was detected.

Serotype distribution between genders did not display any preferences or patterns. HEV-A serotypes were equally distributed between males (20) and females (19). More males than females were infected by serotypes in HEV-B and HEV-C, 107 males, 82 females and 62 males, 32 females respectively. In HEV-D more females were infected than males with 6 females and 3 males infected with EV68. Serotypes only infecting males were CA1, CA2, CA14, CA17, E2, E5, E16, E17, E18, E32, EV71, EV76, EV77 and EV88. Serotypes only infecting females were CA4, CA8, CB6, E31, EV114, and PV1-SABIN. With detection levels below 10 viruses in each of these

serotypes, a much larger study will be needed to determine any infection patterns between genders.

Table 3.5: Serotypes detected in all samples tested, arranged in species groups

HEV-A			
Serotype	Overall detections	Species HEV-A percentage of total samples	Individual percentage
CA2	4		1.28
CA4	1		0.32
CA5	6		1.92
CA6	3		0.96
CA7	2		0.64
CA8	1		0.32
CA10	4		1.28
CA14	2		0.64
CA16	3		0.96
EV71	2		0.64
EV76	1		0.32
EV114	3	10.22	0.96
Total	32		
HEV-B			
Serotype	Overall detections	Species HEV-B percentage of total samples	Individual percentage
CB1	5		1.60
CB2	4		1.28
CB3	15		4.79
CB4	4		1.28
CB5	8		2.56
CB6	1		0.32
CA9	3		0.96
Ec1	3		0.96
Ec2	1		0.32
Ec3	8		2.56
Ec4	2		0.64
Ec5	1		0.32
Ec6	11		3.51
Ec7	6	56.55	1.92

Ec9	7		2.24
Ec11	10		3.19
Ec12	5		1.60
Ec13	11		3.51
Ec14	7		2.24
Ec15	4		1.28
Ec16	3		0.96
Ec17	1		0.32
Ec18	1		0.32
Ec19	9		2.88
Ec20	6		1.92
Ec21	7		2.24
Ec24	6		1.92
Ec25	7		2.24
Ec27	4		1.28
Ec29	4		1.28
Ec30	5		1.60
Ec31	1		0.32
Ec32	1		0.32
EV75	2		0.64
EV77	1		0.32
EV80	2		0.64
EV88	1		0.32
Total	177		
HEV-C			
Serotype	Overall detections	Species HEV-C percentage of total samples	Individual percentage
CA1	2		0.64
CA11	4		1.28
CA13	11		3.51
CA17	4		1.28
CA19	3		0.96
CA20	2		0.64
CA21	2		0.64
CA22	4		1.28
CA24	16		5.11
EV99	27		8.63
EV102	1		0.32
PV1-SABIN	2		0.64
PV2-SABIN	5		1.60
PV3-SABIN	12	30.35	3.83
Total	95		

HEV-D			
Serotype	Overall detections	Species HEV-D percentage of total samples	Individual percentage
EV68	9	2.88	2.88
Total	9		

Species groups dominated in distribution depending on which sample type was tested. Cell culture specimens had a majority of the serotypes originating from HEV-B (90.40%). The stool samples yielded many more HEV-C serotypes (63.64% in the culture negative AFP samples and 47.2% in the Rotavirus samples), and the respiratory samples contained mostly HEV-B serotypes (43.24%) but also had the only HEV-D serotype detected (Table 3.6-3.8).

Table 3.6: Distribution of the serotypes per species for the AFP surveillance program

AFP Culture Positive Samples				AFP Culture Negative Samples			
Species	Serotype	Number of detections	Total detections	Species	Serotype	Number of detections	Total detections
HEV-A	CA2	1	7/125 (5.60%)	HEV-A	CA2	0	4/22 (18.18%)
	CA4	1			CA4	0	
	CA5	0			CA5	1	
	CA7	1			CA7	0	
	CA8	0			CA8	1	
	CA10	0			CA10	1	
	CA16	1			CA16	1	
	EV71	2			EV71	0	
	EV76	1			EV76	0	
HEV-B	CB1	4	113/125 (90.40%)	HEV-B	CB1	0	4/22 (18.18%)
	CB2	4			CB2	0	

CB3	13	CB3	0
CB4	3	CB4	0
CB5	8	CB5	0
CB6	1	CB6	0
CA9	0	CA9	1
Ec1	1	Ec1	0
Ec2	1	Ec2	0
Ec3	6	Ec3	0
Ec4	1	Ec4	1
Ec5	1	Ec5	0
Ec6	12	Ec6	0
Ec7	3	Ec7	0
Ec9	2	Ec9	0
Ec11	6	Ec11	0
Ec12	2	Ec12	0
Ec13	8	Ec13	0
Ec14	2	Ec14	0
Ec15	0	Ec15	1
Ec18	1	Ec18	0
Ec19	7	Ec19	0
Ec20	5	Ec20	0
Ec21	3	Ec21	0
Ec24	6	Ec24	0
Ec25	1	Ec25	1
Ec27	2	Ec27	0
Ec29	3	Ec29	0
Ec30	2	Ec30	0
Ec31	1	Ec31	0
Ec32	1	Ec32	0
EV75	2	EV75	0

	EV77	1			EV77	0	
HEV-C	CA1	0	5/125 (4.00%)	HEV-C	CA1	1	14/22 (63.64%)
	CA11	0			CA11	1	
	CA13	0			CA13	3	
	CA17	0			CA17	3	
	CA21	1			CA21	0	
	CA22	1			CA22	0	
	CA24	0			CA24	2	
	EV99	3			EV99	4	

Table 3.7: Distribution of the serotypes per species for the Rotavirus surveillance program

Rotavirus Surveillance			
Species	Serotype	Number of detections	Total detections
HEV-A	CA2	3	22/161 (13.67%)
	CA5	5	
	CA6	3	
	CA7	2	
	CA10	3	
	CA14	2	
	CA16	1	
	EV114	3	
HEV-B	CB1	1	63/161 (39.13%)
	CB3	2	
	CB4	1	
	CA9	2	
	Ec1	2	
	Ec3	2	
	Ec6	2	
	Ec7	3	
	Ec9	5	
	Ec11	4	
	Ec12	3	

	Ec13	3	
	Ec14	5	
	Ec15	3	
	Ec16	3	
	Ec17	1	
	Ec19	2	
	Ec20	1	
	Ec21	4	
	Ec25	5	
	Ec27	2	
	Ec29	1	
	Ec30	3	
	EV80	2	
	EV88	1	
HEV-C	CA1	1	76/161 (47.20%)
	CA11	3	
	CA13	8	
	CA17	1	
	CA19	3	
	CA20	2	
	CA21	1	
	CA22	3	
	CA24	14	
	EV99	20	
	EV102	1	
	PV1-SABIN	2	
	PV2-SABIN	5	
	PV3-SABIN	12	

Table 3.8: Distribution of the serotypes per species for the SARI surveillance program

SARI Surveillance Samples			
Species	Serotype	Number of detections	Total detections
HEV-A	CA4	1	6/37 (16.22%)
	CA5	3	
	CA6	2	
HEV-B	CB5	5	16/37 (43.24%)
	Ec3	5	

	Ec9	2	
	Ec11	1	
	Ec12	1	
	Ec14	1	
	EV80	1	
HEV-C	EV99	1	6/37 (16.22%)
	PV1-SABIN	3	
	PV2-SABIN	2	
HEV-D	EV68	9	9/37 (24.32%)

3.9 Distribution of EV by province, age, and surveillance program

Enterovirus serotypes were varied and widespread across the country. A geographic distribution of all the samples typed in each province is shown in Figures 3.2 – 3.10. The graphs indicate no distinct distribution pattern in regards to the serotypes found in the different provinces. Diversity and number of viruses are increased in provinces where the Rotavirus Surveillance Program is collecting samples.

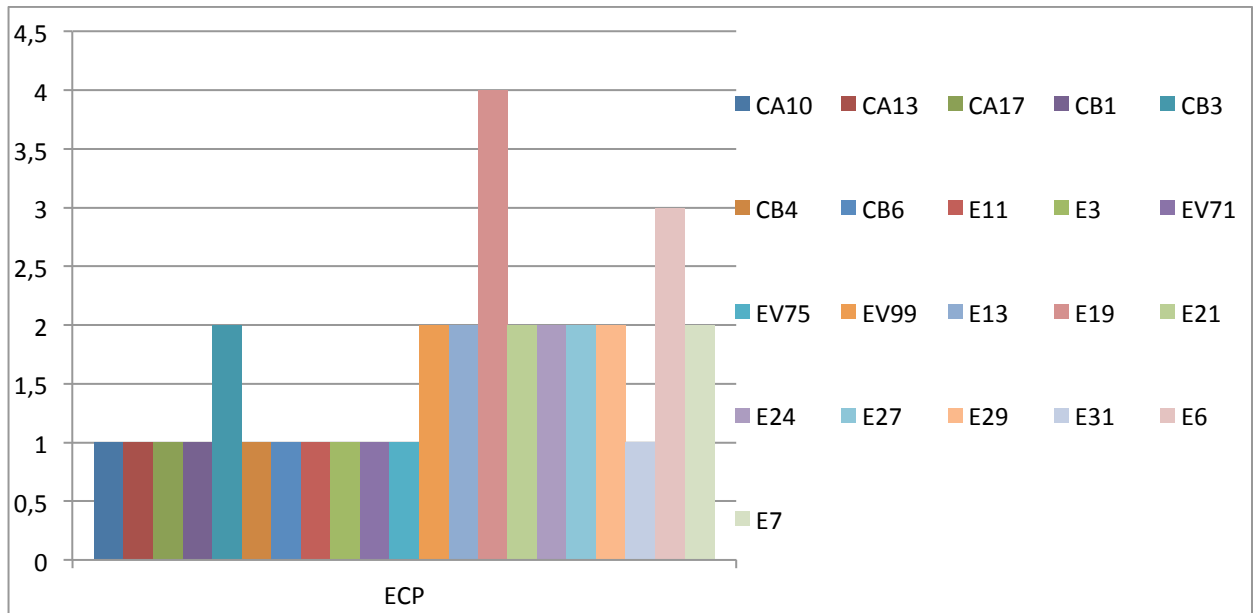


Figure 3.2: Serotype distribution for the Eastern Cape Province. CA – Coxsackievirus

A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus

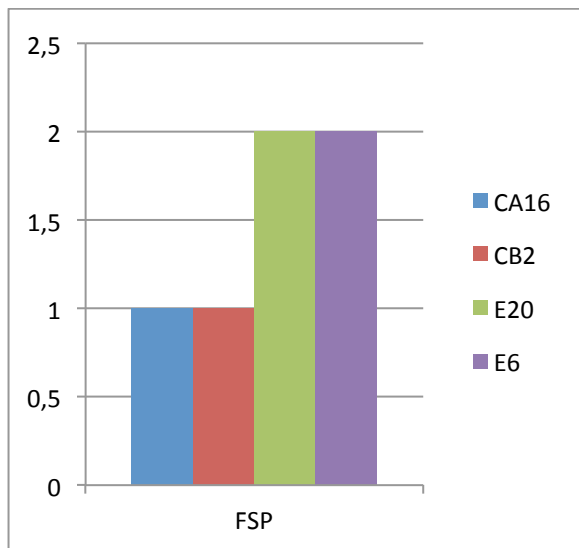


Figure 3.3: Serotype distribution for the Free State Province. CA – Coxsackievirus A,

CB – Coxsackievirus B, E – Echovirus

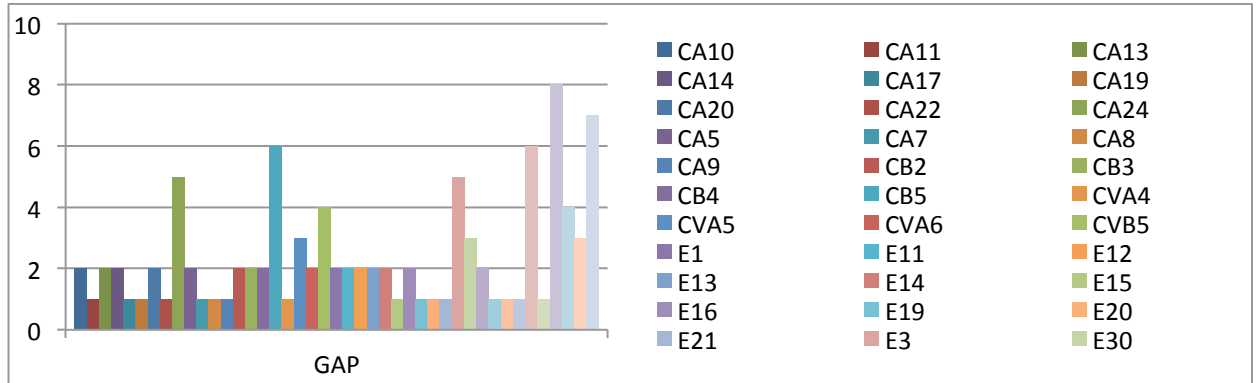


Figure 3.4: Serotype distribution for the Gauteng Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus, PV – Poliovirus

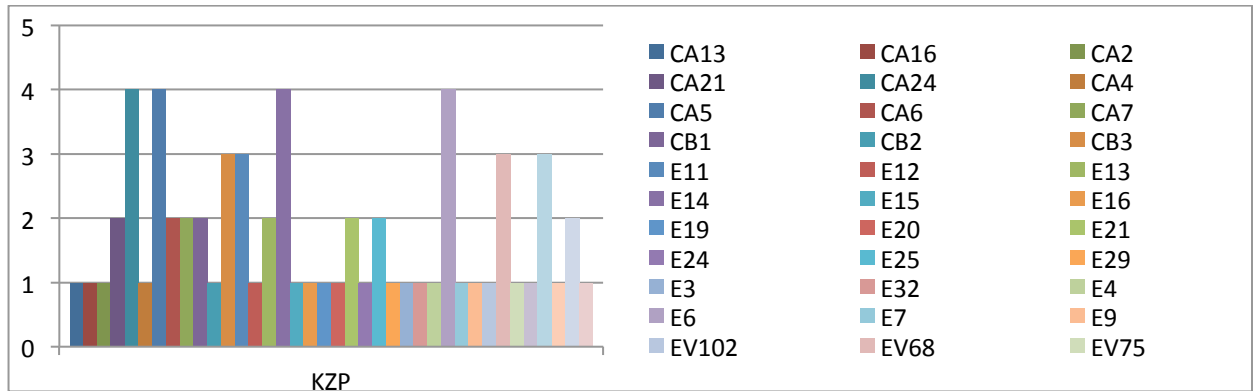


Figure 3.5: Serotype distribution for the Kwa-Zulu Natal Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus, PV – Poliovirus

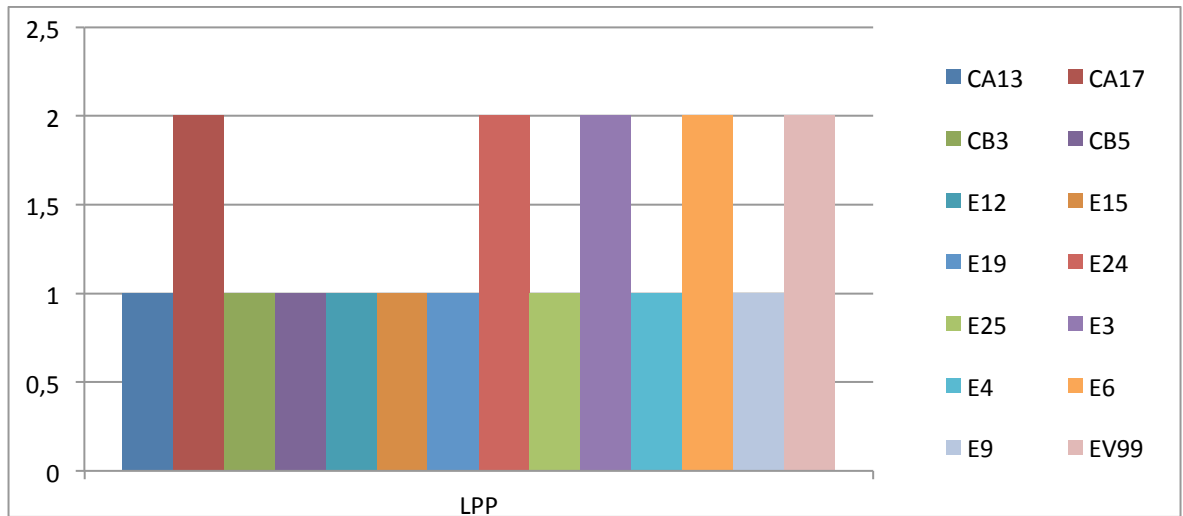


Figure 3.6: Serotype distribution for the Limpopo Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus

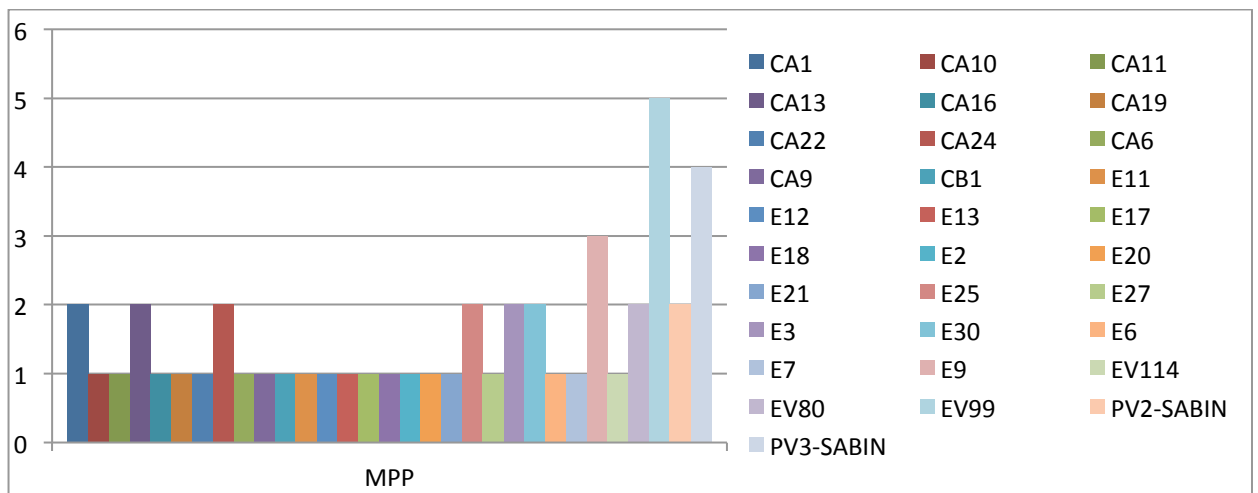


Figure 3.7: Serotype distribution for the Mpumalanga Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus, PV – Poliovirus

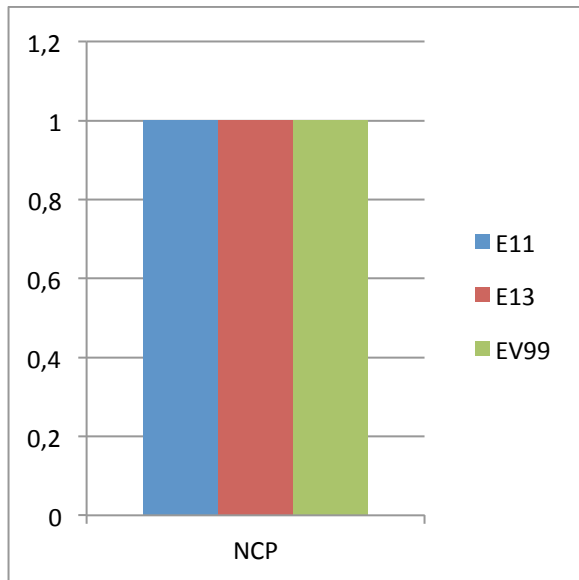


Figure 3.8: Serotype distribution for the Northern Cape Province. E – Echovirus, EV – Enterovirus

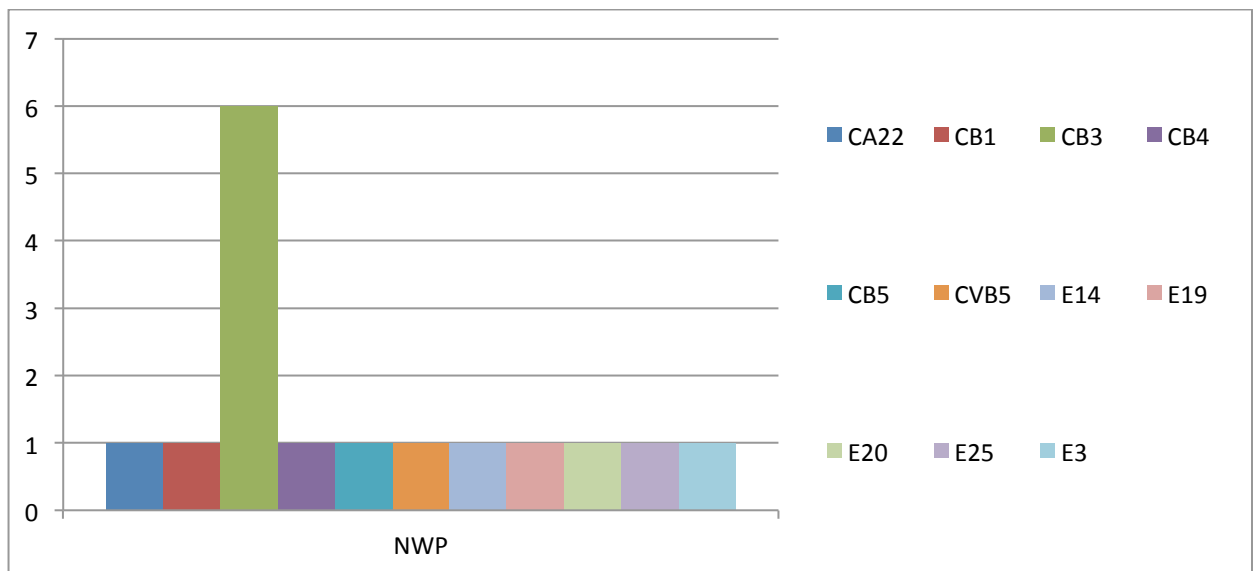


Figure 3.9: Serotype distribution for the North West Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus

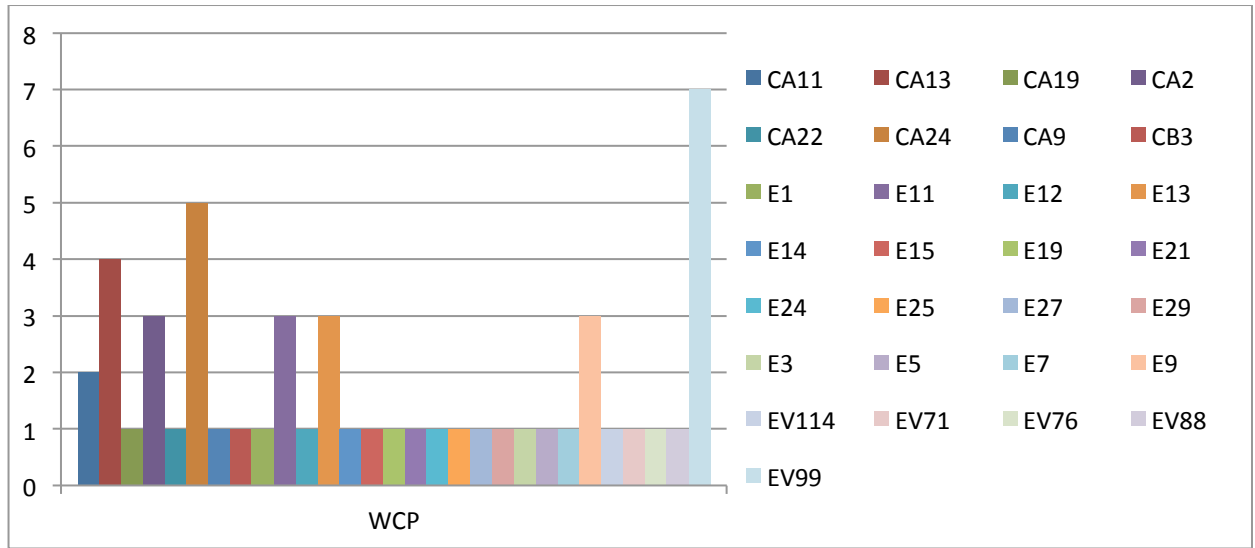


Figure 3.10: Serotype distribution for the Western Cape Province. CA – Coxsackievirus A, CB – Coxsackievirus B, E – Echovirus, EV – Enterovirus

Figure 3.11 displays the ages of the patients in which a positive EV result was obtained on the screening real-time PCR. Over 92% of the samples were in children under the age of 5. This graph shows that of that 92%, almost half are under the age of 1 year, with the 10-12 month age group accounting for 81 (16.77%) samples.

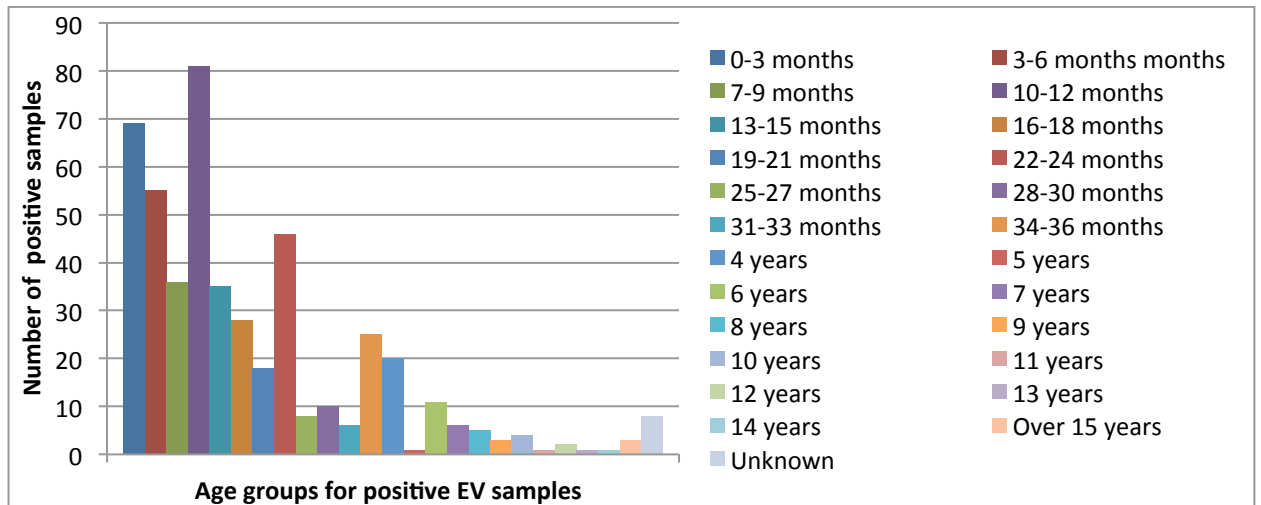


Figure 3.11: Number of samples positive for EV by age distribution. Ages are in months until 36 months, and then ages are in years.

Figures 3.12 and 3.13 show the seasonal distribution of all positive samples detected by month and year. Figure 3.12 contains all the positive EV samples tested in this study and no clear seasonality can be detected. When isolating only the AFP samples, as in Table 3.13, seasonality can be seen with the months January to March having a peak in infections.

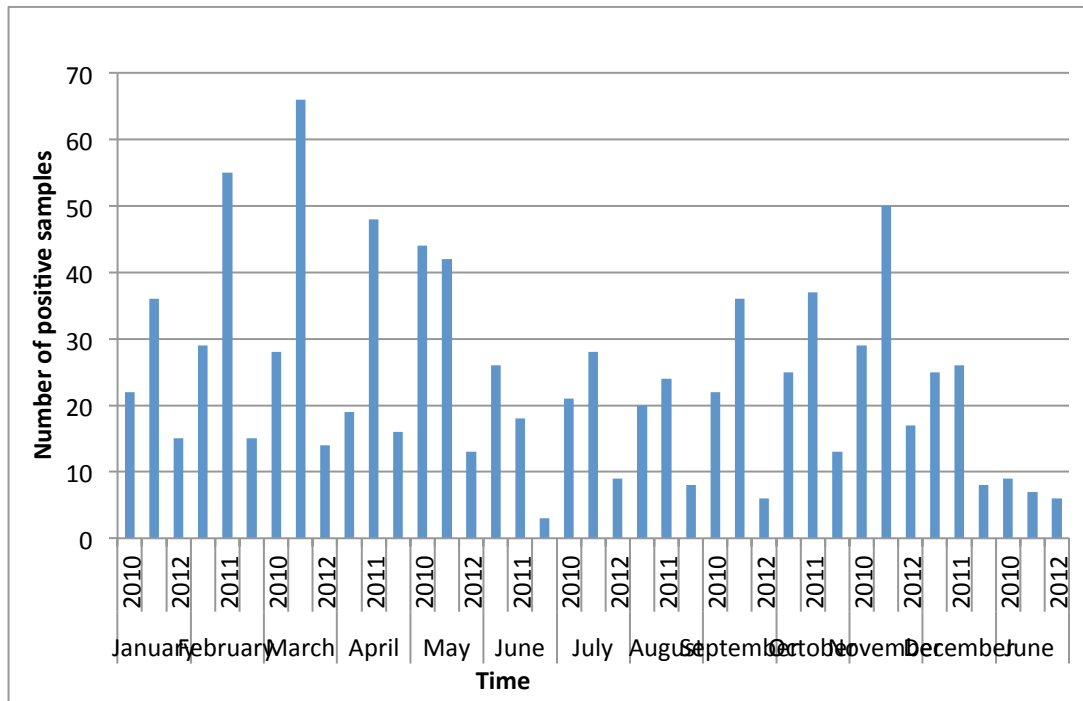


Figure 3.12: Total number of positive samples by month and year

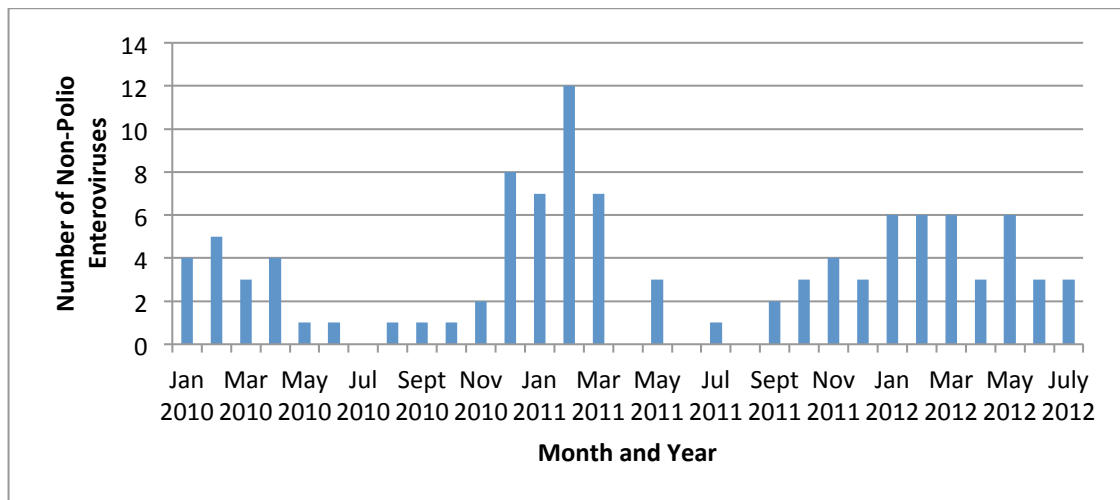


Figure 3.13: Acute Flaccid Paralysis culture positive samples detected by month and year.

3.10 Molecular Typing Assay Comparison With Neutralisation Assays

A comparison is not possible between the Sanger sequencing assay, and the next generation sequencing assay, as results were not obtained using the next generation sequencing assay.

Neutralisation assays were used to type EV for many years before molecular methods became available. The assay was used on viral isolates, and requires anti-sera against all the serotypes for accurate typing. As there are over 100 enteroviruses and more discovered on a regular basis, individual assays are impractical. There have been intersecting anti-sera pools that have helped narrow down the range of viruses before an individual type-specific anti-serum can be used to confirm the type (Lim and Benyesh-Melnick, 1960, Melnick et al., 1973). Unfortunately the method is expensive, time-consuming and labour intensive. The anti-sera pools are also limited, and none exists for those viruses discovered after EV71. 38 of the samples from this study would not be able to be typed using neutralising assays, including the predominant serotype (Table 3.5). Mixed infections and virus aggregates also confound neutralisation assays which prevent a definitive result from being obtained. With the inconsistencies, and the lack of clinical relevance for typing EV, this assay has declined in use over the years, and until recently was only commonly used in the WHO Polio Eradication Initiative, though PCR has also been introduced in those laboratories and has superseded the neutralisation assays (WHO, 2004).

4. Discussion and Conclusion

4.1 EV Surveillance in South Africa and Source Material

The AFP Surveillance Program is in place to detect flaccid paralysis in the country's population, and to determine if the paralysis is caused by one of the three Polioviruses. This is to aid the eradication of Polio and the disease burden from the world's population. The program obtains its samples from all nine provinces which enables this study to get a countrywide indication of the Enteroviruses present in the population. Stool specimens from the patients are sent to the NICD (Johannesburg, South Africa) for investigation (WHO, 2004). Some districts within the provinces may send fewer samples than others due to population density and symptom presentation. South Africa's immunization program has thus far prevented a polio outbreak, whether from a wild virus or a vaccine derived virus. The only case of paralysis that was a result of infection by a poliovirus was in a patient with a immunodeficiency condition and had recently stopped breastfeeding (Gumede et al., 2012). The use of this program as an Enterovirus surveillance tool has a number of advantages. The samples originate from all districts, and the infrastructure for sample collection and transport has already been established. The disadvantages are this is a passive surveillance system, cell culture is used to isolate virus, and only one symptom is used as the trigger for sample collection and testing. The complexity of enterovirus infections confounds the surveillance as many of the symptoms of infection are similar to many pathogenic and non-pathogenic causes, as well as the innumerable asymptomatic infections. Additionally, the South African AFP Surveillance program does not meet all the targets in all districts set by the World Health Organisation, and

this may influence the detection of non-polio enteroviruses in the country. In some districts there have been no cases of AFP reported. The current requirements to ensure sufficient sensitivity of the program is 4 Non-Polio AFP cases per 100 000 individuals in a district. There are a small number of districts that have not reported any AFP cases, and very few that have met their target of 4/100 000. The majority of districts are able to meet the old target of 2/100 000 which was increased for the 2015 year to try and increase the sensitivity of the AFP program in light of the WHO activities planned for the next five years. This is planned to lead to the eradication of polioviruses from the planet (www.polioeradication.org).

The Rotavirus Surveillance Program (Msimang et al., 2013) has sites that cover different regions in the country. Using this surveillance program has the advantage that stool samples are tested directly, without a virus isolation intermediary step. This allows for the detection of viruses that are unable to grow or are difficult to culture on cell cultures. It also allows for the opportunity to detect viruses that may be associated with gastrointestinal disease rather than the associated neurological disease causing viruses that the AFP Surveillance Program may select for. The disadvantage is that results are more difficult to obtain using these sample types. The PCR inhibitors are difficult to remove, and mixed enterovirus infections are a possibility. The major population centres are covered in the Gauteng, Western Cape and Kwa-Zulu Natal provinces, as well as samples from a more rural population in Mpumalanga. These sites are the same sites where the Severe Acute Respiratory Infection (SARI) Surveillance Program obtains its specimens (Cohen et al., 2014). The samples from the SARI program would be able to possibly associate respiratory symptoms with the

EV detected, though other respiratory pathogens may confound this association if there are mixed infections with a known respiratory pathogen and an enterovirus.

4.2 Methods Discussion

The Nix et al (2006) assay was used rather than using the traditional neutralisation assays due to the ease of running the assay, the time it takes to obtain the result from the beginning of the test, and the fact that anti-sera are becoming difficult to obtain as the stock volumes are fixed. If neutralization assays were still in use, the time to obtain a result would be up to three weeks: one to two weeks to culture the virus, and a further 2-5 days to conduct the neutralisation assay itself, furthermore not all Enteroviruses grow in cell culture. The detection of EV in the stool samples that were negative for EV in RD and L20B cells is an example of this. The variety and distribution of the serotypes detected through the species also demonstrated a bias introduced by culturing the virus in cells. The molecular sequencing assay in this study (if only testing 1 sample) can have a result within 3-4 days, and if a 24 hour laboratory is running the assay, even shorter times, due to the evening hours being utilised. As discussed above (Section 3.7), the large number of EV that are known, and the probable discovery of more in the near future, make a universal sequencing assay that is able to detect all the viruses (though sample types may cause some inhibition) a logical choice if looking for the epidemiology of EV, or the unknown cause of a possible EV outbreak, within a population.

The positive culture specimens need very little treatment to yield viral RNA, as there are vast amounts of virus present due to the culturing procedure (Westwood et al., 1960). This makes a standard RNA extraction procedure all that is required for molecular techniques to be successfully conducted on the eluted genetic material. Conversely, stool samples are notorious for being difficult to test using molecular techniques (Widjoatmodjo et al., 1992, Monteiro et al., 1997). The mixture of human, bacterial, parasitic, and viral genetic material complicates designing specific oligonucleotides for primers and probes for PCR and sequencing assays. There are also vast amounts of inhibitory compounds found in stool. Bilirubin, bile salts and complex polysaccharides inhibit and reduce the sensitivity of molecular assays (Monteiro et al., 1997, Widjoatmodjo et al., 1992).

Systems are needed to remove these PCR inhibitors from the sample to increase the sensitivity of the assays. The PCR assay this study was based on (Nix et al., 2006) used a chemical, Vertrel XF, to pre-treat the sample before extraction. This is a chemical cleaner used for removing imperfections and particulates from oils, electrics and optics (www2.dupont.com). Importing this chemical proved to be excessively prohibitive due to the chemical nature of Vertrel XF, and the precautions needed for shipping. An alternative was found from Roche in the form of the Stool Transport And Recovery (S.T.A.R) buffer. This buffer was designed to stabilise pathogens present in stool samples sufficiently such that storage and transport could be conducted for up to seven days at room temperature (Package insert, Roche, Mannheim, Germany). The buffer therefore improves the PCR process through its chemical action. Sequences obtained before treating the samples varied, but were all below 50% of

the positive PCR screens. The samples that were positive on the PCR real-time screen, but negative on the sequencing PCR gel, were then treated with the STAR buffer. The number of samples that were able to be sequenced doubled after this treatment. While not all of the positive samples could be typed, the improvement from 38.3% yield to 60.0% yield shows that the treatment with the buffer is required for the assay and both extraction methods should use samples that have been pre-treated with the STAR buffer.

4.3 Screening Real-Time PCR

The Nijhuis protocol (Nijhuis et al., 2002) uses a forward primer, with two reverse primers and two probes to ensure that all EV are detected, while keeping the specificity of the assay such that no related picornavirus genomes are amplified. The use of a two-step reverse transcriptase PCR, primers and probes that do not contain any non-specific (wobble) bases, and the improvement demonstrated in Table 3.1 (use of the Transcriptor First Strand cDNA Synthesis Kit), combined to provide a stable PCR able to detect EV in multiple sample types.

In Table 3.3 there are a number of samples from the culture positive group that resulted negative on the screening PCR. These are RD cells that showed CPE under the microscope. The specific viral cause of the CPE is unknown, as some viruses may cause a similar CPE to enteroviruses, and needs to be confirmed by another

assay such as PCR or neutralization assays. Toxicity is another possible cause for giving a false positive result as well as large volumes of viral RNA due to the culturing procedure which, after RNA extraction, may also inhibit the PCR and read as a false negative. This is unlikely as the positive control for the PCR and sequencing reactions was a viral culture, and was not inhibited by the high RNA titres. The PCR had been used at NICD, South Africa, for many years on various sample types, and Table 3.1 shows that at least 3 sample types are able to be run on this assay.

The NPENT detection rate was approximately 2% of all samples received in the AFP program (175 samples were positive for Non-polio EV out of the approximately 7500 samples received by the polio network over 2010-2012). The sensitivity of the program requires that the AFP detection rate is 1 AFP case per 100 000 individuals under the age of 15, or 2 per 100 000 individuals in an endemic area. This rate has been approximately 2/100 000 for South Africa for a number of years (www.polioeradication.org). As of 2015, South Africa has attempted to increase the sensitivity of the program to 4/100 000 in light of the impending eradication of Polioviruses. As only stools from the AFP program in 2012 were able to be tested, and assuming the EV circulation rate was constant from year to year, the cultures are missing approximately 30% of the EV infections present in the population, as per Table 3.3 where the detection rate of EV in the culture negative stools was 33.68%. This is expected, as the program has been refined to detect only polioviruses.

4.4 Enterovirus sequencing

Once the samples have been screened for EV using the real-time PCR assay mentioned above, the Nix assay (Nix et al., 2006) can then be applied to the samples. cDNA is produced using short oligonucleotides that target the VP1 gene, as primers for the RNA dependent RNA polymerase. The first set of primers is semi-specific, and targets EV and rhinoviruses. They also amplify many different non-specific products. The semi-nested primers are also semi-specific, though for a smaller amplicon. An electrophoresis gel must then be run to separate the cDNA bands out, and the band corresponding to the product of 348-393 bases is excised and purified from the entire PCR product. The size difference is due to the insertions and deletions causing differences in amino acid composition of the different EV capsids. As shown in Table 3.2, the Promega kit was found to be more effective and easier to use when cleaning up the gel band. The sequencing primers used are identical to the semi-nested PCR primers and they contain a specific 5' end and a variable 3' end. This helps the binding of the primers to the wide variety of EV in the population. If these primers produce a chromatogram that indicated a mix of amplicons, a set of more specific primers can be attempted. These more specific primers are also identical to the semi-nested primers except that they do not contain the variable 3' sequences, and only the specific 5' end.

Despite these steps taken, (electrophoresis of the entire PCR product instead of running a portion thereof, excision of the correct sized band, conducting a PCR

product clean-up instead of a gel clean-up, use of the more specific sequencing primers, use of the Transcriptor reverse transcriptase enzyme instead of the AMV enzyme), many sequences were still unobtainable from the positively screened samples. Reasons for this could include: samples that were negative on the gel electrophoresis may have had a very low virus titre, or had PCR inhibitors that the STAR buffer was unable to remove; cDNA positive samples that were excised and cleaned from the agarose gel and sequenced may have resulted in sequences that were mixed in nature, or matched an organism that was not EV (Plasmids were sometimes a match for samples not matching an EV serotype). A possible method to eliminate these mixed sequences and plasmid occurrences is performing a gel electrophoresis that is much longer than the 30cm gel used in this study. The disadvantage to extending the length of the gel is that it would take much longer to run, extending the processing time of the samples.

4.5 Epidemiology

Using the national surveillance systems in place as a source of samples ensured good population coverage, and a high diversity of HEV was detected. The detection rate in the samples from the Rotavirus Surveillance Programs stool samples (49.29% positive) was higher than in the AFP Surveillance Programs negative stool samples (33.68% positive), though the AFP positive samples have already been screened from these samples. This may be due to the patient pool from which the samples were obtained. The patients from the Rotavirus Surveillance Program were young,

severely ill, and hospitalised which may have been caused by an enterovirus infection which would have increased the detection rate in these samples.

The pattern seen in the enterovirus distribution (Tables 3.3 and 3.4) is consistent with other studies (Benschop et al., 2010, Tryfonos et al., 2011, Tan et al., 2011, Hsu et al., 2011), though previous studies detected HEV from cell culture, with few attempting to test the patient samples directly (Apostol et al., 2012, Odoom et al., 2012, Oyero and Adu, 2010). This is due to the fact that the studies have sourced their samples from the respective AFP surveillance programs in their countries. The results from this study indicate that using the AFP samples may have biased the results from studies only using cell culture samples to detect EV. Mostly HEV-B was detected from culture specimens and this can be seen in the studies mentioned, whereas a more diverse EV presence was obtained from stool samples as seen by the EV distribution in Tables 3.3 and 3.4.

EV99 is the most prevalent virus detected over the 3 years (27 out of 308 samples typed (8.63%)), and within each year is the most prevalent along with E6 (9 viruses, 2010), CB5 and E14 (6 viruses each, 2011) and E13 (10 viruses, 2012). This is unusual as EV99 is not a common virus found in previous epidemiology studies (Smura et al., 2014, Tao et al., 2014). It is also closely related to CA24 in the VP1 region (Smura et al., 2014). Together these 2 viruses accounted for 17.7% of the detections over the 3 years tested. Neither of these viruses has been definitively linked to diseases, though CA24 has been isolated for many years in different studies.

Many different disease-associated viruses were detected, but did not contribute significantly to the number of viruses detected. EV 71 was isolated in the AFP culture positive samples. This virus has been associated with aseptic meningitis (Liu et al., 2000, Ortner et al., 2009), and so the presence of the virus in cultures from patients presenting with neurological symptoms is expected, though only 2 were detected over the 3 years. EV 68 is associated with respiratory infections (Peci et al., 2015) and was the virus detected most in the SARI samples. CA5 and E3 were the next most common SARI viruses found to occur, but they are associated with a variety of symptoms, one of which includes respiratory disease. The old classification of enteroviruses was based on phenotype, infection of certain cell types, and symptoms caused. While some specific viruses, such as those mentioned above, are more commonly associated with specific diseases, those viruses sharing a designation (Echo, Coxsackie A, Coxsackie B) , are more likely to cause similar symptoms, but can be found in aseptic infections, as well as infections causing different symptoms to the ones most commonly found in the old classification groups. This makes associations between serotypes and symptoms very difficult, and sometimes impossible to determine the link between the two. The 'new' numbered enteroviruses can be associated with a symptom type like EV71, but many have no disease association, and have been detected in mixed infections, environmental samples or asymptomatic patients.

In this study, Enterovirus infections did not show a seasonal pattern (Figure 3.12), except the AFP culture positive samples, which show a spring-summer distribution (Figure 3.13). The lack of seasonality is unusual as enteroviruses have a well-established infection pattern with an increased transmission rate in the warmer months (Froeschle et al., 1966).

EV infections followed the usual pattern of infecting young children (Froeschle et al., 1966). Over 90% of the EV positive samples detected in this study were from patients under the age of 5 years old (Figure 3.11).

The distribution of the serotypes over the country did not show a pattern (Figures 3.2 – 3.10). There were many more types detected in Gauteng, Mpumalanga, Kwa-Zulu Natal and Western Cape probably due to the large numbers of samples obtained from these provinces, though to confirm this, further studies will be needed with samples collected more evenly between all nine provinces. What would be needed is a surveillance program tailored to detecting symptoms commonly caused by enteroviruses (Hand, foot and mouth disease, aseptic meningitis, etc.), though this could be confounded by other pathogenic and non-pathogenic causes of similar symptoms. A children's hospital in China is conducting surveillance in this way and are successful in detecting enteroviruses, and associating them with some disease types (Li et al., 2015).

Serotype distribution varies over time within a geographic location (Trallero et al., 2010), as well as over large distances, for example, between continents (Spain's predominant virus was Echovirus 30, (Trallero et al., 2010); India's Enterovirus 71 (Rao et al., 2012); and in this study Enterovirus 99 for South Africa). The higher detection of EV99 than what has been previously reported, may be due to lack of serotyping studies in South Africa, and the use of stool samples instead of viral isolates to type the viruses (Moore, 1982, Kumar et al., Tanel et al., 1996, Grandien M, 1989). EV99 is in HEV-C species group which, in cell cultures, does not grow as readily as viruses from HEV-B.

4.6 Limitations and Way Forward

There was no control group of healthy individuals to compare the data with. Differences seen in serotype distribution between the surveillance programs may have been more due to the sample type, though a clear association between EV68 and respiratory infections is known. The presence of HEV in a population may not be associated with clinical disease and may be part of normal EV circulation. The sample groups utilized in this study cover many of the symptoms that EV may cause. The less severe symptoms types, such as conjunctivitis and hand-foot-and-mouth disease aren't covered by any surveillance group and would need additional permissions, time and man-power to collect, and were thus not included in this study. Similarly, the viruses that cause variations of myocarditis will also have to be studied further with different sample types and patient groups. Outbreaks commonly associated with

enteroviruses would need to be tested more frequently in South Africa for more accurate knowledge on viral association with disease, and virus circulation in the country. An example where this has been done is in China (Li et al., 2015)(Bal et al., 2015, Li et al., 2015) . With the eradication of Polioviruses in the country, studies have been focusing on NPENTs causing disease in children through active surveillance.

Full genome sequencing may possibly shed light on effects of recombination on virulence and infectivity. This may help explain how different EV cause the same symptoms between individuals or how one EV serotype can be associated with many symptom types. This will also become more urgent as wild type and Sabin vaccine strains become eradicated thanks to the Global Polio Eradication Initiative, however, elements of these viruses may still remain in other HEV-C viral genomes due to the recombination procedure.

The failure of the attempts to obtain results from the next generation sequencing may be due to a number of factors. The first attempt in which random cDNA was created to try to cover the entire genome resulted in both Sanger sequencing and one run on the GS Junior (Roche, Mannheim, Germany) producing large amounts of Poly-T oligonucleotides. As random primers are used in the screening PCR, this is confusing, as some other areas of the genome should have been detected. The second attempt with the long cDNA synthesis kit was utilised with the manufacturer helping with troubleshooting. As the control RNA also did not yield any results through the several runs attempted, it is still unknown why long template cDNA was not reliably produced

for the next step of the sequencing process. It is possible further optimisation is required for cDNA to be synthesised, but funding and time constraints limited it for this study.

Further studies in this area may use the same reagents and protocols used with further optimisation to yield results. Other possibilities are attempting different primers to try and create cDNA that covers the whole genome.

4.7 Conclusions

The aims of the study were to develop molecular assays to detect and type EV from patient samples and describe the virus distribution and epidemiology in South Africa. A single EV real-time PCR was chosen over a multiplex PCR able to differentiate between the HEV species. The multiplex PCR was attempted; however, no sample controls were successfully amplified with this PCR. The advantage of the simplex is that it is more robust and more sensitive than multiplex PCRs due to the nature of few primers and probes in the assay. The disadvantage is that the sequencing assay to be based on these results needs to then amplify all EV, instead of more specifically a species per assay. Nix et al developed an assay for this purpose in 2006 (Nix et al., 2006). This assay was able to detect all EV serotypes spread across all 4 HEV species. In this study, more recently classified EV was detected by this assay: EV99, EV102, and EV114. These are little described viruses with no clear disease

association. The epidemiology of EV in South Africa showed a general concordance with other studies (Benschop et al., 2010, Apostol et al., 2012, Dhole et al., 2009). HEV-B and HEV-C contained the most number of typed viruses in the country, though as mentioned, many previous studies were biased due to the use of culture before virus typing, instead of typing directly from patient samples. Echovirus 30 is seen to be the predominant serotype in Europe, but elsewhere in the world, the viral distribution is more even, with serotypes co-circulating (Oberste et al., 1999a). In South Africa, serotypes also co-circulated, but EV99 was seen to be predominant throughout the 2010-2012 periods. Considering the close genetic relationship between EV99 and CA24, adding these virus detections together accounted for almost 18% of all viruses detected over the 3 years. There is no clear reason why EV99 and CA24 are so prominently found in South Africa. Other studies have not definitively linked EV99 to a disease, and so the virus is still relatively unknown. Further investigations are needed to find whether EV99 has any clinical relevance or disease burden.

Neutralization assays are labour intensive; rely on a regular supply of good quality cells, and an antibody pool that is wide and diverse. As most pools are geared towards detecting polio, and the supplies of the antisera are dwindling, this mode of EV typing is not used on a regular basis. Antisera are also sensitive to viral aggregation and cross reactivity, resulting in incorrect serotyping of viruses. While costs may be lower than molecular techniques, trying to use a wide antisera pool, and the time it takes to yield a positive result, makes molecular techniques a clear choice for speed, sensitivity and specificity when typing EV.

Sequencing of the whole EV genome may lead to a greater understanding of the viruses circulating in the country and of any recombination events between serotypes. The attempts to create full genome cDNA failed in this study, but recombination is a common occurrence between EV in the same species (Cammack et al., 1988, King, 1988, Lukashev, 2010). The effect of recombination on viral infectivity, virulence, and disease presentation is not fully understood (Kyriakopoulou et al., 2014, Zhang et al., 2014). This aspect of EV investigation will need to be continued in greater depth to try to discover the nuances of recombination and its effect on EV virology.

This study has given an indication of which enteroviruses are circulating in South Africa, but is not able to indicate a definite link to causality of disease. A longer retrospective period may show how serotypes have shifted in circulation, and if there are more predominant serotypes. Typing EV found in surveillance programs currently operating in the country will help clinicians with patient diagnoses, but typing will be more of use for discovering new viruses, noting recombination (when this aspect has been perfected), or assisting in the development of a new vaccine to lessen the disease burden of serotypes with serious effects on patients, as treatment for EV infection is symptomatic only, and vaccines are only available for the three polioviruses. Further samples can also be sourced from patients suffering from Hand-foot-and-mouth disease, and myocarditis to complete the EV epidemiology in the country.

5. References

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Appendix A

Table 1.1: Members of the Human Enterovirus A species group as of January 2015

(www.picornaviridae.com). CVA – Coxsackievirus A, EV - Enterovirus

Type	Prototype strain	Illness in person with Prototype	Accession number
CVA-2	Fleetwood	Poliomyelitis	AY421760
CVA-3	Olson	Meningitis	AY421761
CVA-4	High Point	Sewage of community with Polio	AY421762
CVA-5	Swartz	Poliomyelitis	AY421763
CVA-6	Gdula	Meningitis	AY421764
CVA-7	Parker	Meningitis	AY421765
CVA-8	Donovan	Poliomyelitis	AY421766
CVA-10	Kowalik	Meningitis	AY421767
CVA-12	Texas-12	Flies in Community with Polio	AY421768
CVA-14	G-14	None	AY421769
CVA-16	G-10	None	U05876
EV-A71	BrCr	Meningitis	U22521
EV-A76	FRA91-10369	Gastroenteritis	AY697458
EV-A89	BAN00-10359	Acute Flaccid Paralysis	AY697459
EV-A90	BAN99-10399	Acute Flaccid Paralysis	AY697460
EV-A91	BAN00-10406	Acute Flaccid Paralysis	AY697461
EV-A114	BAN-11610	Not known	Not known
EV-A119	09C13CMR	Not known	KC787153
EV-A120	184977-PNG-09	Not known	Not known
EV-A121	V13-0682 (India)	Not known	Not known

Table 1.2: Members of the Human Enterovirus B species group as of January 2015

(www.picornaviridae.com). CVB – Coxsackievirus B, CVA – Coxsackievirus A, E –

Echovirus, EV – Enterovirus, SA – Simian Enterovirus.

Type	Prototype strain	Illness in person with Prototype	Accession number
CVB-1	Conn-5	Aseptic Meningitis	M16560
CVB-2	Ohio-1	Summer gripe	AF085363
CVB-3	Nancy	Minor febrile illness	M16572
CVB-4	JVB	Chest and abdominal pain	X05690
CVB-5	Faulkner	Mild paralytic disease with residual atrophy	AF114383
CVB-6	Schmitt	None	AF105342
CVA-9	Bozek	Aseptic Meningitis	D00627
E-1	Farouk	None	AF029859
E-2	Cornelis	Aseptic Meningitis	AY302545
E-3	Morrissey	Aseptic Meningitis	AY302553
E-4	Pesascek	Aseptic Meningitis	AY302557
E-5	Noyce	Aseptic Meningitis	AF083069
E-6	D'Amori	Aseptic Meningitis	AY302558
E-7	Wallace	None	AY302559
E-9	Hill	None	X84981
E-11	Gregory	None	X80059
E-12	Travis	None	X79047
E-13	Del Carmen	None	AY302539
E-14	Tow	Aseptic Meningitis	AY302540
E-15	Charleston	None	AY302541
E-16	Harrington	Aseptic Meningitis	AY302542
E-17	CHHE-29	None	AY302543
E-18	Metcalf	Diarrhoea	AF317694
E-19	Burke	Diarrhoea	AY302544
E-20	JV-1	Fever	AY302546
E-21	Farina	Aseptic Meningitis	AY302547
E-24	DeCamp	Diarrhoea	AY302548

E-25	JV-4	Diarrhoea	AY302549
E-26	Coronel	None	AY302550
E-27	Bacon	None	AY302551
E-29	JV-10	None	AY302552
E-30	Bastianni	Aseptic Meningitis	AF162711
E-31	Caldwell	Aseptic Meningitis	AY302554
E-32	PR-10	Gastroenteritis	AY302555
E-33	Toluca-3	None	AY302556
EV-B69	Toluca-1	None	AY302560
EV-B73	CA55-1988	Not known	AF241359
EV-B74	10213	Not known	AY556057
EV-B75	10219	Not known	AY556070
EV-B77	CF496-99	Not known	AJ493062
EV-B78	W137-126/99	Not known	AY208120
EV-B79	10384	Not known	AY843297
EV-B80	10387	Not known	AY843298
EV-B81	10389	Not known	AY843299
EV-B82	10390	Not known	AY843300
EV-B83	10392	Not known	AY843301
EV-B84	10603	None	DQ902712
EV-B85	10353	Acute Flaccid Paralysis	AY843303
EV-B86	10354	Acute Flaccid Paralysis	AY843304
EV-B87	10396	Acute Flaccid Paralysis	AY843305
EV-B88	10398	Acute Flaccid Paralysis	AY843306
EV-B93	None	Not known	Not known
EV-B97	10355	Acute Flaccid Paralysis	AY843307
EV-B98	None	Not known	Not known
EV-B100	10500	Acute Flaccid Paralysis	DQ902713
EV-B101	10361	None	AY843308
EV-B106	None	Not known	Not known
EV-B107	None	Not known	Not known
EV-B110	None	Not known	Not known
EV-B111	None	Not known	Not known

SA5	None	Not known	Not known
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Table 1.3: Members of the Human Enterovirus C species group as of January 2015

(www.picornaviridae.com). PV – Poliovirus, CVA – Coxsackievirus A, EV -

Enterovirus

Type	Prototype strain	Illness in person with Prototype	Accession number
PV-1	Brunhilde	Paralytic Polio	AY560657
PV-2	Lansing	Fatal Paralytic polio	AY082680
PV-3	Leon	Fatal Paralytic polio	K01392
CVA-1	Tompkins	Paralytic illness	AF499635
CVA-11	Belgium-1	Epidemic myalgia	AF499636
CVA-13	Flores	None	AF499637
CVA-17	G-12	None	AF499639
CVA-19	NIH-8663	Guillain-Barre syndrome	AF499641
CVA-20	IH-35	Infectious hepatitis	AF499642
CVA-21	Kuykendall	Poliomyelitis	AF546702
CVA-22	Chulman	Vomiting and diarrhoea	AF499643
CVA-24	Joseph	None	D90457
EV-C95	None	Not known	Not known
EV-C96	10358	Acute Flaccid Paralysis	Not known
EV-C99	None	Not known	Not known
EV-C102	None	Not known	Not known
EV-C104	None	Not known	Not known
EV-C105	None	Not known	Not known
EV-C109	None	Not known	Not known
EV-C113	None	Not known	Not known
EV-C116	126	Not known	Not known
EV-C117	LIT22	Not known	Not known
EV-C118	ISR10	Not known	Not known

Table 1.4: Members of the Human Enterovirus D species group as of January 2015

(www.picornaviridae.com). EV - Enterovirus

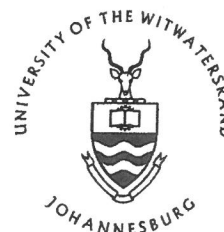
Type	Prototype strain	Illness in person with Prototype	Accession number
EV-D68	Fermon	Pneumonia, bronchiolitis	AY426531
EV-D70	J 670/71	Conjunctivitis	D00820
EV-D94	E210	None	Not known
EV-D111	KK2640	Not known	Not known
EV-D120	6201	Not known	Not known

Appendix B

Human Research Ethics Committee (Medical)

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18 November 2015

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Dear Mr Howard

Re: Protocol Ref no: M111145

Protocol Title: Characterisation of Non-Polio Enteroviruses Identified in Disease Biomes in South Africa

Principal Investigator: Mr Wayne Howard

Protocol Amendment

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the following amendments on the above mentioned protocol, as detailed in your letter dated 28 September 2015.

- Change in a period of samples used. Samples used were from January 2010 to December 2012, and NOT from October 2011 to December 2013 as stated originally.

Thank you for keeping us informed and updated.

Yours Sincerely,

A handwritten signature in black ink, appearing to be 'Rhulani Mkansi', written over a dotted line.

Mr Rhulani Mkansi
Administrative Officer
Human Research Ethics Committee (Medical)



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Wayne Howard

CLEARANCE CERTIFICATE

M111145

PROJECT

Characterisation of Non-Polio Enteroviruses
Identified in Disease Biomes in South Africa

INVESTIGATORS

Mr Wayne Howard.

DEPARTMENT

Medical Virology/Department of Pathology

DATE CONSIDERED

25/11/2011


M1111450DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 01/02/2012

CHAIRPERSON


(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof Adrian Puren

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...



14/2/12



Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

01 February 2012

Mr Wayne Howard
Medical Scientist
Centre for Vaccines and Immunology
National Institute for Communicable Diseases
Sent by e-mail wayneh@nicd.ac.za

Dear Mr Howard

**RE: Protocol M110934: 'The surveillance and Molecular
Characterisation of Viral Neurotropic Agents in Settings of
Meningitis and Encephalitis Cases: A Multi Centre Study in
South Africa
Protocol Amendment**

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved your request to "utilise the residual specimens, that are routinely received for the abovementioned study" as detailed in your letter dated 19 January 2012.

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Academic & Research)

MEMORANDUM

TO: Mr Wayne Howard
Medical Virology & Communicable Disease
EMAIL wayneh@nicd.ac.za

FROM: Ms Anisa Keshav
Secretary: Human Research Ethics Committee (Medical)
Tel 717-1234 fax 011 717 1265
e-mail: anisa.keshav@wits.ac.za

DATE: 15 May 2012

REF: ***R14/49***

The protocol below was considered at a meeting of the Human Research Ethics Committee (Medical) on **Friday 04 May 2012**. **Below please see the Committee's decision:**

M120467

Characterisation of Non-Polio Enteroviruses Identified in Disease Biomes in South Africa

Approved subject to:

-clarifying and providing written permission for residual specimens to be used.

Please submit the above so I may issue your clearance certificate.

Please highlight any changes made and send [two hard copies](#) to this office.



**NATIONAL HEALTH
LABORATORY SERVICE**

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES

Centre for Vaccines and Immunology

1 Modderfontein Road, Sandringham, 2031

Tel: +27 (0)11 555 0398 Fax: +27 (0)11 386 6586

Date: 2012-05-21

To the Human Research Ethics Committee

Reference: R14/49

This letter is to confirm permission to use residual specimens from M091018. *Establishment of Sentinel Surveillance for Rotavirus Diarrhoea Infection in South Africa* in the study M120467 *Characterisation of Non-Polio Enteroviruses Identified in Disease Biomes in South Africa*.

I, Dr Cheryl Cohen, allow residual specimens from the study *Establishment of Sentinel Surveillance for Rotavirus Diarrhoea Infection in South Africa* to be used in the study *Characterisation of Non-Polio Enteroviruses Identified in Disease Biomes in South Africa*.

Dr Cheryl Cohen (PI M091018)

Wayne Howard (PI M120467)



PaperRater

(<http://www.PaperRater.com>)

1. Introduction 1.1 Human Enterovirus 1.1.1 General information and history Human Enteroviruses are part of the Picornaviridae family, in the Enterovirus genus (www.ictvonline.org). They are separated into four species (Human Enterovirus (HEV)-A, B, C and D; Appendix A, Tables 1.1-1.4) that contain the different serotypes. HEV-A contains Coxsackie A viruses and some numbered Enteroviruses. HEV-B is a large species group with 1 Coxsackie A virus, Coxsackie B viruses 1-6, all the Echoviruses as well as numbered Enteroviruses. HEV-C contains the three polioviruses, a number of Coxsackie A viruses and numbered Enteroviruses. HEV-D is the smallest species group currently containing only 5 numbered Enteroviruses. Infections are characteristically in the... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
-A	A, SA, NA, LA, FA
polioviruses	poliovirus
immunodeficient	immunodeficiency
siRNA	mRNA, RNA, tRNA, rRNA

polioviruses	poliovirus
infectiveness	ineffectiveness, inventiveness, effectiveness, defectiveness
intramuscularly	intramuscular
30nm	nm
serotype	serotypes, stereotype, ferrotype, genotype
β-barrel	barrel
form	from
serotype	serotypes, stereotype, ferrotype, genotype
poly-A	poly A
poly-A	poly A

Grammar

Grammar Suggestions

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.3 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and clichés found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 43

This score is based on quality of transitional phrases used within your paper. You did equal or better than **26%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 63

Avg. Length: 19.1 words

Short Sentences (< 17 words): 25 (40%)

Long Sentences (> 35 words): 4 (6%)

Sentence Variation: 9.5 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

 **Style**

Readability Indices

PREMIUM ONLY

This feature is only available to premium subscribers.

Learn more... (<http://www.PaperRater.com/premium>)

 **Style**

Sentence Beginnings

Simple Sentence Starts: 32%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts.

Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Human Enteroviruses are** part...
- **They are** separated...
- **HEV-A contains** Coxsackie...
- **HEV-B is** a...
- **HEV-C contains** the...
- **HEV-D is** the...
- **Infections are** characteristically...
- **Most infections are** asymptomatic...
- **Enteroviruses EV are** also...
- **Most EVs are** transmitted...
- **CVA21 which is** a...
- **Infections are** considered...
- **Small interfering** RNAs...
- **EV history has** been...
- **Poliomyelitis is** believed...
- **Enteroviruses are** spherical...
- **They are** about...
- **Virus stability is** temperature...
- **It contains** the...
- **Nucleotide positions correspond** to...

Starts by Part of Speech:

Adjective: 8%

Adverb: 0%

Article: 0%

Conjunction: 0%

Noun: 35%

Preposition: 17%

Pronoun: 5%

Verb: 2%

Other: 30%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Passive Voice

Passive Voice Sentences: 31.7%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- They **are separated** into four species (Human ...
- ... months, though infections can **be detected** all year round (Pons-Salort et al., ...
- ... (Tapparell et al., 2013), and have **been implicated** in a wide range of acute and ...
- Most EVs **are transmitted** through the oral route, though some ...
- ... major cause of respiratory disease, **is transmitted** through contaminated respiratory ...
- Infections **are considered** acute, though there have been ...
- ... and anti-viral antibodies have **been studied** as possible therapies (Langford et ...
- ... interfering RNAs (siRNA) have also **been investigated** as a possible treatment for EV ...
- EV history has **been dominated** by studies on the three ...
- Many landmarks in virology have also **been based** on PV.
- Poliomyelitis **is believed** to be an ancient disease as ...
- ... of the infectiveness of the disease **was started**.
- While a variety of vaccines **were developed**, 2 vaccines became the most well ...
- The canyon has **been proved** to be the receptor binding site for ...
- The viral genome is infectious since it **is translated** as soon as it **is introduced** ...
- ... has secondary structure and has **been implicated** in controlling RNA synthesis, ...
- If this poly-A tail **is removed**, the resulting RNA is ...
- The translated polyprotein **is processed** to form the individual proteins.
- The full-length polyprotein **is not observed** in natural synthesis as it **is** ...
- The polyprotein **is divided** up into 3 main regions: P1, which



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

⚙️ *Vocabulary Words*

Usage of Academic Vocabulary

Vocabulary Score: 84

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **79%** of the people in your education level.



Vocabulary Word Count: 23

Percentage of Vocab Words: 2.41%

Vocab Words in this Paper (a subset): genus, aseptic, implicated, acute, chronic, dilated, contaminated, potential, inhibited, effective



Excellent work! Your usage of sophisticated words is on par with other well-written papers!

Nevertheless, you may still wish to use our **Vocab Builder**

(http://www.PaperRater.com/vocab_builder/index) to maintain your edge.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

➔ *Grade*

Auto Grader

Grade: 93 A

NOTE: Our grading algorithm changed on 9/23/2015. **More info** (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





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encodes the capsid proteins; P2 and P3, which encode the proteins involved in protein processing (2Apro, 3Cpro, 3CDpro) and genome replication (2B, 2C, 3AB, 3BVPg, 3CDpro, 3Dpol). 1.2 Diversity in Enteroviruses Enteroviruses are dependent on an RNA-dependent RNA polymerase for genome synthesis and, like with other RNA viruses, this enzyme is error prone due its lack of proof-reading capabilities. This results in quasispecies arising within a single infection as error rates can be as high as one misincorporation per 103 to 104 nucleotides (Domingo and Holland, 1997). Enteroviruses have been suggested to exist on the threshold of 'error catastrophe'. Either an increase (Crotty et al., 2000, Gu et al., 2006) or a decrease (Pfeiffer and Kirkegaard, 2005)... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>) .

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
2Apro	Apr, pro, Afro, Apo, Cipro
3Cpro	pro, Ciro, Caro, Cipro, Coro
3CDpro	Cipro
2B	2, 2D, 2 B, B, AB

2C	2, 2D, 2 C, C, PC
3AB	NAB, AB, TAB, VAB, CAB
3BVPg	BHP
3CDpro	Cipro
3Dpol	pol, Dol
due	do
misincorporation	disincorporation
antigenically	antithetically
polioviruses	poliovirus
coxsackie	Coxsackie
echoviruses	choruses, arboviruses, retroviruses
herpangia	hernia, Germania
coxsackie	Coxsackie
coxsackie	Coxsackie
echoviruses	retroviruses, choruses, arboviruses
serologically	serological, geologically, neurologically, ecologically
echoviruses	retroviruses, choruses, arboviruses
antigenically	antithetically
serotype	serotypes, stereotype, genotype, ferrotype
aa	a, at, am, as, an
aa	an, a, a a, a1, aka
2C3CD	CCD
encapsidation	encapsulation
	serotypes, stereotype,

serotype	genotype, ferrotype
serotype	serotypes, stereotype, genotype, ferrotype
aa	an, a, a a, a1, aka
serotype	serotypes, stereotype, genotype, ferrotype
serotypically	stereotypically
2004b	20th, 404, 32000, 68040, 8048
vaccinees	vaccines, vaccine's, vaccine
recombinants	recombinant
polioviruses	poliovirus
serotype	serotypes, stereotype, ferrotype, genotype
noncapsid	noncash, synapsid, capsid
2000b	32000
noncapsid	noncash, capsid, synapsid
serotype	serotypes, stereotype, genotype, ferrotype
2004b	20th, 404, 32000, 68040, 8048
2004a	20th, 404, 32000, 68040, 8048
serotype	serotypes, stereotype, genotype,

	ferrotype
2004c	20th, 404, 32000, 68040, 8048

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 0.7 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **77%** of the people in your education level.



Great job - your score is well above average! You know exactly which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 51

This score is based on quality of transitional phrases used within your paper. You did equal or better than **36%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 43

Avg. Length: 27.1 words

Short Sentences (< 17 words): 9 (21%)

Long Sentences (> 35 words): 11 (26%)

Sentence Variation: 20.2 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 12%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts.

Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Enteroviruses are** dependent...
- **Enteroviruses have** been...
- **Human enteroviruses had** previously...
- **Recombination is** not...
- **It was** noted...

Starts by Part of Speech:

Adjective: 5%

Adverb: 0%

Article: 0%

Conjunction: 2%

Noun: 30%

Preposition: 7%

Pronoun: 5%

Verb: 0%

Other: 40%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Passive Voice

Passive Voice Sentences: 51.2%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- Enteroviruses have **been suggested** to exist on the threshold of 'error ...
- ... within the EV group that have **been categorized** antigenically as different serotypes.
- Human enteroviruses had previously **been classified** as polioviruses, coxsackie A or B ...
- ... identical to echoviruses that **were found** to cause disease in mice and humans.
- ... in the classification **were found** to occur and led to a numbering of ...
- ... more complicated as more viruses **were discovered**, and isolates **were ...**
- ... distinguishes different EVs, it **is still used** currently, though the advent of ...
- ... enteroviruses (HEV-A, B, C and D) **are classified** by (Fauquet, 2005): a) Sharing ...
- ... interactions, and the sequence can **be used** as a surrogate for antigenic typing ...
- ... different RNA genome molecules, has **been found** to occur in Enteroviruses.
- ... to 1% of a growth cycle, and it has **been noted** in vaccinees that sequences from ...
- ... is unknown, but it has **been suggested** that recombinants **are ...**
- ... limited to polioviruses, and has **been demonstrated** in non-polio enteroviruses ...
- The process of recombination has **been demonstrated** to occur during negative strand ...
- Recombination between viruses has **been seen** to be more common in the same ...
- ... the acceptor and the donor, **are shown** as a line and box respectively.
- The RNA polymerase (black oval) **is shown** copying the 3' end of donor RNA and ...
- ... switch, the recombinant RNA shown **is formed** (bottom).
- ... proposal that Enterovirus serotypes **are defined** by the capsid region and that ...
- ... on which region of the genome **is compared**.

- Members of HEV-A and HEV-B have also **been seen** to recombine within their species ...
- It **was noted** that between different species ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Readability Indices

PREMIUM ONLY

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Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 82

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **78%** of the people in your education level.



Vocabulary Word Count: 22

Percentage of Vocab Words: 2.55%

Vocab Words in this Paper (a subset): synthesis, prone, catastrophe, correlates, spectrum, flaccid, problematic, advent, obsolete, significant



Excellent work! Your usage of sophisticated words is on par with other well-written papers!

Nevertheless, you may still wish to use our **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) to maintain your edge.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ **Grade**

Auto Grader

Grade: 94 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

EV infections have a wide variety of clinical presentations, and infect individuals in all age groups. Despite this variation, there are factors that influence disease and produce consistent characteristics. Gender, socioeconomic status, and most importantly, age, have largely predictable effects (Tapparel et al., 2013). Different age groups have different susceptibilities to infection, severity of disease, and clinical manifestations. Generalisations are possible to be made, though understanding these age-effects are complicated by the prior history of EV infection and resulting immunity (Froeschle et al., 1966, Fields, 2007). Most infections are during childhood, due to the high number of infections in the population. The children are then the most... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

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Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
polioviruses	poliovirus
polioviruses	poliovirus
polioviruses	poliovirus
serotype	serotypes, stereotype, genotype, ferrotype

serotype	serotypes, stereotype, genotype, ferrotype
polioviruses	poliovirus
1981b	1581
1981a	1581
transgene	transient, transgender, transgenic, transcend, transience
echovirus	echinus, chorus, choirs, retrovirus
1999a	1394
1999a	1394
endemically	endemicity, genetically, chemically, academically, generically

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.3 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 69

This score is based on quality of transitional phrases used within your paper. You did equal or better than **64%** of the people in your education level.



Good job! Your usage of transitional phrases is above average. Nevertheless, you may still benefit from reading the info below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

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- Adverbs: *also, however, nevertheless*

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For example, you could write:

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The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 53

Avg. Length: 26.3 words

Short Sentences (< 17 words): 16 (30%)

Long Sentences (> 35 words): 12 (23%)

Sentence Variation: 12.5 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Readability Indices

PREMIUM ONLY

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Learn more... (<http://www.PaperRater.com/premium>)

Style

Sentence Beginnings

Simple Sentence Starts: 32%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **EV infections have** a...
- **Different age groups have** different...
- **Generalisations are** possible...
- **Most infections are** during...

- **Cases occur** regularly...
- **Poor socioeconomic status increases** the...
- **Passive case finding** is...
- **Surveillance data collected** from...
- **Incidence is** difficult...
- **PV was** the...
- **Molecular epidemiology has** helped...
- **Older studies were** conducted...
- **E30 was** significant...
- **They used** the...
- **They found** that...
- **Enterovirus specific primers are** used...
- **HEV-C viruses constituted** the...

Starts by Part of Speech:

Adjective: 15%

Adverb: 8%

Article: 0%

Conjunction: 0%

Noun: 30%

Preposition: 21%

Pronoun: 6%

Verb: 0%

Other: 21%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression
(<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Passive Voice

Passive Voice Sentences: 35.8%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- Generalisations are possible to **be made**, though understanding these ...
- ... exposure makes them more likely to **be affected** by serious disease than adults, ...
- Historically, Polio incidence **was found** to be low in ages 0-6 months due to ...
- In these countries, increased incidence **was seen** of paralytic disease in children ...
- ... seen in the different age groups **is also seen** in other EVs, such as EV71 (Komatsu ...
- ... are still being diagnosed that **are not caused** by PV, and may **be caused** by ...
- ... AFP cases, many different EV have **been typed**, and no clear association can **be ...**
- ... particular EV-caused disease can **be obtained** from prospective longitudinal ...
- ... at least 50% of the population has **been infected** by more than one of the viruses ...
- They can **be characterised** by serotype, time, location, and ...
- ... virus for which an infected clone **was constructed** (Racaniello and Baltimore, 1981a), ...
- ... in 1989 (Mendelsohn et al., 1989), **was then generated** in mice carrying the CD155 as a ...
- Older studies **were conducted** using monoclonal antibodies and ...

- Various studies on EV have **been conducted** using molecular sequencing ...
- ... non-polio enteroviruses that **are isolated** in cell culture from the acute ...
- Enterovirus specific primers **are used** to amplify only viral RNA and then ...
- ... these studies is that Enteroviruses **are found** endemically in healthy and diseased ...
- ... and –C, though no HEV-A viruses **were typed**.
- ... from HEV-B. HEV-A and –C viruses **were also detected**.



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 85

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **80%** of the people in your education level.



Vocabulary Word Count: 28

Percentage of Vocab Words: 2.62%

Vocab Words in this Paper (a subset): severity, manifestations, exposure, aseptic, incidence, maternal, hygiene, susceptible, endemic, surveillance



Excellent work! Your usage of sophisticated words is on par with other well-written papers! Nevertheless, you may still wish to use our **Vocab Builder**

(http://www.PaperRater.com/vocab_builder/index) to maintain your edge.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

Grade

Auto Grader

Grade: 95 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

A study conducted over a period of 10 years using various specimen types, and many enterovirus sensitive cell lines, display the variety of enteroviruses circulating in the population, and the change in distribution over time (Trallero et al., 2010). The study also demonstrates how difficult it would be to diagnose a patient without molecular typing, as a number of the different types were found distributed between different symptoms, and specimen types. 1.5 Diagnosis and Surveillance tools Clinical diagnosis is difficult considering the factors of EV infection already described. Symptoms vary widely, and many are similar to other pathogenic and non-pathogenic diseases. Also, as many EV infections are asymptomatic, the detection of the virus in a... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>) .

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
cytopathic	cytoplasmic
antisera	antisexual, artistry, anser, Antisana, anthers
	advisers,

antisera	adviser, antient, antlers, antisexual
antisera	advisers, adviser, antlers, antisexual, artistry
untypable	untaxable, untamable, untenable
intensity	intensity, intensively
inosine	iodine, inside, insane, incline, inline
serotype	serotypes, stereotype, genotype, ferrotype
1999b	1394
serotype	serotypes, stereotype, genotype, ferrotype
florescent	fluorescent, flourescent
lengthed	lengthen

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.8 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and clichés found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 45

This score is based on quality of transitional phrases used within your paper. You did equal or better than **31%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 59

Avg. Length: 22.5 words

Short Sentences (< 17 words): 23 (39%)

Long Sentences (> 35 words): 8 (14%)

Sentence Variation: 13.2 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 24%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Clinical diagnosis is** difficult...
- **Symptoms vary** widely...
- **Preferred samples are** stool...
- **Confirmatory testing is** performed...
- **Individual typing was** impractical...
- **Molecular techniques have** greatly...
- **PCR is** able...
- **Genetic sequencing** has...
- **Current serotyping assays are** based...
- **Modern equipment allows** for...
- **Sanger sequencing** is...
- **EVs have** genomes...
- **Sanger sequencing** of...
- **Outbreak studies have** been...

Starts by Part of Speech:

Adjective: 7%

Adverb: 7%

Article: 0%

Conjunction: 0%

Noun: 22%

Preposition: 17%

Pronoun: 0%
Verb: 2%
Other: 41%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Passive Voice

Passive Voice Sentences: 45.8%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... as a number of the different types **were found** distributed between different ...
- ... or epidemic, clinical diagnoses **are made** easier, as the causative agent is ...
- ... and Drew, 1980) though this may **be complicated** by the intermittent viral shedding ...
- ... of EV using virus isolation have **been described** (Grandien M, 1989).
- If the appropriate cell lines **are used**, isolation of EV from these cells ...
- ... washings, and spinal fluid can also **be used** to inoculate the cells.
- Unfortunately, no single cell line can **be used** to culture all HEV.
- A combination of several cell lines **is commonly used** to detect EV (Chonmaitree et al., ...
- Suckling mice **are then used** to propagate these serotypes.
- ... for PV attachment and entry **are also used** to select for certain serotypes, ...
- Confirmatory testing **is performed** using these cells as there are a ...
- ... in these cells, identification **is typically done** by neutralization with ...
- ... typing was impractical so antisera **were combined** in intersecting pools in such a way ...
- Even using these pools, untypable EV **were still detected** due to mixed infections, aggregates ...
- ... the specificity of the reaction can **be modified** to detect specific EV serotypes ...
- This method has **been used** extensively to target the VP1 gene ...
- ... of PCR and genetic sequencing can **be used** to assign an unknown virus to a ...
- Current serotyping assays **are based** on sequencing the 3' end (to ...
- The sequences **are then used** to characterise the virus based on ...
- ... different sequencing methods that **are commonly used** in current molecular laboratories.
- These oligonucleotides **are terminated** by nucleotides with a 3' carbon ...
- ... for these terminator nucleotides to **be detected** by a laser as the terminators ...
- ... is precise and accurate as primers **are used** to detect a targeted sequence.
- If non-specific sequences **are needed to be sequenced**, then more ...
- ... single nucleotide mutations can **be detected** between quasispecies in a sample.
- The next-generation sequencing methods **are varied** though costly.
- ... Enteroviruses in South Africa have **been focused** on Poliovirus or outbreaks of other ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active->

voice.html)

Style

Readability Indices

PREMIUM ONLY

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Learn more... (<http://www.PaperRater.com/premium>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 55

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **42%** of the people in your education level.



Vocabulary Word Count: 15

Percentage of Vocab Words: 1.62%

Vocab Words in this Paper (a subset): surveillance, aseptic, intermittent, concurrent, appropriate, propagate, aggregates, assays, conventional, synthesis



This paper could benefit from greater usage of vocabulary words. Although your vocabulary score is within the average range for most writers, boosting it will help your paper stand out. Consider using the **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) and set a goal. Try to reach the 60th percentile after revising your text with a thesaurus. Next, keep going! Why not reach the 101st percentile? Is that even possible? There's only one way to find out...

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ *Grade*

Auto Grader

Grade: 93 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

in South Africa was a retrospective study performed in 1993 (McIntyre and Keen, 1993) that focused only on Cape Town. It was found that Coxsackie B viruses were endemic, though the meningitis outbreaks (that were in the summer months) were caused by a number of HEV-B viruses: Echoviruses 4 and 9, and Coxsackievirus A9. While it was stated that enteroviruses were endemic and were the largest cause of meningitis in the population, the study only focused on meningitis. Since PV has been eliminated from South Africa, what is of particular importance is that acute flaccid paralysis cases are still being diagnosed in the country. These cases may be caused by other non-polio enteroviruses and hence the continued surveillance for other enteroviruses should be... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
serotype	serotypes, stereotype, ferrotype, genotype
-B	B, AB, BB, KB, RB

-C	C, PC, DC, EC, NC
assay	essay
cytopathic	cytoplasmic
supernatant	supergiant
c2	cm, c, cc, cf, ch
genotyping	serotyping

Grammar

Grammar Suggestions

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 0.6 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **77%** of the people in your education level.



Great job - your score is well above average! You know exactly which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 49

This score is based on quality of transitional phrases used within your paper. You did equal or better than **34%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences

to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 11

Avg. Length: 103.5 words

Short Sentences (< 17 words): 3 (27%)

Long Sentences (> 35 words): 1 (9%)

Sentence Variation: 274.9 words (std deviation)



Your average sentence length is a little bit high, which may make your writing difficult to follow. Please read the [guide to effective use of sentence length](http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html)

(<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) .

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Passive Voice

Passive Voice Sentences: 54.5%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- It **was found** that Coxsackie B viruses were ...
- While it **was stated** that enteroviruses were endemic and ...
- Since PV has **been eliminated** from South Africa, what is of ...
- These cases may **be caused** by other non-polio enteroviruses ...

- ... serotypes and the diseases they **are associated** with.
- ... sequencing). This can then possibly **be used** as a basis to develop an essay to ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 9%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **It was** found...

Starts by Part of Speech:

Adjective: 0%

Adverb: 0%

Article: 0%

Conjunction: 0%

Noun: 0%

Preposition: 18%

Pronoun: 9%

Verb: 0%

Other: 64%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Readability Indices

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⚙️ *Vocabulary Words*

Usage of Academic Vocabulary

Vocabulary Score: 68

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **64%** of the people in your education level.



Vocabulary Word Count: 17

Percentage of Vocab Words: 1.82%

Vocab Words in this Paper (a subset): retrospective, endemic, acute, flaccid, surveillance, objectives, assay, assays, institute, yield



Not bad! Your usage of sophisticated words is above average! Nevertheless, you may still wish to use our Vocab Builder (http://www.PaperRater.com/vocab_builder/index) to take your writing to the next level.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our Vocabulary Builder (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

➔ *Grade*

Auto Grader

Grade: 92 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

Extractions were conducted on automated as well as manual systems. The automated system used was the Maxwell 16 (Promega, Madison, WI, USA). Manual extractions were conducted using the Qiagen Qiampr Viral Mini Kit (Qiagen, Venlo, Netherlands). The manual and automated extractions on stool samples (not cultures) were preceded by a treatment of the samples with a Stool Transport and Recovery (STAR) buffer (Roche, Mannheim, Germany). This buffer stabilises any pathogens, neutralises nucleases, as well as binding PCR inhibitors present in the sample. Automated extractions were performed using 100 µl of initial stool or culture sample. The stool samples were processed as supplied from the surveillance programs after they had completed routine testing. The STAR... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
nucleases	nucleated, nucleus's
µl	kl, ml, ll, hl, Fl
centrifuged	centrifuges, centrifuge, centrifugal

supernatant	supergiant
aliquoted	aliquot
assay	essay
assay	essay
agarose	arose
amplicon	apricot, amplify, apeiron, amylin, american
amplicons	applicants, applicant, apricots, americans, amplifies
agarose	arose
50µl	586
electrophoresed	electrophoresis
agarose	arose
amplicon	applicant, apricot, american, Amphion, amnion
agarose	arose

Grammar

Grammar Suggestions

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 0.8 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **77%** of the people in your education level.



Great job - your score is well above average! You know exactly which phrases to avoid in your

writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 31

This score is based on quality of transitional phrases used within your paper. You did equal or better than **14%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 41

Avg. Length: 17.9 words

Short Sentences (< 17 words): 18 (44%)

Long Sentences (> 35 words): 1 (2%)

Sentence Variation: 8.7 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Readability Indices

PREMIUM ONLY

This feature is only available to premium subscribers.

Learn more... (<http://www.PaperRater.com/premium>)

Style

Sentence Beginnings

Simple Sentence Starts: 15%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts.

Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Extractions were** conducted...
- **Manual extractions were** conducted...
- **Automated extractions were** performed...
- **Excision was** done...
- **Sanger Sequencing** was...
- **Nucleotide positions correspond** to...

Starts by Part of Speech:

Adjective: 2%

Adverb: 0%

Article: 0%

Conjunction: 0%

Noun: 22%

Preposition: 0%
 Pronoun: 0%
 Verb: 2%
 Other: 59%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression
 (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Passive Voice

Passive Voice Sentences: 65.9%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- Extractions **were conducted** on automated as well as manual ...
- Manual extractions **were conducted** using the Qiagen Qiamp Viral Mini ...
- ... on stool samples (not cultures) **were preceded** by a treatment of the samples with ...
- Automated extractions **were performed** using 100 µl of initial stool or ...
- The stool samples **were processed** as supplied from the surveillance ...
- The STAR buffer treated stool samples **were centrifuged** at 1000 rpm in a microcentrifuge ...
- ... Kit (Promega ,Madison, WI, USA) **was used** to extract the viral RNA.
- The initial screening PCR **was conducted** on the extracted material and ...
- ... failed to yield a useable sequence, **were then extracted** manually and rerun on the ...
- ... Kit (Qiagen, Venlo, Netherlands) **was used** for manual extractions of the viral ...
- ... used in the automated extraction **was used** for the manual extraction.
- ... al., 2002) PCR protocol (Table 2.2) **was used** to screen samples for the presence ...
- This protocol had **been used** diagnostically within the NICD for ...
- ... the screening assay A comparison **was conducted** between two cDNA synthesis kits.
- This **was compared** with the Transcriptor First Strand ...
- The Transcriptor Kit **was used** for the final assay.
- ... sites to ensure that all viruses **were detected** (Figure 2.1, Table 2.2).
- Using this protocol, the primers **were tested** and validated on QCMD control ...
- An agarose gel **was run** using the PCR product to separate ...
- This **was performed** using a 30cm gel of 1.5% agarose ...
- The agarose gel **was visualised** under UV light and the molecular ...
- Excision **was done** using a scalpel to remove the band ...
- ... System (Promega, Madison, WI, USA) **was used** as per the manufacturer's ...
- Sanger Sequencing **was then conducted** as per the BigDye Terminator ...
- ... Carlsbad, CA, USA) can **be found** in Figure 2.1.
- ... and sequencing PCR reactions **are described** in Table 2.2.
- The sequences **were analysed** on the 3130 analyser (Life ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

⚙️ *Vocabulary Words*

Usage of Academic Vocabulary

Vocabulary Score: 66

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **61%** of the people in your education level.



Vocabulary Word Count: 10

Percentage of Vocab Words: 1.74%

Vocab Words in this Paper (a subset): surveillance, centrifuged, yield, assay, protocol, synthesis, validated, excised, schematic, probes



Not bad! Your usage of sophisticated words is above average! Nevertheless, you may still wish to use our Vocab Builder (http://www.PaperRater.com/vocab_builder/index) to take your writing to the next level.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our Vocabulary Builder (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

➔ *Grade*

Auto Grader

Grade: 90 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

2.6 Next Generation Sequencing Culture positive samples from the AFP network were selected for Next Generation Sequencing according to their species. HEV-C viruses were selected based on their increased likelihood to have recombined with the Polio Sabin vaccine strains. Primers were sourced from a study by (Boot et al., 2004) which covered the entire EV genome and were specific to HEV-C. cDNA was synthesised using three combinations: 1) anchored primers only, 2) random primer and anchored primers, 3) and anchored primers only. PCR was then conducted with the sourced primers and the Expand Long Template PCR System (Roche, Mannheim, Germany) as per the manufacturer's instructions. This kit contained a Taq polymerase enzyme able to synthesize long strands of... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
assay	essay
serotyped	serotypes, stereotyped
genotyping	serotyping
user-	user, users

chromatogram	chromatography, chromatographs
--------------	-----------------------------------

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 0.8 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **77%** of the people in your education level.



Great job - your score is well above average! You know exactly which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 41

This score is based on quality of transitional phrases used within your paper. You did equal or better than **21%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text

and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 40

Avg. Length: 19.8 words

Short Sentences (< 17 words): 14 (35%)

Long Sentences (> 35 words): 3 (8%)

Sentence Variation: 10.4 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Passive Voice

Passive Voice Sentences: 55.0%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... samples from the AFP network **were selected** for Next Generation Sequencing ...
- HEV-C viruses **were selected** based on their increased likelihood ...
- Primers **were sourced** from a study by (Boot et al., 2004) ...
- PCR **was then conducted** with the sourced primers and the ...
- ... Junior (Roche, Mannheim, Germany) **were conducted** and the results compared.
- A second method **was attempted** to create double stranded cDNA.

- ... System (Roche, Mannheim, Germany) **was utilized** for this assay as per the ...
- ... random and specific primers can **be used** with this kit and **was tested** ...
- ... of the different primers **were used** attempted to obtain usable cDNA for ...
- ... assays, all specimen controls **were serotyped** correctly by genotyping as per ...
- ... Information (NCBI) database **was used** as a source to BLAST (Basic Local ...
- ... was similar to the BLAST tool, but **was found** to be more user- friendly.
- ... analysis and visual checking **was conducted** using Sequencher 4.10.1 (Gene Codes ...
- Consensus sequences **were exported** into a text file that **was** ...
- Microsoft Excel **was used** to sort data and create tables and ...
- Epi Info (CDC, Atlanta, USA) **was used** to create the country distribution ...
- Ethics **was obtained** from the University of ...
- After the comparison **was taken** into account, and the treatment of ...
- ... market, the Transcriptor enzyme, **was made** available and **was tested** and ...
- ... RNA to the point where it could not **be detected**.
- ... that other sample types could **be used**, and would yield similar results.
- The Transcriptor Kit **was then implemented** in the screening assay to improve ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 18%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **HEV-C viruses were** selected...
- **Primers were** sourced...
- **PCR was** then...
- **Sanger sequencing** on...
- **Consensus sequences were** exported...
- **Microsoft Excel was** used...
- **Ethics was** obtained...

Starts by Part of Speech:

Adjective: 3%

Adverb: 0%

Article: 0%

Conjunction: 0%

Noun: 35%

Preposition: 10%

Pronoun: 0%

Verb: 0%

Other: 40%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Readability Indices

PREMIUM ONLY

This feature is only available to premium subscribers.

Learn more... (<http://www.PaperRater.com/premium>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 65

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **57%** of the people in your education level.



Vocabulary Word Count: 11

Percentage of Vocab Words: 1.82%

Vocab Words in this Paper (a subset): synthesised, synthesis, assay, assays, criteria, institute, consensus, yield, surveillance, protocol



Not bad! Your usage of sophisticated words is above average! Nevertheless, you may still wish to use our Vocab Builder (http://www.PaperRater.com/vocab_builder/index) to take your writing to the next level.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our Vocabulary Builder (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ GradeAuto Grader**Grade:** 91 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

Initial investigations to detect enterovirus nucleic acids from the extracted samples used primers targeting the 3' Untranslated region of Enteroviruses with the aim of distinguishing the samples by species, as this region is conserved within a species, but differs with viruses of another species. A method described by (Oberste et al., 2006) described these primers. The results from their PCR were then used to determine species specific primers that gave sequences able to be used for typing. Using the protocol described, no product was detected from the extracted samples using the Roche LightCycler FastStart DNA Master SYBR Green I Kit (Mannheim, Germany). Extracted RNA of an enterovirus control provided a constant starting nucleic acid concentration, and... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
360C	360s, 6309, S60
460C	404, S60, 360s
05C	DC, C
agarose	arose
dye	die

dye	die
spectrophotometer	spectrophotometry
10ng	long
μl	ll, ml, Cl, El, l
amplicon	applicant, apricot, american, amnion, apeiron
agarose	arose
spectrophotometer	spectrophotometry
μl	kl, cl, l, Tl, al
260λ	S60, 360s
230λ	6309
260λ	S60, 360s
230λ	6309
spectrophotometer	spectrophotometry
1μl	oil
10μl	16000, 1802, 1541, 1581, 1394
50μl	586
electrophoresed	electrophoresis
spectrophotometer	spectrophotometry
eluent	fluent
agarose	arose
μM	M, GM, AM, NM, IM
μM	M, GM, AM, NM, IM
μM	GM, M, IM, FM, AM
μM	M, DM, EM, FM, GM

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.1 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and clichés found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 45

This score is based on quality of transitional phrases used within your paper. You did equal or better than **31%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 43

Avg. Length: 24.9 words

Short Sentences (< 17 words): 12 (28%)

Long Sentences (> 35 words): 10 (23%)

Sentence Variation: 14.5 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 7%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Conventional PCR was** also...
- **Anchored primers did** not...
- **Non-specific smudges were** seen...

Starts by Part of Speech:

Adjective: 9%

Adverb: 5%

Article: 0%

Conjunction: 0%

Noun: 14%

Preposition: 9%

Pronoun: 0%

Verb: 7%

Other: 33%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

StylePassive Voice**Passive Voice Sentences:** 55.8%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... samples by species, as this region **is conserved** within a species, but differs with ...
- The results from their PCR **were then used** to determine species specific ...
- ... the protocol described, no product **was detected** from the extracted samples using ...
- ... acid concentration, and attempts **were made** to improve the protocol by varying ...
- No amplification **was seen** throughout these trials, though ...
- ... primer-dimer formation, and it **was not observed** in any other aspect of the trials.
- Conventional PCR **was also attempted** with these primers, though they had ...
- The molecular weight marker **was loaded** with dye as a reference.
- ... resultant cDNA concentrations **were measured** using a mass spectrophotometer and ...
- ... indeed contain virus, an experiment **was performed** using the routine Enterovirus ...
- ... clean sequences that were able to **be typed** on the BLAST programs, a comparison ...
- 1 sample **was amplified** in separate tubes, and 3 tubes ...
- ... sequencing primers from that study **were not used**, as the species type **was** ...
- ... two or more Enteroviruses cannot **be typed** though, as the Nix primers would ...
- ... Transcriptase package insert), **was found** to improve PCR yields if added in ...
- ... 10µl of sequencing PCR product **was run** on the gel and the remainder **was** ...
- Subsequently, it **was found** that using this cleaned product for ...
- Using a spectrophotometer, RNA presence **was confirmed** on the eluent from the sample ...
- 3 samples **were obtained** from cell culture and in HEV-C: a ...
- ... Kit (Roche, Mannheim, Germany) **was used** with three combinations of primers ...
- ... detecting the HEV-C samples that **were typed**.
- Non-specific smudges **were seen** on the gel after nested PCR ...
- ... variations of deoxynucleotides **were then attempted**.
- ... 700 µM and 1000 µM concentrations **were attempted** in the 2 PCR reactions that



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Readability Indices

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This feature is only available to premium subscribers.

Learn more... (<http://www.PaperRater.com/premium>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 45

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **36%** of the people in your education level.



Vocabulary Word Count: 11

Percentage of Vocab Words: 1.25%

Vocab Words in this Paper (a subset): protocol, increments, conventional, yield, excised, genus, amplify, yields, synthesis, yielded



Your usage of sophisticated vocabulary words used is LESS than average. Aim for a higher vocabulary score and it will show in your writing. Please use the **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) tool and set a goal. Try to reach the 60th percentile after revising your text with a thesaurus. Next, keep going! Why not reach the 101st percentile? Is that even possible? There's only one way to find out...

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ *Grade*

Auto Grader

Grade: 93 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

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PaperRater

(<http://www.PaperRater.com>)

constituted the nested reactions. A smudge was detected on the agarose gel using a 300 μM concentration in the first round PCR and 500 μM in the second round PCR. This smudge excised from the gel and cleaned up as described. The DNA was then sequenced using Sanger sequencing, and on the GS Junior (Roche, Mannheim, Germany), and next generation sequencer. On both instruments only Poly-T sequence was obtained. Attempts using the cDNA Synthesis System Kit (Roche, Mannheim, Germany) were done using the kit instructions. When no band was obtained in the control RNA reaction, concentrations of starting RNA, Mg^{2+} , and different combinations of anchored, random and specific primers were attempted. Still no control band was obtained, and consultations with the... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
agarose	arose
μM	M, DM, EM, FM, GM
μM	GM, M, DM, AM, LM
Poly-T	Poly T

assay	essay
pools	pulls
assay	essay
serotype	serotypes, stereotype, genotype, ferrotype
-C	PC, C, EC, DC, AC
serotype	serotypes, stereotype, ferrotype, genotype
serotype	serotypes, stereotype, ferrotype, genotype

Grammar

Grammar Suggestions

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.1 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 61

This score is based on quality of transitional phrases used within your paper. You did equal or better than **51%** of the people in your education level.



Good job! Your usage of transitional phrases is within an acceptable range! Nevertheless, you may still benefit from reading the info below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 47

Avg. Length: 19.1 words

Short Sentences (< 17 words): 21 (45%)

Long Sentences (> 35 words): 3 (6%)

Sentence Variation: 10.2 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

 StyleSentence Beginnings**Simple Sentence Starts:** 11%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Attempts using** the...
- **Neutralisation assays were** used...
- **Male patients constituted** 55.51...
- **Samples originated** from...
- **HEV-A serotypes were** equally...

Starts by Part of Speech:

Adjective: 6%

Adverb: 9%

Article: 0%

Conjunction: 0%

Noun: 21%

Preposition: 17%

Pronoun: 0%

Verb: 2%

Other: 32%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

 StylePassive Voice**Passive Voice Sentences:** 59.6%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- A smudge **was detected** on the agarose gel using a 300 µM ...
- The DNA **was then sequenced** using Sanger sequencing, and on the ...
- ... instruments only Poly-T sequence **was obtained**.

- ... Kit (Roche, Mannheim, Germany) **were done** using the kit instructions.
- When no band **was obtained** in the control RNA reaction, ...
- Still no control band **was obtained**, and consultations with the ...
- Further studies will need to **be conducted** to find a method that is able to ...
- ... and the Dutch Blast tool from RVIM **were used** to type results.
- For the control specimens it **was found** that the identification had a 100% ...
- ... samples, slight typing differences **were observed** between the 2 tools (e.g. viruses ...
- ... sequencing assay, as results **were not obtained** using the next generation ...
- Neutralisation assays **were used** to type EV for many years before ...
- The assay **was used** on viral isolates, and requires ...
- ... type-specific anti-serum can **be used** to confirm the type (Lim and ...
- ... Initiative, though PCR has also **been introduced** in those laboratories and has made ...
- ... samples A total of 832 samples **were tested** from the AFP Surveillance Program, ...
- From the AFP program, 175 samples **were screened** after being classified as a ...
- In addition, ninety five samples **were obtained** from the culture negative stools ...
- ... Program yielded 562 samples to **be screened** (Table 3.3).
- ... samples yielded sequences able to **be compared** to the NCBI database and typed.
- An additional 37 sequences **were obtained** from the Severe Acute Respiratory ...
- ... samples (including the SARI data) **are indicated** in Table 3.4.
- Sixty four (64) serotypes **were detected** from all four species groups, HEV-A ...
- ... and HEV-B. Only 1 HEV-D serotype **was detected**.
- HEV-A serotypes **were equally distributed** between males (20) and females (19).
- More males than females **were infected** by serotypes in HEV-B and HEV-C, ...
- In HEV-D more females **were infected** than males with 6 females and 3 ...
- ... serotypes, a much larger study will **be needed** to determine any gender patterns.



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Readability Indices

PREMIUM ONLY

This feature is only available to premium subscribers.

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⚙️ *Vocabulary Words*

Usage of Academic Vocabulary

Vocabulary Score: 94

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **84%** of the people in your education level.



Vocabulary Word Count: 18

Percentage of Vocab Words: 2.59%

Vocab Words in this Paper (a subset): smudge, excised, synthesis, yielded, constraints, amplify, correlation, assay, assays, aggregates



Excellent work! Your usage of sophisticated words is on par with other well-written papers! Nevertheless, you may still wish to use our **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) to maintain your edge.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

➔ *Grade*

Auto Grader

Grade: 92 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

A comparison is not possible between the Sanger sequencing assay, and the next generation sequencing assay, as results were not obtained using the next generation sequencing assay. Neutralisation assays were used to type EV for many years before molecular methods became available. The assay was used on viral isolates, and requires anti-sera against all the serotypes for accurate typing. As there are over 100 enteroviruses, and more discovered on a regular basis, individual assays are impractical. There have been intersecting anti-sera pools that have helped narrow down the range of viruses before an individual type-specific anti-serum can be used to confirm the type (Lim and Benyesh-Melnick, 1960, Melnick et al., 1973). Unfortunately the method is... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>) .

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases
were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
assay	essay
pools	pulls
serotype	serotypes, stereotype, ferrotype, genotype

assay	essay
polioviruses	poliovirus
assay	essay
assay	essay

Grammar

Grammar Suggestions

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.5 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and clichés found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 50

This score is based on quality of transitional phrases used within your paper. You did equal or better than **35%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 44

Avg. Length: 22.0 words

Short Sentences (< 17 words): 15 (34%)

Long Sentences (> 35 words): 5 (11%)

Sentence Variation: 11.5 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Passive Voice

Passive Voice Sentences: 36.4%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... sequencing assay, as results **were not obtained** using the next generation ...
- Neutralisation assays **were used** to type EV for many years before ...
- The assay **was used** on viral isolates, and requires ...
- ... type-specific anti-serum can **be used** to confirm the type (Lim and ...
- ... this study would not be able to **be typed** using neutralising assays, ...
- ... Initiative, though PCR has also **been introduced** in those laboratories and has ...
- ... and to determine if the paralysis **is caused** by one of the three Polioviruses.
- ... and transport has already **been established**.
- ... surveillance system, cell culture **is used** to isolate virus, and only one ...

- ... the old target of 2/100 000 which **was increased** for the 2015 year to try increase ...
- This **is planned** to lead to the eradication of ...
- ... the advantage that stool samples **are tested** directly, without a virus isolation ...
- ... to detect viruses that may **be associated** with gastrointestinal disease ...
- The major population centres **are covered** in the Gauteng, Western Cape and ...
- ... confound this association if there **are mixed** infections with a known respiratory ...
- The Nix et al (2006) assay **was used** rather than using the traditional ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 2%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Neutralisation assays were** used...

Starts by Part of Speech:

Adjective: 0%

Adverb: 7%

Article: 0%

Conjunction: 0%

Noun: 11%

Preposition: 7%

Pronoun: 2%

Verb: 2%

Other: 64%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Readability Indices

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Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 71

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **69%** of the people in your education level.



Vocabulary Word Count: 13

Percentage of Vocab Words: 1.74%

Vocab Words in this Paper (a subset): assay, assays, aggregates, confound, definitive, superseded, surveillance, flaccid, passive, confounds



Not bad! Your usage of sophisticated words is above average! Nevertheless, you may still wish to use our **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) to take your writing to the next level.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

Grade

Auto Grader

Grade: 92 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

serotypes detected through the species also demonstrated a bias introduced by culturing the virus in cells. The molecular sequencing assay in this study (if only testing 1 sample) can have a result within 3-4 days, and if a 24 hour laboratory is running the assay, even shorter times, due to the evening hours being utilised. As discussed above (Section 3.7), the large number of EV that are known, and the probable discovery of more in the near future, make a universal sequencing assay that is able to detect all the viruses (though sample type may cause some inhibition) a logical choice if looking for the epidemiology of EV, or the unknown cause of a possible EV outbreak, within a population. The positive culture specimens need very little treatment to yield... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>) .

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
assay	essay
assay	essay
assay	essay
eluted	elated, eluded, fluted

assay	essay
picornavirus	coronavirus
assay	essay
assay	essay
polioviruses	poliovirus
rhinoviruses	rhinovirus
amplicon	applicant, apricot, american, Amphion, amnion
capsids	capsid
chromatogram	chromatography, chromatographs
amplicons	applicants, applicant, apricots, americans, antlions
agarose	arose
serotype	serotypes, stereotype, ferrotype, genotype

Grammar

Grammar Suggestions

Error	Suggestion
not unexpected	expected

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.4 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and clichés found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 44

This score is based on quality of transitional phrases used within your paper. You did equal or better than **29%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 52

Avg. Length: 26.2 words

Short Sentences (< 17 words): 15 (29%)

Long Sentences (> 35 words): 9 (17%)

Sentence Variation: 16.4 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 8%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts.

Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **Systems are** needed...
- **Sequences obtained** before...
- **Toxicity is** another...
- **They contain a...**

Starts by Part of Speech:

Adjective: 0%

Adverb: 4%

Article: 0%

Conjunction: 0%

Noun: 10%

Preposition: 15%

Pronoun: 4%

Verb: 2%

Other: 58%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

 **Style**Passive Voice

Passive Voice Sentences: 38.5%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... 3.7), the large number of EV that **are known**, and the probable discovery of more ...
- ... RNA extraction procedure all that **is required** for molecular techniques to **be** ...
- Systems **are needed** to remove these PCR inhibitors from ...
- The PCR assay this study **was based** on (Nix et al., 2006) used a ...
- An alternative **was found** from Roche in the form of the Stool ...
- This buffer **was designed** to stabilise pathogens present in ...
- ... negative on the sequencing PCR gel, **were then treated** with the STAR buffer.
- The number of samples that were able to **be sequenced** doubled after this treatment.
- ... all of positive samples could not **be typed**, the improvement from 38.3% yield ...
- ... two probes to ensure that all EV **are detected**, while keeping the specificity of ...
- ... CPE to enteroviruses, and needs to **be confirmed** by another assay such as PCR or ...
- ... reactions was a viral culture, and **was not inhibited** by the high RNA titres.
- The PCR had **been used** at NICD, South Africa, for many ...
- ... AFP program in 2012 were able to **be tested**, and assuming the EV circulation ...
- Once the samples have **been screened** for EV using the real-time PCR ...
- cDNA **is produced** using short oligonucleotides, that ...
- An electrophoresis gel must then **be run** to separate the cDNA bands out, and ...
- As shown in Table 3.2, the Promega kit **was found** to be more effective at cleaning up ...
- ... a set of more specific primers can **be attempted**.
- ... remove; cDNA positive samples that **were excised** and cleaned from the agarose gel ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

 **Style**Readability Indices**PREMIUM ONLY**

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Learn more... (<http://www.PaperRater.com/premium>)

⚙️ *Vocabulary Words*

Usage of Academic Vocabulary

Vocabulary Score: 55

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **42%** of the people in your education level.



Vocabulary Word Count: 16

Percentage of Vocab Words: 1.47%

Vocab Words in this Paper (a subset): assay, universal, yield, probes, assays, compounds, inhibit, prohibitive, protocol, forward



This paper could benefit from greater usage of vocabulary words. Although your vocabulary score is within the average range for most writers, boosting it will help your paper stand out. Consider using the **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) and set a goal. Try to reach the 60th percentile after revising your text with a thesaurus. Next, keep going! Why not reach the 101st percentile? Is that even possible? There's only one way to find out...

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

➔ *Grade*

Auto Grader

Grade: 93 A

NOTE: Our grading algorithm changed on 9/23/2015. [More info \(http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html\)](http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are



supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.



PaperRater

(<http://www.PaperRater.com>)

Using the national surveillance systems in place as a source of samples ensured good population coverage, and a high diversity of HEV was detected. The detection rate in the samples from the Rotavirus Surveillance Programs stool samples (49.29% positive) was higher than in the AFP Surveillance Programs negative stool samples (33.68% positive), though the AFP positive samples have already been screened from these samples. This may be due to the patient pool from which the samples were obtained. The patients from the Rotavirus Surveillance Program were young, severely ill, and hospitalised which may have been caused by enterovirus infection which would have increased the detection rate in these samples. The pattern seen in the enterovirus distribution... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>).

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
2000a	32000
serotype	serotypes, stereotype, ferrotype, genotype
	serotypes,

serotype	stereotype, ferrotype, genotype
Poly-T	Poly T

Grammar

Grammar Suggestions

Error	Suggestion
not unexpected	expected

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 1.8 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 46

This score is based on quality of transitional phrases used within your paper. You did equal or better than **32%** of the people in your education level.



Your usage of transitional phrases is below average. Please review the writing tips below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 49

Avg. Length: 27.2 words

Short Sentences (< 17 words): 10 (20%)

Long Sentences (> 35 words): 12 (24%)

Sentence Variation: 12.1 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Passive Voice

Passive Voice Sentences: 53.1%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- ... and a high diversity of HEV **was detected**.
- ... AFP positive samples have already **been screened** from these samples.
- ... patient pool from which the samples **were obtained**.
- ... and hospitalised which may have **been caused** by enterovirus infection which ...
- Mostly HEV-B **was detected** from culture specimens and this can ...
- Neither of these viruses has **been definitively linked** to diseases, though CA24 has ...
- ... disease-associated viruses **were detected**, but did not contribute ...
- EV 71 **was isolated** in the AFP culture positive samples.
- This virus has **been associated** with aseptic meningitis (Liu et ...
- EV 68 **is associated** with respiratory infections (Peci ...
- ... viruses found to occur, but they **are associated** with a variety of symptoms, one of ...
- The old classification of enteroviruses **was based** on phenotype, infection of certain ...
- ... to cause similar symptoms, but can **be found** in aseptic infections, as well as ...
- The 'new' numbered enteroviruses can **be associated** with a symptom type like EV71, but ...
- ... confirm this, further studies will **be needed** with samples collected more evenly ...
- What would **be needed** is a surveillance program tailored ...
- ... detection of EV99 than what has **been previously reported**, may be due to lack of serotyping ...
- ... EV68 and respiratory infections **is known**.
- ... of HEV in a population may not **be associated** with clinical disease and be part ...
- ... of myocarditis will also have to **be studied** further with different sample types ...
- ... with enteroviruses would need to **be tested** more frequently in South Africa for ...
- An example where this has **been done** is in China (Li et al., 2015).
- ... or how one EV serotype can **be associated** with many symptom types.
- The first attempt in which random cDNA **was created** to try to cover the entire genome ...
- As random primers **are used** in the screening PCR, this is ...
- ... with the long cDNA synthesis kit **was utilised** with the manufacturer helping with



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 10%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to

pay attention to sentence starts and deliberately edit for variety if necessary. Here are some simple sentence starts that we found in your text:

- **It is** also...
- **EV infections followed** the...
- **Serotype distribution varies** over...
- **Differences seen** in...
- **Full genome sequencing** may...

Starts by Part of Speech:

Adjective: 4%

Adverb: 6%

Article: 0%

Conjunction: 2%

Noun: 14%

Preposition: 10%

Pronoun: 4%

Verb: 2%

Other: 57%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Readability Indices

PREMIUM ONLY

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Learn more... (<http://www.PaperRater.com/premium>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 36

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **28%** of the people in your education level.



Vocabulary Word Count: 12

Percentage of Vocab Words: 1.17%

Vocab Words in this Paper (a subset): surveillance, diverse, prevalent, aseptic, seasonal, natal, collected, confounded, forward, healthy



Your usage of sophisticated vocabulary words used is LESS than average. Aim for a higher vocabulary score and it will show in your writing. Please use the **Vocab Builder** (http://www.PaperRater.com/vocab_builder/index) tool and set a goal. Try to reach the 60th percentile after revising your text with a thesaurus. Next, keep going! Why not reach the 101st percentile? Is that even possible? There's only one way to find out...

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ *Grade*

Auto Grader

Grade: 92 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.





PaperRater

(<http://www.PaperRater.com>)

troubleshooting. As the control RNA also did not yield any results through the several runs attempted, it is still unknown why long template cDNA was not reliably produced for the next step of the sequencing process. It is possible further optimisation is required for cDNA to be synthesised, but funding and time constraints limited it for this study. Further studies in this area may use the same reagents and protocols used with further optimisation to yield results. Other possibilities are attempting different primers to try and create cDNA that covers the whole genome. 4.7 Conclusions The aims of the study were to develop molecular assays to detect and type EV from patient samples and describe the virus distribution and epidemiology in South... (only first 800 chars shown)



Analysis complete. Our feedback is listed below in printable form. Some of the items have been truncated or removed to provide better print compatibility.

Plagiarism Detection

Original Work

Originality: 100%



No sign of plagiarism was found. That's what we like to see!

A low originality percentage is indicative of plagiarized papers. Sometimes the score is lower due to long quotations within a document, so please make sure that you use proper citations if this is the case. For more information on our originality scoring process, [click here](http://www.PaperRater.com/page/plagiarism-detection) (<http://www.PaperRater.com/page/plagiarism-detection>) .

Upgrade to premium
(<http://www.PaperRater.com/pricing>) to see which phrases were found to be un-original

Spelling

Spelling Suggestions

Error	Suggestion
assay	essay
assay	essay
assay	essay
serotype	serotypes, stereotype, ferrotype, genotype

1999a	1394
antisera	advisers, adviser, antlers, anti-war, anthers
antisera	antiseptical, artistry, anser, Antisana, anthers
polioviruses	poliovirus

Grammar

Grammar Suggestions

Error	Suggestion
try and	try to

Word Choice

Word Choice Suggestions

Suggestions for improving word choice appear in the text underlined in blue. Select this text to view the tips.

Word Choice

Usage of Bad Phrases

Bad Phrase Score: 2.0 (lower is better)

The Bad Phrase Score is based on the quality and quantity of trite or inappropriate words, phrases, and cliches found in your paper. You did equal or better than **46%** of the people in your education level.



Your score is within an acceptable range, which shows that you have a solid grasp on which phrases to avoid in your writing.

Style

Usage of Transitional Phrases

Transitional Words Score: 59

This score is based on quality of transitional phrases used within your paper. You did equal or better than **47%** of the people in your education level.



Good job! Your usage of transitional phrases is within an acceptable range! Nevertheless, you may still benefit from reading the info below.

One sign of an excellent writer is the use of transitional phrases. Transitional words and phrases (e.g. therefore, consequently, furthermore) contribute to the cohesiveness of a text and allow the sentences to flow smoothly. Without transitional phrases, a text will often seem disorganized and will most likely be difficult to understand. When these special words are used, they provide organization within a text and lead to greater understanding and enjoyment on the part of the reader.

These words and phrases fall under a few grammatical categories:

- Conjunctions: *but, provided, and, although*
- Prepositional phrases: *in addition to, in conclusion*
- Adverbs: *also, however, nevertheless*

Transitional phrases may be used in various places in a text:

- between paragraphs
- between sentences
- between sentence parts
- within sentence parts

For example, you could write:

Form and function are central themes in Biology. However, knowing the structure of something does not necessarily reveal its function.

The word '**however**' contributes to greater unity or cohesion between sentences.

Style

Sentence Length Info

Total Sentences: 35

Avg. Length: 22.5 words

Short Sentences (< 17 words): 12 (34%)

Long Sentences (> 35 words): 3 (9%)

Sentence Variation: 12.4 words (std deviation)



Your average sentence length is within an acceptable range, but consider that effective use of sentence length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>) cannot be easily measured.

Line chart of the length of each sentence (first 50 sentences). A jagged chart indicates variation.



Helpful Resources:

- Effective Use of Sentence Length (<http://blog.paperrater.com/2015/05/effective-use-of-sentence-length.html>)

Style

Passive Voice

Passive Voice Sentences: 40.0%

Many writers feel that passive voice represents poor writing form, as it allows the object of an action to be the subject of a sentence. The following sentences were detected as having passive voice:

- It is possible further optimisation **is required** for cDNA to **be synthesised**, ...
- A single EV real-time PCR **was chosen** over a multiplex PCR able to ...
- The multiplex PCR **was attempted**, however, no sample controls ...
- ... is that the sequencing assay to **be based** on these results needs to then ...
- ... study, more recently classified EV **were detected** by this assay: EV99, EV102, and EV114.
- These **are little described** viruses with no clear disease ...
- ... as mentioned, many previous studies **were biased** due to the use of culture before ...
- Echovirus 30 **is seen** to be the predominant serotype in ...
- ... also co-circulated, but EV99 **was seen** to be predominant throughout the ...
- Further investigations **are needed** to find whether EV99 has any ...
- As most pools **are geared** towards detecting polio, and the ...
- ... of EV investigation will need to **be continued** in greater depth to try to discover ...
- ... recombination (when this aspect has **been perfected**), or assisting in the development ...
- Further samples can also **be sourced** from patients suffering from ...



Helpful Resources:

- Active vs Passive Voice (<http://blog.paperrater.com/2015/05/passive-voice-vs-active-voice.html>)

Style

Sentence Beginnings

Simple Sentence Starts: 20%

Variety is the hallmark of a good writer and this is especially true in regards to sentence starts. Creatively arranging sentence beginnings breaks up the monotony and choppy style associated with a simple noun phrase followed by a verb. This does NOT mean that all sentences should begin with prepositional phrases, transitions, or adverbial phrases, but it does mean that you should be certain to pay attention to sentence starts and deliberately edit for variety if necessary.

Here are some simple sentence starts that we found in your text:

- **It is** possible...
- **Other possibilities are** attempting...
- **Other studies have** not...
- **Further investigations are** needed...
- **Neutralization assays are** labour...
- **Anitiseras are** also...

- **Typing EV found in...**

Starts by Part of Speech:

Adjective: 9%

Adverb: 6%

Article: 0%

Conjunction: 0%

Noun: 17%

Preposition: 14%

Pronoun: 3%

Verb: 6%

Other: 40%



Helpful Resources:

- Sentence Beginnings - your sentences' first impression (<http://blog.paperrater.com/2015/06/sentence-beginnings.html>)

Style

Readability Indices

PREMIUM ONLY

This feature is only available to premium subscribers.

Learn more... (<http://www.PaperRater.com/premium>)

Vocabulary Words

Usage of Academic Vocabulary

Vocabulary Score: 82

This score is based on the quantity and quality of scholarly vocab words found in the text. You did equal or better than **78%** of the people in your education level.



Vocabulary Word Count: 14

Percentage of Vocab Words: 2.31%

Vocab Words in this Paper (a subset): yield, synthesised, constraints, protocols, assays, robust, probes, assay, amplify, diverse



Excellent work! Your usage of sophisticated words is on par with other well-written papers! Nevertheless, you may still wish to use our **Vocab Builder**

(http://www.PaperRater.com/vocab_builder/index) to maintain your edge.

Tips

Whether you are writing for a school assignment or professionally, it is imperative that you have a vocabulary that will provide for clear communication of your ideas and thoughts. You need to know the type and level of your audience and adjust your vocabulary accordingly. It is worthwhile to constantly work at improving your knowledge of words. To help with this task, please consider using our **Vocabulary Builder** (http://www.PaperRater.com/vocab_builder/index) to improve your comprehension and usage of words.

→ **Grade**

Auto Grader

Grade: 93 A

NOTE: Our grading algorithm changed on 9/23/2015. More info (<http://blog.paperrater.com/2015/09/automated-essay-scoring-updates.html>) ...

The grade above is NOT complete! We do not actually use a crystal ball to generate your grade. Instead, this grade takes into account spelling, grammar, word choice, style, vocabulary, and more; but it does NOT examine the *meaning* of your words, how your ideas are structured, or how well your arguments are supported. We should also mention that our automated grader doesn't **always** get things right. So, please consider this grade to be one facet of your paper's overall grade.

