DETECTION AND IDENTIFICATION OF NON-CULTURABLE ENTEROVIRUSES IN FAECAL SAMPLES FROM ACUTE FLACCID PARALYSIS CASES IN SOUTHWESTERN NIGERIA

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Abstract

This study revealed the detection and identification of the species of non-culturable enteroviruses present in faecal samples of children with Acute Flaccid Paralysis and characterized the genotype of the enteroviruses by Phylogenetic analysis.Non-culturable enteroviruses are very common, distributed worldwide and go unnoticed and sometimes caused outbreaks in which a larger than usual number of patients develop clinical disease, sometimes with serious consequences.Enteroviruses (EVs) are human pathogens that are very important, associated with various clinical syndromes of which their infections are common in humans across the globe and remain to be a vital public health problem. A total number of sixty (30 cases) culture negative stool suspensions from Southwestern Nigeria were analyzedfor the presence of non-polio Enteroviruses. The study location of six States were included in this study with the children's age under 15. Viral RNA was extracted from each stool suspension using Total RNA purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Eluted Ribonucleic acid (RNA) was used to synthesize complimentary Deoxyribonucleic acid (cDNA) using Script cDNA synthesis kit (Jena Bioscience, Germany) according to the manufacturers' instructions. Polymerase Chain Reaction (PCR) was carried out using each newly synthesized cDNA. First round PCR was carried out with 35 cycles of amplification and the second round PCR for semi-nested amplification was carried out in three different assays using a constant reverse Primer AN88 and different forward enterovirus species-specific primers AN89, 189 and I87 for Pan-enteroviruses, species A and C Enteroviruses and species B, respectively. Five microliter of the reaction product was separated and visualized on 2 % agarose gel containing 10 ul Ethidium bromide and viewed using a transilluminator. The resulting DNA amplicon was purified and sequenced using the second round primers used for the semi-nested amplification reaction. Generated sequences were manually edited using MEGA 6.06

software. Phylogenetic analysis of the sequenced viruses was done for virus identification by comparing with many references sequences from GenBank. Reference sequences of species A, B and C Enteroviruses was retrieved from GenBank, aligned with generated sequences using the CLUSTAL W programme in MEGA 6.06 software and a maximum Likelihood (ML) tree with 1000 bootstrap replicates constructed using MEGA 6.06 software.

Results showed that of the 30 samples screened, only one was positive showing the expected band size approximately 350bp for enteroviruses VP1 gene. The sample was recovered from a male patient from Oyo State. Only one species (Species B) of enterovirus was recovered from all the samples. Phylogenetic analysis strain showed the sequence as serotype Echovirus 21 (E-21).

In conclusion, this study revealed the prevalence of Enteroviruses (EVs)in south-western Nigeria is low, it furthered generated results about the detection and identification of enterovirus species present in the patient samples which were negative during culturing method. Hence emphasizing the necessity of combination of cultural and molecular method for the detection of non culturable enteroviruses. Also, it documents the first molecular sequence data on Echovirus 21 (E-21) in Oyo State Nigeria and finally characterize the genotype of the enteroviruses by phylogenetic analysis thereby establishing the recovery Echovirus 21 (E-21) of species B in a male child in Oyo State.

Keywords : detection and identification, non-culturable enteroviruses, Faecal sample, Acute Flaccid paralysis, Phylogenetic analysis

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CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Global Polio Eradication Initiative (GPEI), has a network of about 150 laboratories across the globe (Global Polio Laboratory Network (GPLN)) that isolate non-polio enteroviruses (NPEVs) as a by-product of poliovirus surveillance. GPEI emerged as a result of the World Health Assembly's (WHA) resolution in the year 1988 to eradicate poliomyelitis by the year 2000 and were able to achieve a decrease of cases of poliomyelitis from 350000 in 1988 to 416 in the year 2013 (WHO, 1988; Kew *et al*., 2003). With this mandate, wild Polio Virus was eliminated all over the world except from countries like Nigeria, Afghanistan and Parkistan (Kew *et al*., 2003). Oral poliovirus vaccine (OPV), a live attenuated vaccine containing Sabin polioviruses was one of the primary instruments used by the GPEI to achieve this purpose.

The epidemiological pattern of enteroviruses differ by geographical region and climate, but the occurrence of infection is higher in the summer and autumn months in temperate climates while remaining prevalent year-round in the tropical climates. Non-Polio enteroviruses are very common and distributed across the globe and go unnoticed, these viruses are also associated with occasional outbreaks in which a larger number of patients develop clinical disease, sometimes with fatal consequences (Noyce*et al*., 2011). A person gets infected with non-polio enterovirus by having close contact with an infected person, touching objects or surfaces that have the virus on them, touching mouth, nose or eyes. The virus may be excreted in the stool for many weeks (NCIRD, 2014).

Enteroviruses (EVs), being a group of more than 250 naked icosahedral virus serotypes, have a diameter of 28–30 nm, are categorized as members of the family Picornaviridae, genus

Enterovirus and order Picornavirales. Within the genus are 12 species and these have been identified with human infection and disease. These include (enterovirus A–D (EV-A, EV-B, EV-C, and EV-D)) and human rhinovirus (HRV) A-C (HRv-A, HRv-B, HRv-C)) (http://www.picornaviridae, 2016). Within the naked (non-enveloped) icosahedral capsid of an enterovirus is a positive-sense, protein-linked, single-stranded, approximately 7.5 kb RNA genome, of a single open reading frame (ORF). The ORF is flanked on both sides (5^1 and 3^1 ends) by untranslated regions (UTRs) and translated into an approximately 250-kDa polyprotein. This polyprotein is auto-catalytically cleaved into P1, P2 and P3 polyproteins, which are further cleaved into VP1–VP4 (VPI, VP2, VP3, VP4), 2A–2C (2A, 2B, 2C) and 3A–3D (3A, 3B, 3C, 3D), respectively (http://www.picornaviridae 2016).

The capsid is formed as VP4 is buried (submerged) within the virion and VP1, VP2 and VP3 are exposed on the virion outer surface. That is VPI – VP4 forms the capsid. The other seven polyproteins (2A–2C and 3A–3D), are nonstructural proteins and are crucial in enterovirus replication. Enteroviruses have been associated with a host of clinical conditions. The replication of enterovirus begins right in the gastrointestinal or respiratory tract and once the virus is present in the blood stream, infection may affect various tissues and organs, causing a variety of diseases (Tassin *et al*., 2013)**.**

Enteroviruses are associated with specific syndromes for example, the viruses that are within the *Human enterovirus* speciesA, most of the time cause hand-foot-mouth disease (rash especially on the soles and palms with vesicular eruption and inflammation of the mouth). While those within the *Human enterovirus B species,* cause meningitis or myopericarditis. Besides the association of different enterovirus types with the same clinical manifestation, the same enterovirus type has also been known with different clinical manifestations.

In addition, because more than 90% of enterovirus infections are asymptomatic**,** most of the infections with clinical manifestation represent less than 10% of enterovirus infections. (Nathanson *et al*.,2010). The majority of infections are asymptomatic or mild in nature, the most common effect usually is a non-specific illness, with fever. Other manifestations include exanthems (rashes), herpangina (vesicular eruption and inflammation of the throat), pleurodynia, gastroenteritis, acute respiratory disease, pancreatitis, hepatitis, type 1 diabetes, conjunctivitis, aseptic meningitis, encephalitis (inflammation of the brain), myopericarditis (inflammation of the heart tissue), upper and lower respiratory tract diseases and occasionally, paralysis or myelitis (Tapparel*et al*.,2013).

Moreover, anyone can get infected with non-polio enteroviruses but young children (infants) are most susceptible to infection. Infants and people with impaired immune system have a greater chance of having complications and infection of the heart or brain or paralysis. In less developed areas, children may become infected during early infancy while in more socio-economically advanced areas, first infection may not occur until adolescence (NCIRD, 2014). These groups are mostly infected because they do not have immunity. For instance, the Centre for Disease Control (CDC) reported high increase of children across the United States who developed Neurologic illness called Acute Flaccid Myelitis in 2014 and 2015. Males most of the time develop clinically-recognizable diseases than females (NCIRD, 2014).

Besides the clinical manifestation, another dimension to the enterovirus diversity landscape is in

their receptor usage. The prevailing paradigm is that a particular enterovirus serotype uses one or a defined set of receptors (and co-receptors). In human *enterovirus species* A, two receptors SCARB2 and PSGL1 are used. Studies have now recently shown that CD150 is not the only cell surface receptor for measles virus (Tatsuo*et al*., 2000)**.** When being transferred

from lymphocytes to epithelial cells, measles viruses use poliovirus receptor-like 4 (PVRL4) as their receptor to enter into epithelial cellsof the host (Noyce*et al.,*2011).

In similar light, CV- A20 strains exist and can independently use either Intercellular Adhesion Molecule 1(ICAM 1) or another yet to described cell surface molecule as the receptor (Arita*et al*.,2013).

1.2 STATEMENT OF RESEARCH PROBLEM

Over the years, there has been under-reporting of non-cultural enteroviruses in Nigeria as a whole most especially Southwestern in Nigeria. Despite increasing number of cases of Acute Flaccid Paralysis detection of enterovirus in cell culture is low. It has been revealed that lack of unique cell line for all enteroviruses accounts for this hence the study**.**

1.3 RESEARCH JUSTIFICATION

Despite the declaration of WHO that Nigeria has been removed from the list of Polio endemic countries, Acute Flaccid Paralysis (AFP) caused by enteroviruses remain an important clinical presentation in the country. Most isolates recovered from the Nigeria vaccine derived poliovirus serotype 2 (VDPV2) outbreak were recombinant with nonstructural region of Non-Poliovirus enterovirus Species C (NPESC) origin. Most human Enterovirus (hEV) infections have no symptoms and consequently go unnoticed. The symptomatic infections represent approximately 1 in 100 to 1 in 1000 infections depending on the serotype being considered (Nathanson and Kew, 2010).

Reports from previous works showed that enteroviruses isolated using RD cell line were usually not the complete picture. Most of the time, other enteroviruses are present in the sample that will not grow on the Rhabdomyosarcoma (RD) cell line. Even when a clinical sample is negative for enteroviruses by the pan-enterovirus VP1 screen based on primers (Nix *et al*., 2006) usually, the species-specific screen still detects, sometimes different

serotypes in the same sample. An enterovirus serotype that was not detected in the clinical specimen even after repeated screening will show up after cell culture (http://www.picornaviridae.com, 2016).

Furthermore, studies that documented the isolation of NPESC members made use of isolation protocols slightly different from the R-L protocol. Such studies included Hep-2 cell surface expression of ICAM-1 and this has greatly improved the recovery of NPESC members, more importantly the coxsackievirus (Huang *et al*., 2000). Several studiescarried out on enteroviruses in countries like Pakistan, Afghanistan and Nigeria have immensely contributed to knowledge by throwing more light to the importanceenteroviruses which is the etiological agent of AFP. Further work and effort is required to identify the Non-Polio enterovirus species, to resolve members of different species and, more specifically, members of the same species present in the same sample. Molecular methods have not been routinely used to detect and characterize non-culturable enteroviruses in feacal samples from patients with Acute Flaccid Paralysis, therefore the study focused on detection and identification of non-culturable species of enteroviruses using a negative feacal sample of poliomyelitis which were collected from patients with Acute Flaccid Paralysis(Arita*et al*., 2005).

1.4 OBJECTIVES OF THE STUDY

The general objective of the study is to determine the presence and prevalence of non-culturable Species A, B and C enteroviruses in the feacal samples collected from patients with Acute Flaccid Paralysis in the National Polio Laboratory in Ibadan Oyo State Nigeria.

The specific objectives of this study are:

- To detect and identify enteroviruses species in patients samples with Acute Flaccid Paralysis.
- To characterize the genotype of the enteroviruses by phylogenetic analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF ENTEROVIRUSES

Enteroviruses (EVs) are human pathogens that are very important and are associated with various clinical syndromes of which their infections are common in humans across the globe. Till date, EV infections are of great importance and remain a vital public health problem. Enteroviruses belong to the family PICORNAVIRIDAE(Landsteiner *et al*., 1908). This family comprises five genera this include, rhinoviruses, hepatoviruses, cardioviruses, aphthoviruses and enteroviruses (Blomqvist and Roivainen, 2016).They cause disorders with various clinical manifestations, which include cutaneous, visceral, and neurological diseases. The study of Enteroviruses began as a result of the dreaded disease poliomyelitis. This was revealed in 1908 when enteroviruses was shown to be a "filterable agent". Afterword the concept of poliovirus (PV) started taking form.

Consequent to its isolation in tissue culture and the demonstration of its serological types (Kessel*et al*., 1949). Poliovirus started off the field of enterovirology. This occured because, in addition to polioviruses, other enteric viruses were found to be present in the faeces of children that have "paralytic disease" indistinguishable from poliomyelitis.These other viruses include coxsackievirus A (CV-A) and B (CV-B), echoviruses (Es) and the numbered enteroviruses. These viruses were isolated and identified using a combination of histopathology in newborn mice, cytopathic effect (CPE) in cell culture and serology (Dalldorf, 1949; Melnik *et al*.,1950).

For many years polioviruses were the most important Enteroviruses, since they caused great outbreaks of paralytic disease. Though known and dreaded for its paralysis-causing ability.

The concept of poliovirus (PV) started taking shape after its isolation in tissue culture and the demonstration of its serological types (Kessel *et al*., 1949).

2.1.1 New Emergence of EV-D68 and other Respiratory EVS

In the year 1962 in California, USA, EV-D68 a member of the small EV-D species was first isolated in respiratory samples of 4 children that have respiratory diseases (Schieble *et al*., 1967). Enterovirus D has the characteristics of both RV and EV, such as acid lability and optimal temperature of 33^0C (Oberste *et al*., 2004). Being isolated from respiratory samples, many strains of EV-D68 were classified initially into RV genus as RV87. Later, studies based on genetics and antigenicity revealed that they were similar to EV-D68 strains. Finally, all RV87 strains were reclassified as EV-D68 type (Blomqvist *et al*., 2002) which was not common until year 2000. Some cases of EV-68 were continually reported in various parts of the world in the last decade and were accounted to mild and chronic respiratory illness (Imamura *et al*., 2011; Tokarz *et al*., 2011; Meijer *et al*., 2012). In 2014, there was a serious outbreak of EV-68 in the USA during autumn with unprecedented levels of circulation across the nation most especially, in the pediatric population. During this time, a total number of 1153 individuals in 49 states and the District of Columbia tested positive for EV-68, very common in children, some with report of wheezing or asthma (CDC, 2014; CDC, 2015).

The rapid emergence of this virus (EV-68) over the years was first believed to be as a result of new methods of detection and to former misidentification as a RV leading to an underestimated prevalence test based on retrospective findings which affirmed the increment of prevalence (Ikeda *et al*., 2012, 2016). Recently, the phylogenetic analysis revealed an increased diversity in VP1 sequences of the recently discovered EV-68 strains. These discovered strains have different genetic lineages that are obviously different from the prototype strains (Khetsuriani *et al*., 2006;Kreuter *et al*., 2011; Langereis *et al*., 2014). Aside

respiratory tropism of EV-68, its infections have also been highly associated with neurologic disease and occurrence of acute flaccid paralysis (Khan, 2015; Lang *et al*., 2014; Pfeiffer *et al*., 2014, 2015) hence, reflecting the link of Viruses.

EV-D68 makes use of Sialic acids (SA) as a receptor binding molecule, this is the same with many other viruses that have great affinity for -2-6-linked SA more than -2-3-linked SA. The sialytated glycans are found on the other cell membranes of human respiratory track (Imamura *et al*., 2014). Apart from this, EV-70 and coxsackievirus A24 variant (CVA24v, member of EV-C) (Zocher *et al*., 2014) also have affinity for sialic acid and are responsible for symptoms in the upper respiratory tract and acute hemorrhagic conjunctivitis. Furthermore, they are the causative agent of neurological impairment such as acute flaccid paralysis, and are considered to have a pandemic potential. These 3 viruses (EV-67, EV-68 and CV A24v) have different pathogenesis but use the same receptor and this suggests the same mechanism that needs to be investigated the more (Nilsson *et al*., 2008).

EV-C104, EV-C105, EV-C109 , EV-C117 , and EV-C118 are the other non-RV EVs that were discovered to emerge from species C as these viruses are widely distributed across the globe they cause diseases that vary in their severity beginning without symptoms which could either be mild respiratory infections to complicated ones like pneumonia (Tokarz *et al*., 2013). Besides, another EV-C, coxsackievirus-A21 is said is to cause mild respiratory illness (Xiang *et al*., 2012) and it uses ICAM-1 as its receptor which is the main receptor used by the group of RV (Xiao *et al*., 2005).

2.1.2 ORIGIN AND CLASSIFICATION OF ENTEROVIRUS

Enteroviruses work began in 1908 when it was revealed that it is a filterable agent and has an outcome or manifestation of poliomyelitis disease which has ability to cause paralysis (Landsteiner *et al*., 1908). Then the concept of poliovirus (PV) began to take form. Before enterovirus tissue sub-culturing and application of its serological types, poliovirus made the

field of enterovirology to emerge (Kessel *et al*., 1949). This occurred because aside from polioviruses found in the feces of children with paralysis, other enteroviruses like coxsackievirus A (CV-A) and B (CV-B), echoviruses (Es) and the numbered enteroviruses were found and these are indistinguishable from poliomyelitis. Isolation and identification of these viruses was carried out using serology and cytopathic effect (CPE) in cell culture and histopathology in newborne mice (Melnik, *et al*., 1950).

Further studies in experimental animals revealed that CAVs affect skeletal and heart muscle, while CBVs do replicate in a wide range of tissues including the central nervous system, liver, exocrine pancreas, brown fat and striated muscle. The adoption of culture techniques into virus laboratories enabled the isolation of viruses which did not replicate in experimental animals. Echoviruses however were isolated from stool samples where polioviruses and coxsackieviruses are usually also found, but did not possess the pathogenic properties of these subgroups in experimental animals. The name echovirus was chosen as a result of its association with human disease which was at the time of discovery unknown ECHO enteric, cytopathogenetic, human, associated disease (Tao *et al*., 2014).

The Committee of ECHO Viruses postulated in 1955 that whenever an echovirus was affirmed as the etiological agent of a clinically specific disease, it would be removed from the group. However, it soon became evident that individual serotypes cannot be directly associated with individual illness but with a wide range of clinical manifestation. Wherefore, echoviruses are still classified together and they form the largest enterovirus subgroup which consists of several serotypes (Oyero *et al*., 2010).

Early in the year 1950, monkey kidney cell cultures was used for the growth of polioviruses and this revealed the presence of latent simian viruses. Kalter *et al*.,1980furthered the investigations that revealed that some of these polioviruses possess properties of

enteroviruses and consequently 18 serotypes were described. Investigations of viruses that were infecting domesticated animals in the late 1950s revealed the presence of enteroviruses in both pigs (*Sus scrofa*) and cattle (*Bos taurus*). Afterward, about 13 porcine enterovirus (PEV) serotypes (Honda *et al*., 1990; Auerbach *et al*., 1994) and bovine enterovirus (BEV) serotypes (Urakawa and Shingu, 1987) were described. Enteroviruses isolated from African buffalo (*Syncerus caffer*), water buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*), deer (*Cervus nippon*) and impala (*Aepyceros melampus*) have been shown to be related to BEV-1 (Urakawa and Shingu, 1987).

Implicit in the history of enterovirus classification is the existence of antigenically distinct viruses which can be said to constitute serotypes. An operational definition for a serotype includes the notion that a strain represents a new serotype if it is not neutralized to a significant extent by antisera to previously characterized viruses and if it is unable to induce significant levels of neutralizing antibodies to these viruses (Committee on Enteroviruses, 1962). Unfortunately, in practice, some virus serotypes tend to include strains occurring as an antigenic continuum which multiplex the use of reference antisera. This lead to difficulty in serotyping some isolates due to poor identification by reference antisera and the observation of extensive cross reactivity between some serotypes, identification of enterovirus was primarily based on serotypic differentiation, and these difficulties were significant for epidemiology and diagnosis. On the other hand, distinction between the three serotypes of poliovirus is clear, and inclusion of one representative of each serotype is necessary and sufficient in poliovirus vaccines.

Few years later, when enterovirology began, neutralization assay was employed for isolation and identification of isolate and this prevented the manifestation of cytopathic effect of enteroviruses (Minor *et al*., 1983; Emini *et al*.; 1983; Evans *et al*., 1983., Chow, 1985) in cell culture and was seen altered after antibodies elicited against PVI protein had neutralizing

ability. Consequently, the binding sites of these antibodies which have the neutralization power were localized to certain epitopes in the protein product of the VP1 gene.

As a result of Global Polio Eradication Initiative (GPEI's) activities, annual cases of poliomyelitis decreased from 350,000 in 1988 to 416 in 2013 (www.polioeradication.org) and poliomyelitis has been eliminated globally except in countries like; Nigeria, Pakistan and Afghanistan (WHO, 2012). The method used to accomplish this goal include active poliovirus surveillance programme in addition with massive vaccination campaigns World Health Organization (2004). The inactivated poliovirus vaccine (IPV) and oral poliovirus vaccine (OPV) were the workhorses used during the vaccination campaigns. The OPV has been the vaccine of choice in developing countries because, they are not expensive and hence more affordable, as opposed to IPV confers mucosal immunity to vaccines, thereby reducing the number of susceptible individuals that can participate in poliovirus transmission (Nathanson, 2010).

Furthermore, various studies have confirmed silent circulation of enteroviruses in the environment even though in the absence of associated clinical conditions in the community. Most hEV (human enteroviruses) infections are asymptomatic, and go unnoticed. The symptomatic infection could be represented approximately 1 in 100 to 1 in 1000 infections and this will depend on the serotype that is being considered (Nathanson and Kew, 2010). As a result of this gap, symptomatic hEV infection surveillance is considered inadequate when knowledge of hEV circulation in any population is requested. Thus, environmental surveillance (ES) approach has been one of the tools used in the study of enteroviruses as it is seen to be more dependable to generate adequate information on hEV circulation in a population. Environmental surveillance for hEVs is a method for rapidly documenting circulating strains of hEV in any population by sampling sewage or sewage-contaminated water (SCW) within the community in a particular geographical area.

In 1988, great effort was made by the World Health Assembly to resolved and eradicate poliomyelitis by the year 2000 (WHO, 1988) and there was an establishment of Global Polio Eradication Initiative (GPEI) with this mandate. Prior to molecular identification, it was difficult to type these non-polio enteroviruses (NPEVs) hence, some types were not known because the access-able panel of antisera could mainly recognize just 40 of the first 66 enteroviruses that were characterized (Oberste *et al*., 2000). As a result of this, there was an array of unidentified enteroviruses which are referred to as the 'untypables'. There has been a great improvement with the help of molecular identification and many of the previous untypable enterovirus isolates have been discovered and identified.

Hence, the use of ES has been recommended as a central part of the Global Polio Eradication Initiative (GPEI) in other to monitor the presence of poliovirus in high-risk environment most especially when gaps are suspected in the Acute flaccid paralysis (AFP) sentinel surveillance system (WHO, 2003). Aside poliovirus, which is the target pathogen of AFP, isolation, and identification of non-polio enteroviruses (NPEVs) was also done by neutralization test using pools of antisera which can only identify limited number of NPEVs (Apostol *et al*., 2012).

More recently, non-polio enteroviruses became the center of many studies, where recombination was recognized as a frequent event and was linked with the appearance of new enterovirus origins and types. The accumulation of both inter- and intra-typic recombination events could also show light on the series of successive emergences and disappearances of specific enterovirus types that could in turn explain the epidemic profile of spreading of several types (Kyriakopoulou *et al*., 2015).

2.2 CLASSIFICATION OF ENTEROVIRUSES

The human enteroviruses (EV) family consist of up to 100 immunologically and genetically distinct types. They are Polioviruses, coxsackieviruses A and B, echoviruses, and the more

recently characterized 43 EV types. Constant recombinations and mutations observed in enteroviruses have been recognized as the major mechanisms for the resulted high rate of evolution, thus enhancing them to rapidly respond and adapt to new environmental factors. The first signs of genetic exchanges between enteroviruses came from polioviruses some years back, and ever since the recombination has been observed along with mutations, as the major cause for reversion of vaccine strains to neurovirulence (Kyriakopoulou *et al*., 2015).

Before the use of molecular method for the purpose of identification, enteroviruses were classified based on historical lines as Polio viruses (PVs), Coxsackievirus A (CV-A) and Coxsackievirus B (CV-B), Es and numbered EVs. However, identification through the molecular method and phylogenetic process or analysis revealed that human enteroviruses could mainly be classified into four (4) specific species (EVA, EVB, EVC and EVD). This further proceeded in the incorporation of poliovirus into the EV-C and the reclassification of the existing CV-A15, CV-A18, HRV-87 and the swine vesicular disease virus (SVDV to CV-A11, CV-A13, EV-D68 and CV-B5 respectively (Brown *et al*., 2013).

Enterovirus species A (EV-A) contain 25 serotypes which is made up of CV-As and a few numbered enteroviruses. In Enterovirus virus species B (EV-B). There are 63 serotypes found containing CV-A and CV-B, the echovirus and some numbered enteroviruses. Enterovirus species C (EV-C) consists of 23 serotypes containing the remaining CV-As, the three poliovirus (PV1, PV2, and PV3) serotypes and some numbered enteroviruses. Enterovirus D (EV-D) contain just five serotypes which consist only of the numbered enteroviruses (http://www.picornaviridae.com 2016).

According to the International Committee on Taxonomy of Viruses (ICTV), enteroviruses (EVs) belong to the Enterovirus genus of the family Picornaviridae and have been classified into twelve main species of which only five species infect animals (EVE–J), the remaining

seven species are known to infect humans. Species A–D and Rhinovirus species A–C are specifically the only species in the genus that have been reported to infect humans. Within each species are several serotypes and the genus, as a whole, comprises of more than 200 serotypes such as polioviruses (PV), coxsackieviruses A and B (CV-A and -B), echoviruses, numbered EVs, and human rhinovirus (HRVs), among them including several important human pathogens such as PV, CV-A16, CV-B3, EV-A71, EV-D68, and HRV (http:// www picornaviridae, 2015).

2.3 ENTEROVIRUSES (EV) GENOME AND VIRAL PROTEINS (VP)

The enterovirus virion is a non-envelop, icosahedral symmetry with diameter of 27 to 30nm (27-30nm). The enterovirus genome is a positive-sense single-stranded RNA molecule having 7000–8000 nucleotides comprising a single open reading frame (ORF) with a 5^1-untranslated region (5^1-UTR) and a 3^1-UTR. The 5^1-UTR contains an internal ribosomal entry site (IRES) which is useful for the binding of the 40S ribosomal subunit to initiate cap-independent translation, while the 3^1-untranslated region (UTR) contains a pseudoknot and a poly (A) tail. The open reading frame (ORF) encodes a large polyprotein precursor, which comprises of P1, P2, and P3 regions which are further cleaved to make 11 proteins. In enterovirus (EV) infected cells, this polyprotein precursor is initially cleaved between P1 and P2 by viral 2A proteinase, while the P2-P3 point is cleaved by 3C proteinase (Racaniello, 2013).

Finally, this precursor is processed into mature viral proteins, including four structural proteins that give rise to the four viral capsid (VP1, VP2, VP3, VP4) and seven non-structural proteins (2A–2C and 3A–3D)(Figure 2.1). The 2A and 3C proteinase in particular have profound effects on host cells by modulating proteins related with translation, apoptosis, natural or innate immunity, RNA processing and polyadenylation (Weng *et al*., 2009; Lei *et*

al., 2010; Wang *et al*., 2013). A link has been affirmed between VP1 nucleotide sequences and distinct serotype(Oberste *et al*., 1999;Oberste *et al*., 2005).

Moreover, VP1 nucleotide sequences have become a tool used in typing enterovirus isolates into serotypes (Oberste *et al*., 1999; Oyero *et al*., 2014; Adeniji and Faleye, 2014). Furthermore, studies have affirmed that all regions of the enterovirus genome except the 5^{I}-UTR can be used for typing enteroviruses into various species (Oberste and Pallansch, 2005;Oberste *et al*., 2006).

2.3.1 The Value of VP1 in Enterovirus Identification

At the beginning of enterovirology, identification of isolate was done by neutralization assays, and this prevented the development of the enterovirus-specific cytopathic effect in cell culture.

Later, it became clear that antibodies elicited against the VP1 protein had neutralizing activity (Chow*et al*.,1982; Wychowski*et al*.,1983)**.** Consequently, the binding sites of these neutralizing antibodies were localized (Emini *et al*., 1983; Evans *et al*., 1983; Minor *et al*., 1983; Chow*et al*., 1985) to specific epitopes in the protein product of the VP1 gene.

TABLE 2.1 Classification of Enteroviruses Enterovirus Species and Type

Enterovirus Species	Type
Human enterovirus A	
Human coxsackievirus	A2–8, 10, 12, 14, 16
Human enterovirus	71, 76, 89–92, 114, 119–121
Human enterovirus B	

Human coxsackievirus	A-9, B1-6
Human echovirus	1–9, 11–21, 24–27, 29–33
Human enterovirus	69, 73–75, 77–88, 93, 97–101, 106, 107, 110–113.
Human enterovirus C	
Human coxsackievirus	A1, 11, 13, 17, 19–22, 24.
Human poliovirus	1–3
Human enterovirus	95, 96, 99, 102, 104, 105, 109, 116–118.
Human enterovirus D	
Human enterovirus	68, 70, 94, 111, 120.
Rhinovirus A	
Human rhinovirus A	1, 2, 7–13, 15, 16, 18–25, 28–34, 36, 38–41, 43, 45–47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94, 96, 100–109.
Rhinovirus B	
Human rhinovirus B	3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91–93, 97, 99, 100–106
Rhinovirus C	
Human rhinovirus C	1-55

(http://www.picornaviridae, 2015).

Enterovirus genome organization

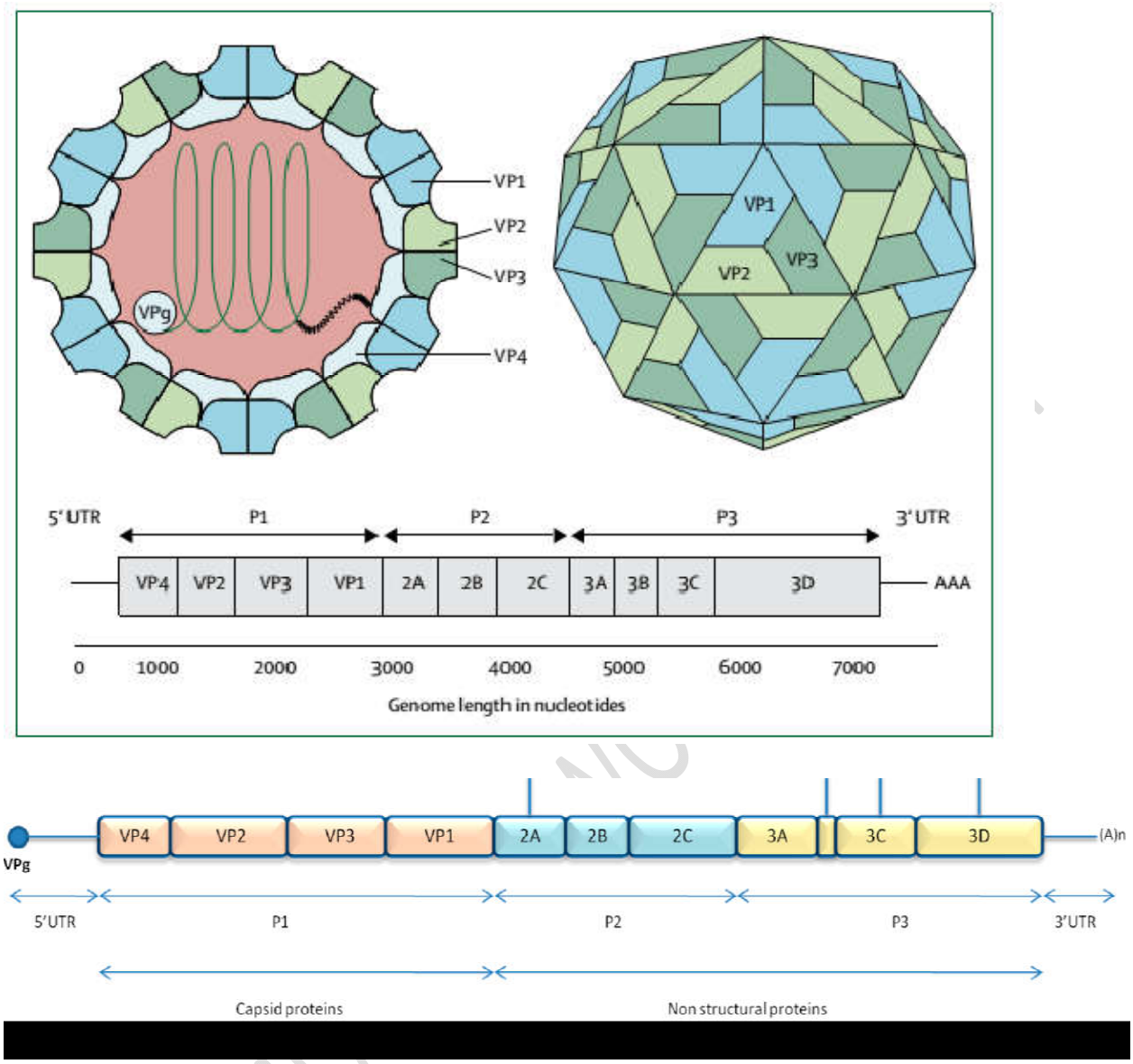


FIGURE 2.1 **Viral and genome structure of enterovirus(Brown and Pallansch, 1995).**

2.3.2 Serotypes and Genotypes of Enteroviruses

The confluence of four things encouraged enterovirus molecular identification, these include the existence of previously neutralized and identified pure cultures of enterovirus reference strains, discovery that the VP1 gene was greatly responsible for defining or identifying enterovirus serotype, the mainstreaming of primer synthesis and polymerase chain reaction (PCR) finally the automation of Sanger sequencing.

With all the above in place, Oberste *et al*. (1999) showed the relationship between VP1 sequence data and enterovirus serotypes (Casas*et al*.,2001; Caro*et al*.,2001; Norder*et al*.,2001; Oberste*et al*., 2001;Oberste*et al*., 2003; Thoelen*et al*.,2003; Blomqvist*et al*.,2008). This was independently affirmed and asserted by various investigators applying both previously-neutralized pure cultures and field strains.

Moreover, enterovirus identification became synonymous with VP1 amplification and sequencing (molecular identification). Before molecular identification, enteroviruses were recognized and classified as PVs, CV-A and CV-B, Es and numbered EVs based on history. However, the consequent phylogenetic analysis and molecular identification revealed that "human" enteroviruses could be unequivocally classified into four (4) different species (EV-A–EV-D). This in progression resulted in the incorporation of polioviruses into the EV-C and the reclassification of former CV-A15, CV-A18, HRV-87 and Swine Vesicular Disease virus (SVDV) to CV-A11, CV-A13, EV-D68 and CV-B5, respectively (Brown *et al*., 2003).
EV-A contains 25 serotypes made up of some CV-As and some numbered enteroviruses. EV-B contains 63 serotypes consisting of CV-A and CV-B, the echoviruses and some numbered enteroviruses. EV-C contained 23 serotypes consisting of the remaining CV-As, the three poliovirus serotypes and some numbered enteroviruses. EV-D contains five serotypes consisting only of numbered enteroviruses (http://www.picornaviridae.com. 2016).

2.3.3 Poliovirus versus Non-Polio Enterovirus

There has been several reports on poliovirus as human enterovirus contains over 100 types that are classified into 4 species, EV-A to EV-D. Poliovirus is known to be associated with Acute Flaccid Paralysis (poliomyelitis). This virus has tropism for epithelial cells of the alimentary tract and the central nervous system. It causes spinal and bulbar poliomyelitis currently. Usually, paralysis is irreversible and all the three polioviruses PV1, PV2, PV3 can give rise to paralysis. Following the experience in the year 1988 when World Health Organization (WHO) recommended a standard approach for polio surveillance in investigating, discovery and detecting of acute flaccid paralysis in cases that include standardized virological analysis of feacal samples of patient and sometimes those from contacts, China, in 1994 were able to discover a lot of non-polio enteroviruses (NPEVs) asides PVs.

Furthermore, other studies affirmed the existence of non-polio enterovirus species C, which is about 20 apart from the poiloviruses (http://www.picornaviridae.com 2016). It has also been reported that there are three serological types of poliovirus (PV1, PV2, PV3) attenuated versions that were developed as oral polio vaccines (OPV) and are used for immunization campaigns. The vaccine virus can revert to wild-type virulence and transmissibility. The genomic nature of such isolates, termed circulating vaccine-derived polioviruses (cVDPVs), show them to be mostly recombinant with OPV/NPEV-C structural and non-structural region sides or region respectively (Combelas *et al*., 2011; Burns *et al*., 2013).In addition, in Nigeria 403 cases of cVDPV 2 were confirmed from 2005 to 2011 and seven (7) out of all were OPV2/NPEV-C recombinants and were resolved into (23) independent occurrences most of which took place in Northern part of Nigeria.

The global eradication of polio has been set by 2018 with strategic objectives specific to the post-eradication era. These objective have special implications for countries that are already

polio free-free. Part of the objectives is to detect and interrupt transmission of wild poliovirus by 2014 and new outbreaks due to cVDPV within 120 days of confirmation of the index case. Improvement of surveillance and immunization campaigns, laboratory containment and destruction of polioviruses and ensuring rapid outbreak responses are the key activities required to maintain our polio-free status.

2.3.4 Cases of Non-Polio Enteroviruses Isolation in other Countries

The Acute Flaccid Paralysis surveillance was conducted in all Shandong Province 138 countries all together and this involved over 600 sentinel hospitals. Patients less than 15 years of age were hospitalized of which male to female ratio was 1:9:1 for all cases right from year 1990 to 2013. The data for the surveillance in the years 1988 and 1989 were unavailable but NPEV strains were isolated during the same period. Between 1990 and 2013, a total of 9263 cases of AFP were reported (Zhang *et al.,* 2011). Isolation of EV was performed on all stool specimens and 788 NPEV strains were isolated from the stool specimens of these cases. In addition, specimens from 1059 contacts of AFP were collected during the same period of which 170 NPEVs were isolated.

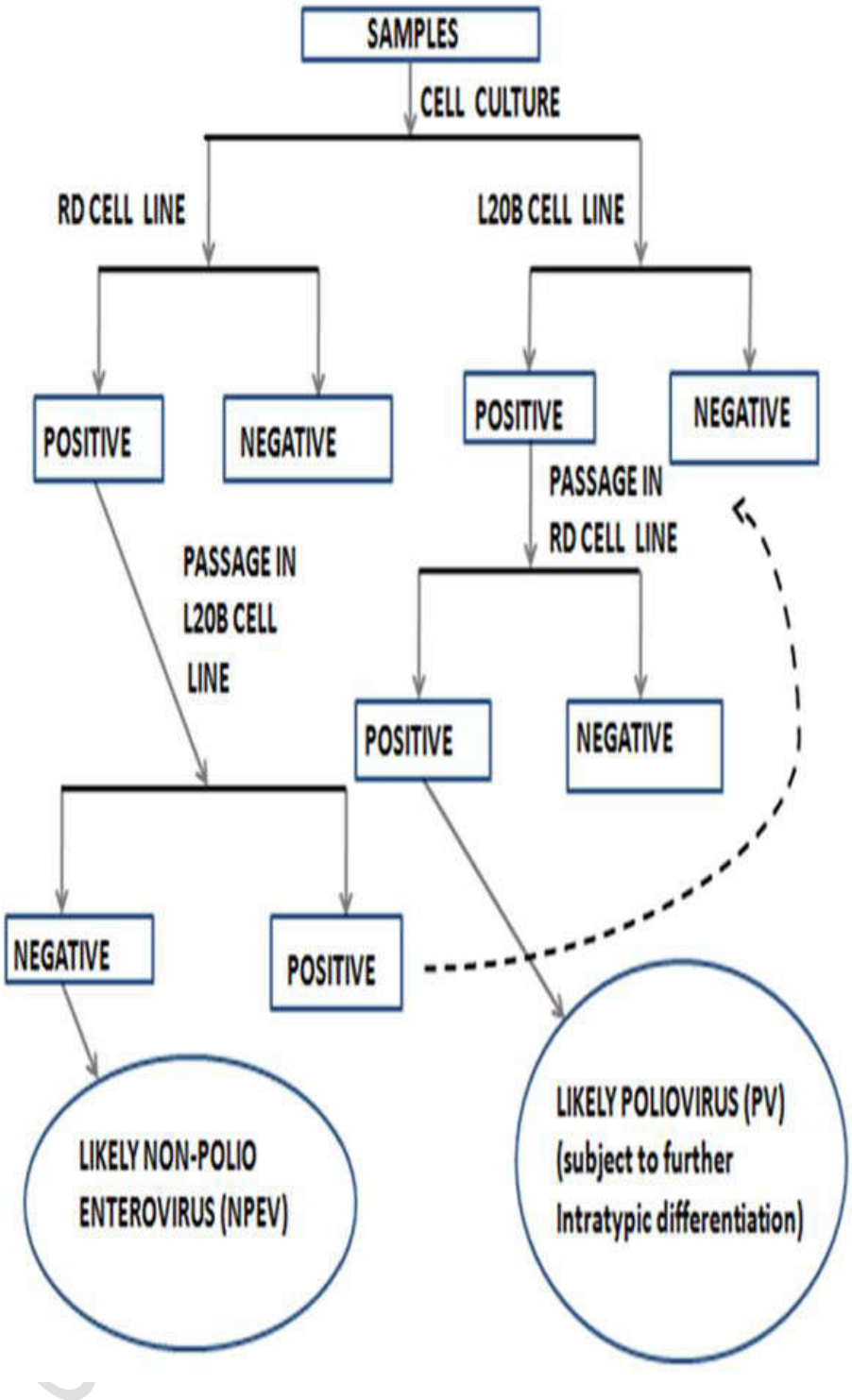


FIGURE 2.2 Poliovirus (PV) isolation algorithm as recommended by the World Health Organization (2003, 2004).

In 1990s, AFP surveillance in China was greatly increased but earlier reports before 1995 was not active and this resulted into low level of NPEV isolation before 2007. AFP reports got increased in summer and autumn months with a peak in the month of July with 1100 (11.9%) of 9255 reports with known month between 1990 and 2013. However, a more prominent seasonality of NPEV detection from AFP cases was recorded within June and October accounting for 79.3% (629 of 792) of isolation with known month of specimen collection (Tao *et al*., 2012).

2.4.0 LIFE CYCLE AND REPLICATION OF ENTEROVIRUSES

The entry of the virus in to the cell is determined by the cell surface molecule used as receptor, likewise as putative attachment receptors which is not the same in different enteroviruses. Depending on the type of virus and the type of the host cell, viral uptake can easily be mediated either by clathrin-dependent or-independent endocytosis or through macropinocytosis (Fuchs *et al*., 2010). Human beings are the only known natural hosts of human enteroviruses. The replication cycle of all species of enteroviruses is similar.Virions will then be faced by the drastic change in environment caused by the drop in PH or by receptor binding which give rise to uncovering of the hydrophobic domains, and this leads to pore-mediated release of the genome in the cytoplasm.

Afterward, a cap-independentinternal ribosomal entry site (IRES) mediated polyprotein synthesis is then mediated through the host cell ribosomal machinery, the viral polyprotein precursor acquired which is about 2000 amino acids then cleaved by the viral proteases into several viral proteins. Finally, the genome will be replicated in many folds in association with the cytoplasmic membranes (Belov *et al*., 2014).Replication of the enterovirus begins in the gastrointestinal or respiratory tract the moment the virus is present in the blood stream,

infection may affect various tissues and organs, causing a variety of diseases (Tassin *et al*., 2013).

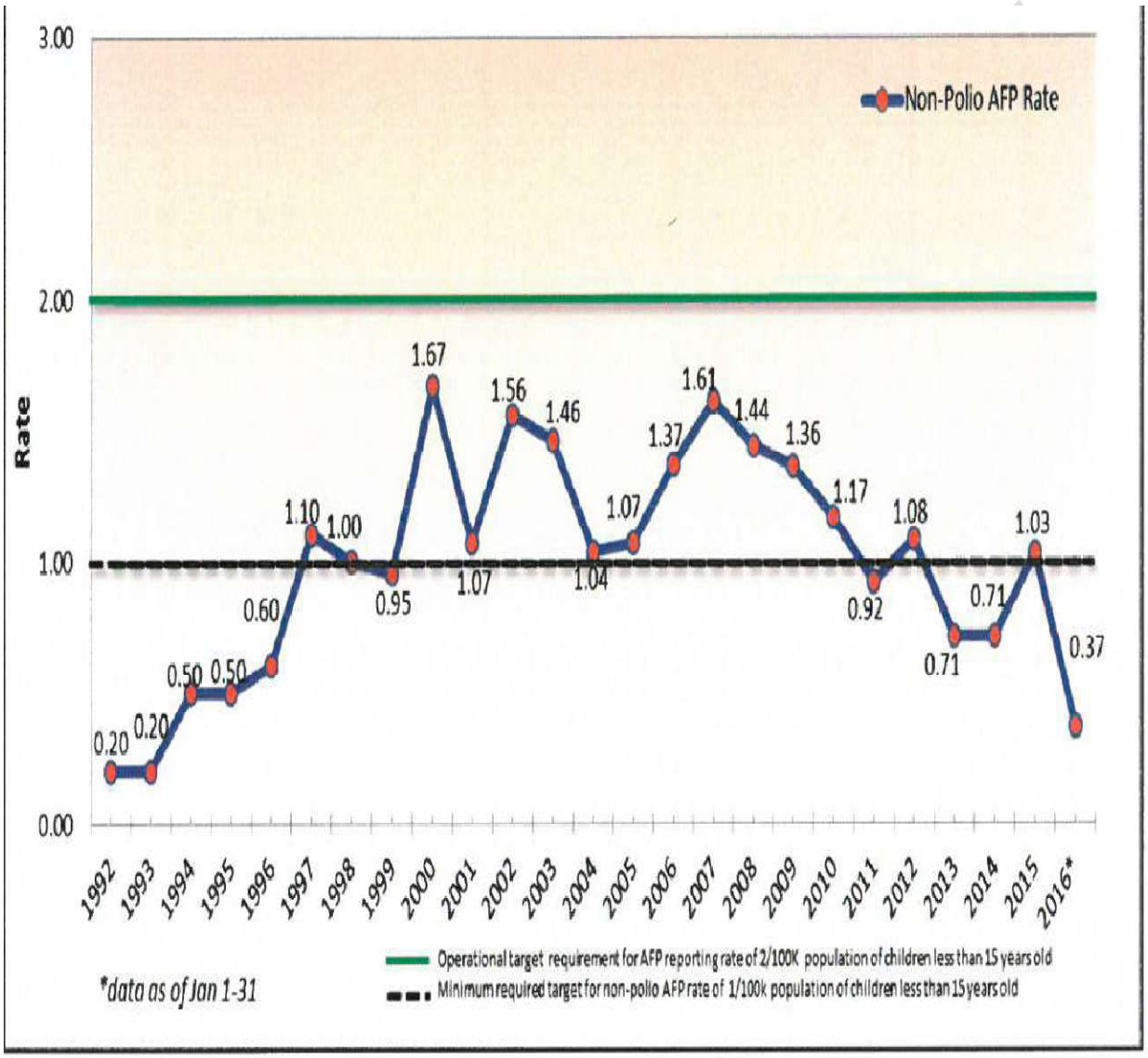


FIGURE 2.3 Trend of non-Polio AFP Rate in the Philippines 1992-2016

Enterovirus Species and Type (http:// www.picornaviridae, 2015).

It has been revealed that some enteroviruses use more than one receptor to infect a host cell. Several receptors for species A, B and C have been identified, but a ubiquitously expressed cellular receptor, scavenger receptor B2, and a functional receptor, human P-selectin glycoprotein ligand-1, found on white blood cells, are specific for EV71. Sialic-acid-linked glycan, expressed in abundance in the respiratory and gastrointestinal tracts, and dendritic-cell-specific intercellular adhesion-molecule 3-grabbing non-integrin (CD209), found exclusively in dendritic cells in lymphoid tissues, have also been identified (Yang *et al*., 2009). While some EVs are capable of utilizing multiple receptors, PV uses only one, CD155, an adhesion molecule also known as the human PV receptor (hPVR). In an in vitro blood-brain barrier (BBB) model, PV has been show to enter human brain microvascular endothelial cells (hBMECs) using hPVR in a dynamin and caveolin-dependent manner. Without any element of doubt, receptor expression on potential target cells defines the first barrier to virus entry (Yang *et al*., 2009). While the machinery of the host cellular protein synthesis is shut down by viral protease 2A, viral protein synthesis remains unaffected. An infectious virus particle is formed after the packaging of a progeny viral RNA into a virus capsid in the cytoplasm of the infected cells. Mature infectious virus particles are released when an infected cell is lysed(Tassin *et al*., 2013).

2.5 PATHOGENESIS OF ENTEROVIRUS INFECTIONS

The virus gains entry through the mouth or nose and when this happen, Enterovirus infects and replicates in the nasopharyngeal epithelium and regional lymphoid tissues, conjunctiva, intestines, mesenteric nodes, and the reticuloendothelial system.Enteroviruses can resist stomach acid and bile and are able to penetrate to the lower intestine, where they infect and

multiply in the intestinal epithelium and mesenteric lymph nodes. When viremia results; this leads to further multiplication of virus in the reticuloendothelial system. At that point, the virus can be carried by the bloodstream to target organs such as the spinal cord, brain meninges, heart, liver and skin. From the central nervous system the virus can travel via neural pathways to skeletal and heart muscles. It can be transferred by fingers and inanimate objects, such as towels and handkerchiefs to the eye, where it may replicate in the conjunctival epithelium and cornea (http:// www picornaviridae, 2009).

Individuals that are infected with Human enterovirus shed great amounts of hEV in faeces for many weeks irrespective of whether the infection is symptomatic or not (Ranta *et al*., 2001).The sensitivity of ES in detecting the circulation of different serotypes of hEVs in the absence of symptomatic infections in the community has been confirmed and documented by various studies. (El Bassioni *et al*., 2003; Sedmak *et al*., 2003; Manor *et al*., 2007;Kargar *et al*., 2009; Iwai *et al*., 2011).

Human EV infections are widespread and are the most common cause of aseptic meningitis, pericarditis, myocarditis and respiratory infections. Studies have confirmed EV infections as asymptomatic with about 50 – 80 causing clinically mild and self-limited infections. However, some species are able to cause severe and potentially fatal infections (http:// www picornaviridae, 2009).For instance, PV can invade the nervous system and cause poliomyelitis in children, the most significant disease caused by an enterovirus. CV can cause acute clinical manifestations ranging from mild febrile illness to more chronic conditions including meningo-encephalitis, pancreatitis, and fulminate sepsis in neonates (Tebruegge and Curtis, 2009). Some chronic diseases are also caused by CV, such as chronic myocarditis and type I diabetes (Whitton *et al*., 2005; Precechtelova *et al*., 2014).

2.6 IMMUNITY

2.6.1 Shut-comings of Host Protein Synthesis on the Evasion of EVs to Innate Immunity

Many species of enteroviruses tend to evade IFN responses in other to replicate or survive within the host cell. As a result, this has made the innate immune response a critical thing in controlling EV infection**.** PV infection results in the shutoff of host RNA and protein synthesis. During this process, theeukaryotic translation initiation factor 4G (eIF4G) in conjunction with the p220 subunit of the cap-binding protein complex cleaved by PV 2A proteinase (Lloyd *et al*., 1988). These revealed that PV 2A is related to diminished IFN production.

Moreover, eIF4G cleavage only partially induces translation shutoff, which implies that additional mechanisms are involved in EV-induced inhibition of translation (Kuyumcu-Martinez *et al*., 2004). Also, EV3C- mediated cleavage of poly (A)-binding protein (PABP) contribute a vital role in translation arrest.Furthermore, PV infection has the ability to hinder host cell RNA polymerase II-mediated transcription by 3C proteinase mediated cleavage of TATA-binding protein (TBP) and cyclic AMP-responsive element-consisting protein (CREB) (Weidman *et al*., 2001). These evidences demonstrated that EVs hinders the IFN signaling pathways, in some areas by the shutoff of host mRNA transcription and translation through 2A and 3C proteinase.

2.6.2 Interference by Pathogen Recognition Receptors (PRRs) Recognition

Detection of enteroviruses is done by Pathogen Recognition Receptors on cell surfaces and also in the cytosol, therefore EVs have developed mechanisms to antagonize these receptors. For instance, PV and HRV 1α infection helps the degradation of MDA5 in a proteasome and caspase-dependent manner. Moreover, degradation of this receptor is independent of 2A and

3C protease that is responsible for the inhibition of IFN production against the infection caused by enteroviruses (Barral *et al*., 2007). On the other hand, CV-B3, EVA71 and PV 2A proteinases directly cleave in a caspase-dependent manner.Apart from MDA5, RIG-I is another special or critical target for CV-B3 and EV-A71 and their infections can cause RIG-I cleavage by 3C proteinases (Feng *et al*., 2012; 2016; Wang *et al*., 2014; 2015). These demonstrated that MAD5 and RIG-I are common targets for EVs evasion against innate immune responses.

Moreover, results obtained in 2010 revealed that 3C inhibits RIG-I and the adaptor molecule MAVS instead of cleaving RIG-I directly (Lei *et al*., 2010). When there is infection by EV-A71 NLRP3 inflammasome is activated and takes up the role of protection against EV-A71 infection (Wang *et al*., 2015). As a result of this, EV-A71 infection inhibits NLRP3 inflammasome activation by cleaving NLRP3 with 2A and 3C proteinases (Wang *et al*., 2015). With the fact that TLRs has a vital role on antiviral responses against EVs, studies is yet to confirm the ability of enteroviruses to directly target TLRs to invade innate immunity.

2.6.3 Roles of Innate Immunity Evasion in EV Pathogenesis

As natural or innate immunity is the first line of defense against viral infections, viruses have to develop mechanisms to overcome or avoid innate immune responses in other to survive. Innate immunity is important for the control of the EV infection at the early stage, as studies show that EV-induced morbidity and mortality is increased in type I IFNs- or IFNAR-knockout mice (Ida-Hosonuma *et al*., 2005; Liu *et al*., 2005).

In addition, treatment with neutralizing antibody of type I IFN tends to increase viral loads and EV-A71-induced lethality while type I IFN treatment increases the survival rate of mice (Liu *et al*., 2005).These findings revealed the fact that the innate immune responses are closely associated with EV pathogenesis. However, there are a few direct evidence

demonstrating how these mechanisms to antagonize and evade innate immunity are related to EV pathogenesis. Further works is required as to gaining further insights into these mechanisms, to verify whether recombinant EV contains mutated 2A, 3C, or 2C, which are unable to antagonize the described targets, can induce stronger IFN response compared with wild type virus.

2.7 HOST DEFENSES

Interferon and virus-specific IgA, IgM, and IgG antibodies are important in the host defense. Neutralizing antibody confers serotype-specific immunity**.** Not long after infection of the respiratory or alimentary tract occur there will be increase in amounts of interferon and subsequently virus-specific IgA-antibody are detected in the saliva and the respiratory and gut secretions. Interferon hinders virus multiplication, and IgA complexes with extracellular virus. The complexing of virus by IgA not only inhibits the spread of virus to susceptible epithelial cells but also affect by decreasing the oral and fecal shedding of infectious virus (Wang *et al*., 2014 and 2015).

The first serum antibody to appear as a result of picornavirus infection is IgM, this will occur when it is about 2 weeks, afterward IgM will be overtaken by IgG. The IgG response gets to the highest point at about 2 to 3 weeks and remains at that point for a few weeks, before it begins to fall. The IgG elicited but some enterovirus infections will remain detectable for several years. This neutralizing IgG confers serotype-specific immunity (Hosonuma *et al*., 2005). Both IgG and IgM can complex with invading virus and prevent the spread of virus through the bloodstream to a target or specific organs. Virus-antibody complexes are eliminated by phagocytosis, digestion, and excretion.

2.8.CLINICAL MANIFESTATION AND TRANSMISSION

2.8.1 Clinical Manifestation

Enteroviruses are the causative agent of many diseases, including undifferentiated febrile illnesses, upper and lower respiratory tract infections, gastrointestinal disturbances, conjunctivitis, skin and mucous membrane lesions, and diseases of the central nervous system, muscles, heart, and liver. EVs have been associated with a host of clinical conditions, which include intrauterine Enterovirus transmission with fatal outcome (Tassin *et al*., 2013) encephalitis, meningitis, pleurodynia, herpangina, conjunctivitis, gastroenteritis, myopericarditis, pancreatitis, hepatitis, type 1 diabetes, hand, foot and mouth disease, upper and lower respiratory tract diseases and paralysis or myelitis (Tapparel *et al.,* 2013).

Enterovirus infections are asymptomatic, most of the infections with clinical manifestation represent less than 10% of Enterovirus infections (Nathanson and Kew, 2013).In rear cases, enteroviruses are associated with generalized neonatal infections, diabetes mellitus, pancreatitis, orchitis, and occasionally hemolytic-uremic syndrome and intrauterine infections. (Tapparel *et al*., 2013). Prevention or treatment of EV infections is greatly affected by the diversity of species that cause the similar illness. For example, HRV infection can result in respiratory diseases including the common cold as well as more serious lower respiratory infections that exacerbate asthma (Hammond *et al*.; 2015; Stone *et al*.; 2015). However, there are three species with more than 100 HRV serotypes that can give rise to the same infections. Thus, developing a single vaccine against so many serotypes is nearly impossible (Glanville *et al*., 2015).

Also, according to the study carried out in Califonia, USA in 1962 (Oberste *et al*., 2004) it was observed that similar respiratory illnesses including mild upper respiratory infections, pneumonia, and acute flaccid myelitis can also be caused by EV-D68 (Greninger *et al*.,

2015; Khan, 2015; Waghmare *et al*., 2015). Although, EV-D68 has been rarely reported in the past 40 years, it has recently caused an epidemic in 2014 in the United States (Khan, *et al*., 2015) leading to more than one-thousand (1000) cases of severe respiratory disease.

Similarly, not less than 23 EV serotypes can cause hand, foot, and mouth disease (HFMD), a common infectious disease of infants and children.For instance, the viruses withinHuman enterovirus A (Zhang *et al*., 2010; Zhang, *et al*., 2011; Tapparel *et al*., 2013; Puenpa, *et al*., 2014). Meanwhile, EV-A71 and CV-A16 are the major causative agents of this disease, these viruses can also cause other maladies (McMinn, *et al*., 2001; Chan., *et al*., 2003; Xing, *et al*., 2014). For example, it is common of EV-A71 to cause severe central nervous system diseases, such as encephalitis and aseptic meningitis (Wang *et al*., 2008; WHO, 2011), while CV-A16 is associated with causing more mild HFMD cases. Recently, CV-A6 is becoming the major causative agent in some geographical locations (CDC, 2011; CDC, 2012).

Furthermore, the neurological syndromes associated with enterovirus 71 infection include encephalitis, especially brainstem, acute flaccid paralysis (anterior myelitis), encephalomyelitis, and aseptic meningitis occur frequently with EV A-71. Again, Cerebellar ataxia is infrequent, transverse myelitis is rare while neurological and systemic manifestations brainstem encephalitis with cardiorespiratory failure is frequent. Manifestations indicative of immune-mediated mechanisms such as guillain-Barré syndrome is infrequent, Opsoclonus-myoclonus syndrome and benign intracranial hypertension is rare (McMinn, 2002).

2.8.2 Transmission of Enteroviruses

Enteroviruses are not exempted when it comes to the issue of transmission of viruses. EVs can be found in an infected person's feces (stool), eyes, nose, and mouth secretions (such as saliva, nasal mucus, or sputum), or blister fluid. An individual can easily get exposed to the

virus through maintaining a close contact, such as touching or shaking hands, with an individual that is infected, touching objects or the surfaces that contain the virus on them, and then touching an individual's eyes, nose, or mouth before cleaning or washing the hands, changing diapers of an individual that is infected and then touching the eyes, nose, or mouth before washing of the hands, drinking water that contain the virus in it (Ranta *et al*., 2001; Noyce *et al*., 2011).

Enteroviruses are transmitted mainly via the fecal-oral route. However, transmission via the respiratory route and through conjunctival fluid have also been documented (Nathanson and Kew, 2013). For example, since its discovery in 1962, the transmission of EV68 in most cases had been described to be via the respiratory route. In the United States EV68 was found in 2 of 5 children during 2012/13 cluster of polio-like disease in California (Brown, 2003).

2.9 DIAGNOSIS OF ENTEROVIRUSES

Enteroviruses are among the most common causes of infections in humans, which are often asymptomatic. There are about 64 enterovirusserotypes that infect humans and more commonly severe disease (encephalitis, AFP and myocarditis), therefore any specimen suspected of containing them should be handled with caution and treated as pathogenic. The need for laboratory diagnosis is of great importance and antiviral therapy may be used if more virulent serotypes such as enterovirus 71 are identified, and epidemiological surveillance is important so as to manage the outbreaks (McMinn, 2001) most especially when enteroviruses are able to cause serious infections.

Laboratory diagnosis is established primarily through virus isolation or molecular detection of the virus nucleic acid in appropriate clinical specimens. This method is important as it distinguishes EV71 from other enteroviruses, such as coxsackievirus type A 16.

2.9.1 Choice of Sample

Samples for laboratory investigation should be selected according to the disease manifestations. Possibilities include throat, rectal, and ulcer swabs, and samples of serum, urine, CSF, and fluid from vesicles. The sensitivity, specificity, and usefulness of findings vary according to the sample. In particular, virus detection in samples from sterile sites, such as vesicular fluid, CSF, serum, urine, or those gathered at autopsy, is more reliable than those samples from non-sterile sites, like throat or rectum, where the present virus might merely indicate coincidental carriage. Viral shedding from the gastrointestinal tract through the throat, rectum or in stools might continue after complete resolution of the symptoms of EVs infection. A study in Taiwan showed that EV71 can be detected in the throat up to two weeks after recovery from HFMD or herpangina, and in stools it can be detected up to 11 weeks after recovery. Furthermore, when an enterovirus is detected in non-sterile sites, it is always different from that isolated in samples from sterile sites in 10% of throat swabs and 20% of rectal swabs.

However, the viral load is always very low in some of the sterile sites. As for poliomyelitis; for instance, virus is detected in 0 to 5% of CSF samples from patients with neurological disease. The yield for serum is similarly low. When vesicular fluid is present, it is preferable, but care must be taken during collection. A study revealed that the most efficient approach was to examine throat swabs for all patients, plus swabs from at least two vesicles or from the rectum for patients that has no vesicles (WHO, 2004).

2.9.2 Sample collection and transportation

Specimens are to be transported in well labelled and sealable containers. All specimens are transported in sealed plastic bags or pouches, accompanied by completed laboratory request forms. Diagnostic specimens other than stools and CSF are always transported to the laboratory in an appropriate virus transport medium. Stools and CSF specimens are collected

and transported in sterile, screw-capped containers, preferably plastic rather than glass. Specimens should be transported to the laboratory immediately after collection or within 24 hours. If delayed the specimens should be refrigerated at 4° to 8°C. If samples are stored or refrigerated, repeated freezing and thawing should be avoided and every effort should be made to ensure that specimens stored frozen remain frozen until they can be processed in the laboratory.

2.9.3 Isolation of Enterovirus

The gold standard for diagnosis of enterovirus infection is virus isolation. Several human and non-human primate cell lines can be used, including rhabdomyosarcoma, which is most efficient, human lung fibroblast cells, and African green monkey kidney cells. All specimens should be inoculated in duplicate in each cell line chosen, preferably in tube culture, or in microplate culture if cross-contamination of plate cultures can be avoided. In rhabdomyosarcoma cells, a characteristic cytopathic effect is observed typically 7–10 days after inoculation. In other to improve the yield, blind passage might be necessary before cytopathic effects become apparent (Pallansch, 2001). Once a cytopathic effect is observed, the virus is identified by neutralization tests in intersecting pools of type-specific antisera or by an indirect immunofluorescence assay with the virus specific monoclonal antibodies.

In a situation where characteristic enterovirus CPE such as rounded, refractile cells detaching from the substrate surface appears, incubation should be allowed to develop to at least 75% of the cells are affected (+++ CPE). Then they should be subjected to subsequent passages but if CPE does not occur after seven days, a blind passage can be performed for an additional seven days (WHO, 2003 and 2004). Contents of replicate cell cultures from an individual case should not be pooled for passage that is individual cell cultures should be passaged separately.

2.9.4 Enterovirus Serotyping

Recently, enterovirus laboratories use to employ antibody-based screening assays in determining the identity of viruses in CPE-positive cultures. A few of these assays include components for detecting poliovirus-positive cultures. A molecular serotyping approach developed has been towards amplification of part of the VP1gene of the cultured virus, use of PCR and panenterovirus specific primers, and sequencing of the product. Antisera have been raised in animals against many enteroviruses, but the large number of viruses make it impractical to routinely perform individual neutralization tests. To put an end to thisproblem, antisera have been pooled in an overlapping scheme that allows many viruses to be identified using about nine tests (Xiao *et al*., 2009).

Interpretation of the results is then carried out with the help of a list of the neutralization patterns of individual viruses. Type-specific monoclonal antibodies have also been used. These permit virus confirmation and differentiation by indirect immunofluorescence.Pooled horse antisera against the most frequently isolated echoviruses and coxsackieviruses have been prepared at the National Institute of Public Health and are supplied free of charge to WHO Polio Laboratory Network laboratories (WHO, 2004). Other typing reagents have been prepared and include the Lim and Benyesh-Melnick (LBM) combination neutralizing serum pools, together with commercially available monoclonal antibody reagents.

Also, several sets of primers directed at different regions of the VP1gene of enteroviruses have been developed. Enteroviruses specific primers are used to perform PCR directly on clinical samples. The advantage of this method is that it is faster than virus culture. Speed can be especially important given the explosive nature of some enteroviruses outbreaks and the need for urgent or quick public health interventions. The disadvantage is that only the virus of focus in detection can be detected and, therefore, other unexpected viruses will not be

identified (Xiao *et al*., 2009). DNA microarray is a powerful, although expensive, tool designed to detect multiple pathogen targets by hybridization of pathogen specific probes. Two groups have reported its use to distinguish EV71 and coxsackievirus type A 16 infection in primary clinical specimens, with diagnostic accuracy of about 90% (Xiao *et al*., 2009).

2.9.5 Molecular Method of Identification.

The technique was said to overcome the effect of cell culture bias as it is capable of identifying enterovirus from clinical specimen with the use of reverse transcriptase semi-nested PCR (RT-snPCR) assay (Nix *et al*., 2006). Several studies have reported the effectiveness of molecular technique in the identification of enteroviruses (WHO, 2004). As a result, the strategy has been adopted officially by WHO (WHO, 2004) in preference to the cell culture-based method for enterovirus surveillance (WHO, 2016).

During enteroviruses co-infections, the genomic concentration has an influence on the final identity on mixture of enterovirus. Studies reported that enterovirus with the highest titer in clinical samples could frequently be identified and the mixed isolates showing the identity of only one member of the mixture (Faleye and Adeniji, 2015). Application of the Nix *et al*., 2006 protocol directly to clinical samples helped in the detection of the enterovirus isolates that cannot be detected when the samples were first subjected to cell culture with the use of a susceptible and permissive cell line. The influence of genomic concentration on the enterovirus serotype identified by Nix *et al*., 2006 protocol revealed that molecular identification helps to find and identify enteroviruses that would have not been identified, hence a decision to forgo cell culture-based algorithm behind must be carefully handled to avoid being bias about enterovirus diversity landscape as confirmed by Arita *et al.*, 2015 andAdeniji 2015.

However, part of the strategies used to tackle this challenge was addition of species-specific primers screen using primers 187, 188 and 189 (Oberste *et al*., 2003; WHO, 2015) to the second round Polymerase Chain Reaction (PCR) of the Nix *et al*. protocol. Of which instead of single second round PCR, four different second round PCR assays that use similar first round PCR product as a template were obtained (Nix *et al*., 2006). Adeniji 2015 and Faleye *et al.,*2015, used this strategy to screen feacal samples and RD cell line isolates, paired and unpaired and discovered that the Nix *et al*., 2006 protocol is very sensitive for detecting enterovirus genomes but always mask the presence of more than one enterovirus isolate per sample.

The phenomenon was said to have been inherited from primers 292 and 222 (Faleye and Adeniji, 2015) as these were upgraded to AN89 and AN88. However, primer 292 and by extention AN89 is a consensus of primers 187, 188 and 189 (Oberste *et al*., 2003) the addition of these three primers helped in the restoration of the strength of the assay (Faleye *et al*., 2015). Hence by modifying the WHO recommended Nix *et al.*protocol to singly use primers AN89, 187, 188 and 189 (forward primers) besides AN88 (reverse primer) for the second round PCR, the assay maintained its sensitivity for enterovirus detection and is presently of great value for identification due to its mixed isolate-resolving ability.

In addition, studies have reported that whatever isolate revealed on the cell line is not always the total or complete picture. Often, other enteroviruses are present in the sample that will not grow on the RD cell line (Adeniji, 2015). Hence, studies depending on the RD cell line (or others with their various biases unaccounted for) that have linked some enterovirus strains to certain clinical conditions (Tao *et al*., 2014a; 2014b) need to be interpreted with caution because there is possibility that not all of the enteroviruses in the sample were detected and identified. Therefore, considering the fact that most enteroviruses are without symptoms, go unnoticed and we hardly truly exhaustively catalogue the enterovirus diversity landscape of a

sample, it is hard to conclude the type of enterovirus or combination of enterovirus types which are truly associated with the clinical manifestation(Rao *et al*., 2012).

Furthermore, studies revealed that even when a clinical sample is not positive (negative) for enteroviruses by the pan-enterovirus VP1 screen based on primers 224, 222, AN89 and AN88 (Nix *et al*., 2006), often, the species-specific screen (using the first round product of 224 and 222 as template) still detects sometimes like two different serotypes in the similar sample.

2.9.6 Effect of Cell line on Enterovirus Diversity Landscape

There has been a report about increase in the preponderance of EV-B due to the detection of former untypable Non-Polio Enterovirus (NPEV). This greatly intensified the notion that EV-B were the most diverse and further strengthened the belief on enterovirus divergence that favoured EV-B. It was later discovered that the diversity observed in EV-Bs might not be because of the fact that they are the most evolutionarily successful. On the other hand, two factors was said to have resulted to this.

To start with, some enteroviruses cannot, presently be isolated in cell culture (Brown *et al*., 2003). Although, some enteroviruses will grow in a range of mammalian cell lines which include among many others primary African green, cynomolgus or rhesus monkey kidney cells (AGMK, CMK, RMK), Madin Darby canine kidney (MDCK), human diploid cell lines (MRC-5, WI-38, SF), human embryonic kidney (HEK), human embryonic firoblast (HEF), human epithelial carcinoma (HEP-2), human rhabdomyosarcoma (RD). Enteroviruses vary in their ability to grow in different cell line. To improve the chance of successful virus isolation, several cell lines should routinely be used. Effective enterovirus laboratories usually inoculate specimens into a minimum of the three cell lines, and some may use five or six cell

lines. Polioviruses are the only species C enteroviruses currently known to replicate in L20B cell line. Based on the recommendation World Health Organization (WHO) (figure 2.2), most laboratories in the GPLN use the RD-L20B isolation protocol for poliovirus isolation (WHO 2003, 2004). The RD and L20B isolation protocol is built around two different cell lines, RD and L20B. The RD cell line is from a human rhabdomyosarcoma (a straight muscle cancer) and was shown to be quite sensitive for enteroviruses most especially poliovirus isolation (WHO, 2003).

Also, the RD cell line that GPLN use seems to enhance the replication of Enteroviruses even when the other members of enterovirus species are present (Faleye and Adeniji, 2015). There was an observable change and improvement in the detection of EV-C members when other cell lines such as Hep 2C and MCF-7 were added in the enterovirus protocols. For instance, most of the time, when the same sample is inoculated into RD and MCF-7 cell lines, the two will individually isolate EV-Bs and EV-Cs , respectively (Faleye and Adeniji, 2015). Studies revealed that in cases where both EV-B and EV-C are present in an isolate recovered on the RD cell line, direct molecular identification without first separating the mixture always selectively reveals EV-B component of the mixed isolate (Faleye and Adeniji, 2015). Behind this impression of RD cell line EV-B bias, it appears as if biology of the cell line enhances the propagation of EV-Bs. This might end up with increased titre or relative genome concentration, which is later multiplied, using molecular method of identification.

2.10 TREATMENTS OF ENTEROVIRUS INFECTIONS

2.10.1 Pleconaril

Pleconaril is an antiviral drug that inhibits the entry of several enteroviruses into cells by blocking viral attachment and uncoating. This has been used in clinical trials of aseptic meningitis (Wu *et al*., 2009). This drug is not, however, active against Enterovirus 71.

Several other capsid function inhibitors have been investigated, and many have shown promising activities against EV 71 in preclinical studies. In-vitro and in-vivo studies have shown that both ribavirin and interferons might also be useful and RNA interference approaches are being explored (Wu *et al*., 2009).

2.10.2 Intra-venous Immunoglobulin

In Asia, during the initial large outbreaks of EV71, clinicians in Sarawak and Taiwan made use of intravenous immunoglobulin on the presumptive basis with the hope that it would neutralize the virus and have non-specific anti-inflammatory properties. Retrospective comparisons of patients who did and did not receive immunoglobulin suggest a benefit from this treatment if given early (Ooi *et al*., 2005) For instance, among children with EV71 assessed in Sarawak over the period of three seasons, 204 (95%) of 215 survivors who had severe central nervous system (CNS) complications had received intravenous immunoglobulin treatment (Wu *et al*., 2010).

Intravenous immunoglobulin has become more routinely used for the treatment of severe EV71 disease and has been a standard therapy for aseptic meningitis caused by EV infection. In Taiwan (IVIg) has been introduced into the national treatment guidelines (Wang *et al.*, 2006). However, uncertainty remains, over whether this expensive human blood product treatment is really effective and randomized, placebo-controlled, phase 2 trials are needed. Such trials would be logistically and ethically challenging to establish because the treatment is widely used, and would require careful design with surrogate endpoints of disease progression, such as failure of resolution of tachycardia.

2.10.3 Serology

Serological diagnosis of an acute virus infection basically relies on a fourfold increase being revealed in the concentrations of a specific neutralizing antibody between the acute and

convalescent phases. Considering the case of EV71, very high concentrations of neutralizing antibodies are frequently detectable within the first few days of the onset of illness, and thus such a difference will not be seen (Pallansch *et al*., 2001). Furthermore, homologous antibodies are produced when young children encounter their first enterovirus infection, while heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different enterovirus serotypes. The usefulness of this test, thereby decreases with increase in age.

2.11 VACCINES AGAINST ENTEROVIRUSES

The effectiveness of immunization in protecting the host against EV infections was demonstrated by the historical Salk and Sabin vaccines against PV. Some effective vaccines have been designed and are able to stimulate both the innate and adaptive immune response in order to combat the remaining clinically relevant EVs. To combat EV infection, the adaptive immune response requires both T cells and antibodies in the clearance of the virus. Hence, the most successful vaccines activate both humoral and cell-mediated immunity and induce long viral immunity (Kew *et al*., 2003).

2.11.1 Coxsackievirus Vaccines

Several effective vaccines have been developed by some researchers against Coxsackievirus. Although, no clinically available vaccine currently exists but there has been improvement in using various vaccine techniques utilizing inactivated virus, live attenuated forms of virus or DNA plasmids expressing viral proteins.

2.11.2 Poliovirus Vaccines

The original formalin-inactivated form of the vaccine (IPV) was developed by Jonas Salk and licensed in 1955 (Salk *et al*., 1954). Both the inactivated and attenuated forms of the historical PV vaccines have been used to confer immunity to the virus. Meanwhile, the original formalin-inactivated form of the vaccine (IPV) was developed by Jonas Salk and

licensed in 1955 (Salk *et al*., 1954). An orally administered live attenuated PV vaccine (OPV) was formulated in 1963, (Sabin, 1957). The occurrence of poliomyelitis drastically reduced worldwide during the usage of these vaccines and optimized versions. Although the pathogen has yet to be eradicated worldwide. The preference for the administration of IPV over OPV was due to the discovery of circulating vaccine derived PVs (cVDPV) that is associated with the OPV, cVDPVs are virulent PVs derived from OPV that occur in a small minority of vaccine recipients. The reversion of cVDPVs from attenuation to virulence is a direct consequence of the genetic instability of the vaccine. Circulating vaccine derived PVs cVDPVs were responsible for a polio outbreak in the Dominican Republic and Haiti in 2000 (Kew *et al*., 2003). Sequencing determined that the cVDPVs were recombinant viruses and the derivation of neurovirulent polio strains from the OPV has led to the "OPV paradox," and this is based upon the idea that complete eradication of poliomyelitis is contingent upon the elimination of the attenuated form of the vaccine. Attenuation of EVs via mutations in the genome has historically led to efficient vaccine production. Perhaps the most well-known attenuated form of PV is that used for the Sabin vaccine, which has decreased neurovirulence in part controlled by two stem loops in the viral internal ribosome entry site (IRES) (Gromeier *et al*., 1999). Other mutation that can cause CNS attenuation is located between the 5′ NTR cloverleaf and IRES and reduces the binding of polypyrimidine tract-binding protein.

Studies have made recombinant viruses that use the IRES from human rhinovirus type 2 (HRV2) to attenuate neurovirulence in the Sabin vaccine strain of PV as well as in herpes simplex virus type 1. Somewhat alarmingly, a vaccine-derived PV and coxsackievirus A17 recombinant has been generated in the laboratory, hence demonstrating the possibility of such events occurring naturally.

2.12 ACUTE FLACCID PARALYSIS

Acute flaccid paralysis (AFP) is a clinical syndrome characterized by rapid or sudden onset of weakness, including (less frequently) weakness of the muscles of respiration and swallowing, progressing to maximum severity within several days to weeks.The term "flaccid" indicates the absence of spasticity or other signs of disordered central nervous system motor tracts such as hyperreflexia, clonus, or extensor plantar responses (Alberta Government Health and Wellness, 2005).

Paralysis implies loss of contraction due to interruption of motor pathways from the cortex to the muscle fiber when applied to voluntary muscles. It is preferable to use the term "paresis" for slight loss of motor strength while "paralysis" or "plegia" is better use for chronic loss of motor strength.The differential diagnosis of AFP varies considerably with age. This abnormal condition may be caused by disease or by trauma affecting the nerves associated with the involved muscles. If the somatic nerves to a skeletal muscle are severed for instance, then the muscle will exhibit flaccid paralysis. When muscles enter this state, they become limp and cannot contract. This condition can become fatal if it affects the respiratory muscles, posing the threat of suffocation. (Saladin *et al*., 2012).

AFP is the most common sign of acute polio, and used for surveillance during polio outbreaks.

It is also caused with a number of other pathogenic agents including enteroviruses, echoviruses, West Nile virus, and adenoviruses, among others.Non-polio enteroviruses have been associated with polio-like paralytic disease, commonly followed by other clinical syndromes, such as hand-foot-and-mouth disease, aseptic meningitis and acute hemorrhagic conjunctivitis. Coxsackieviruses A and B, echovirus, enterovirus 70, and enterovirus 71 have been implicated as the causative agents of polio-like paralytic disease. (Kelly *et al*., 2006).

In some studies, outbreaks of acute hemorrhagic conjunctivitis with radiculomyelitis and paralytic illness in India, Taiwan, Thailand, and Panama were etiologically linked to enterovirus 70. Muscle weakness and wasting resulted from enterovirus 70 is always severe and permanent. In Califonia, it was first described in 1969 to 1973 that enterovirus 71 among all other non-polio enteroviruses, as the major cause of the outbreaks of central nervous system disease and AFP. Children under 15 years of age were greatly affected. There was Global attention on enterovirus 71 as a result of severe epidemics of central nervous system disease that occurred in Japan in 1973 and in Bulgaria in 1975 (57). Of 705 patients infected with enterovirus 71 in Bulgaria, 149 (21 percent) developed paralysis, and 44 (29 percent) of those persons died. (Kelly *et al*., 2006)**.**

2.12.1 History of Acute Flaccid Paralysis.

Paralytic poliomyelitis occurred in the ancient times, it was not distinguished as a distinct disease entity with infectious or epidemic potentialities until the end of 18th century when its epidemic potential began to show up (Paul, 1954).Acute flaccid paralysis has a long history, dating back to the Egyptian eighteenth dynasty (1580-1350 BC). The historical record of polio is very fragmentary and isolated cases of poliomyelitis and acute paralysis in children have been occurring since the biblical times. Great number of epidemics were accompanied by various hysterical reactions. A striking feature of paralytic poliomyelitis has been its ever-changing epidemiology. Poliomyelitis is one of the major health problem which was first described by a physician, Michael Underwood from Britain, in 1789 (Drutz and Ligon, 2000).

Despite chronic shortage of resources of developing countries, a number of destabilizing health problems such as poliomyelitis is being experienced.Poliomyelitis is derived from two words ofGreek origin, polio which implies gray and myelon which means marrow, indicating the spinal cord. Therefore poliomyelitis, is often referred to as polio or infantile paralysis. It

is an acute viral infection that influences the motor neurons within the spinal cord and brain leading to the classic manifestations of flaccid paralysis (Kumar and Taunk, 2014).Poliomyelitis being one of the major ofhealth problem is described as the debility of the lower extremities. Poliomyelitis existed across the globe before the eradication initiative was undertaken in 1988 which marked the launch of the Global Polio Eradication Initiative (GPEI), organized by WHO, Rotary International, the US Centers for Disease Control and Prevention (CDC) and the United Nations Children's Fund (UNICEF) (WHO 2012).

2.13 EPIDEMIOLOGY OF ENTEROVIRUSES

Picornaviruses are widely prevalent.Enteroviruses are transmitted mostly by the fecal-oral route, they can also be transmitted by salivary and respiratory droplets. Some serotypes are spread by conjunctival secretions and from skin lesions exudates **(**L'Huillier *et al*., 2015**)**. Non-Polio enterovirus are widely distributed and are associated with occasional outbreak in which a larger number of patients develop clinical diseases.In temperate countries, outbreaks of enterovirus illnesses occur most frequently in summer and autumn. Enteroviruses in excreta that contaminate the soil are carried by surface waters to lakes, beaches, vegetation, and community water supplies (Noyce *et al*., 2011). These sources may serve as foci of infection. Likewise, shellfish that feed in freshwater or seawater beds contaminated by excreta become carrier of enteroviruses. Likewise, cockroaches in sewage pipelines and flies that pet on excreta may act as transient vectors. Immunity is serotype specific and the epidemiological pattern differ by climate and geographical region.

Enteroviruses contain more than 250 naked icosahedral virus serotypes categorize as members of family piconaviridae, genus enterovirus and order piconavirales and have diameter of 28-30 nm within the non-enveloped icosahedral capsid of an enterovirus is a positive-sense, protein-linked, single-stranded approximately 7.5 kb RNA genome, of a single open reading frame (ORF) flanked on both sides 5^1 and 3^1 ends by untranslated regions

and translated into an approximately 250-kDa polyprotein. This polyprotein is auto catalytically cleaved into P1, P2 and P3 polyproteins which further cleaved into VP1-VP4, 2A-2C and 3A-3D, respectively (http://www.piconaviridae 2016). The replication of enterovirus begins in the respiratory or gastrointestinal tract, and once the virus is evident or present in the blood stream, infection may affect different tissues and organs causing different types of diseases. Enteroviruses are associated with specific syndromes for instance, Human enteroviruses A cause hand-foot-mouth disease (HFMD) (Tassin*et al*., 2013).

2.13.1 Epidemiology of Enterovirus-A71

Enterovirus 71 is known to be a virulent serotype of the enteroviruses that has a wide variety of clinicalmanifestations, although CNS infection and HFMD arethe two features most frequently seen. Being one of the enterovirus serotype it was first isolated from a child of in 1969 in California, USA, with phylogenetic evidence that revealed that it was present in the Netherlands as far back as 1963 (Van der Sanden *et al*., 2008). Subsequently, there was an outbreak with neurological infections caused by EV-A71 reported in Australia, Japan, Sweden and the United State of America. Its effect in 1975 in Bulgaria during two large outbreaks and in Hungary three years later resulted into high fatality among the children.There was a report of 44 fatalities amongst 451 children with non-specific febrile illness or neurological disease in Bulgaria (Chumakov *et al*., 1979) and 47 deaths amongst 1550 children, 826 aseptic meningitis and 724 encephalitis inHungary (Nagy *et al*., 1978).

In Sarawak in the year 1997, there was an outbreak of HFMD cases as a result 34 deaths were reported. Later on, Taiwan reported the highest HFMD outbreak that involved 1.5 million cases with 78 deaths in 1998 (Ho *et al*., 1999). A great outbreak of HFMD took place in Singapore in the year 2000. This involved 3790 patients of which 5 deaths were recorded, 3 due to HFMD and 2 to non-HFMD (Chong *et al*., 2003). Since then, HFMD is recognized as

an endemic mild disease in both Malaysia and Singapore. China was the next country to report a large HFMD outbreak of 490,000 infections with 126 deaths in 2008, since 2009, the number of HFMD infections in China had greatly increased and there were 2,819,581 HFMD cases reported with 394 deaths in 2014 while Vietnam reported 4265 cases of HFMD with two deaths in 2015 (WHO , 2015). Aside the Asia Pacific region, there was an outbreaks or sporadic infections in Europe with no fatality or low fatalities (Hassel *et al*., 2015).

2.14 PREGNANCY AND NON-POLIO ENTEROVIRUS INFECTION

Pregnant women who are infected with a non-polio enterovirus shortly before delivery can pass the virus to their babies. These babies usually have only mild illness, but in rare cases they may have severe infection. Non-polio enteroviruses are very common, so a pregnant woman is likely to be exposed at some point in her pregnancy to someone who is infected, especially in the summer and fall. But most pregnant women, like other adults, have immunity (protection) from previous exposures to non-polio enteroviruses. So if she does get infected she will likely not have symptoms or will only have mild illness. Pregnant women without immunity to non-polio enteroviruses havea greater chance of getting infected and having symptoms (NCIRD, 2016).

There is no clear evidence that non-polio enterovirus infection during pregnancy increases the risk of severe complications like miscarriage, stillbirth, or congenital defects. Non-polio enteroviruses can be shed (passed from a person's body into the environment) in the stool for several weeks or longer after one has been infected. The virus can be shed from respiratory tract for 1 to 3 weeks or less. Infected people can shed the virus even if they do not have symptoms (NCIRD, 2016). Commonly, infants, children, and teenagers are most likely to get infected with enteroviruses and become ill this occur because they do not yet have immunity (protection) from previous exposures to these viruses. This is also believed to be true for EV-

D68. Adults can get infected with enteroviruses but they are more likely to have no symptoms or mild symptoms.

2.15 PREVENTION AND CONTROL

Most people who are infected with non-polio enteroviruses do not have symptoms, but can still spread the virus to other people. This makes it is difficult to prevent them from spreading. But the best way to help protect oneself and others from non-polio enterovirus infections is to wash your hands often with soap and water, especially after using the toilet and changing diapers, avoid close contact, such as touching and shaking hands, with people who are sick, clean and disinfecting frequently touched surfaces.Poliomyelitis can be prevented by Salk-type (inactivated) and Sabin-type (live) attenuated poliovirus vaccines, control can be achieved via public education on transmission modes and personal hygiene, adequate sewage disposal and uncontaminated water supplies are critical for prevention of enteroviral infections, there is no specific therapy.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection and Processing

This study adopted a cross sectional design with a purposive consecutive sampling method and was carried out in the World Health Organization (WHO) Polio Laboratory, Virology Department, University College Hospital, Ibadan, Oyo State with ethical approval. Sixty stool suspensions were analyzed as thirty cases (Two stool suspensions from one person), which were selected from feacal samples from children with Acute Flaccid Paralysis (AFP) cases in South-West Nigeria.

These samples were collected from patients in the month of August, year 2015 by WHO National Polio Laboratory, Ibadan, Oyo State, within 14 -15 days ofthe onset of the paralysis as described in the World Health Organization Polio Laboratory Manual, 4th edition (WHO, 2004). The children's age involved in this study 15 years and below. The samples were previously screened using culturing method and showed no cytopathic effectand were said to be negative for Poliovirus and other enteroviruses. Also, the feacal specimen was made into 20% suspension in a balanced salt solution with antibiotics and were treated with chloroform after to which enteroviruses were resistant.

In addition to removing bacteria and fungi, this method removed potentially cytotoxic lipids and dissociates virus aggregates (Adeniji, 2014). After the treatment with chloroform and vortexing, the suspension was clarified by centrifugation at 1500xg for 10 minutes and clarified suspensions were achieved. The samples were taken from six States in Nigeria these include Lagos, Oyo, Osun, Ekiti, Ondo and Ogun State. Considering that the samples were collected from states in Southwest Nigeria, (SW), they are referred to in this study as SW samples.

3.2 METHODS

3.2.1 Viral RNA Extraction

The extraction of viral Ribonucleic acid (RNA) was done for each stool suspension (supernatant) by using total Jena Bioscience RNA kit according to the manufactures' instruction. These include cell lysis that was done using an enzyme Protease and detergent. Column activation, with the use of buffer AW1 W2, column loading with the use of primary and secondary washing. Finally, elution of RNA that was done using elution buffer of RNA.

All reagent were mixed over leaf and label vials, 500 µL of lysis buffer 2 ME (Macfty Ethanol) already added to all approximately labelled vials. Elution buffer of 100 µL was added to each labelled vial containing the sample, vortex for 10 minutes, incubated on a bench for 10 minutes and centrifuged at 10,000 xg for 5 minutes. After this the supernatant was transferred into a new properly labelled vail. A spin column was placed into a 2 mL collection tube, 100 µL of extraction buffer was added into the spin column, centrifuge at 10,000 rmp for 30 seconds and flow through was discarded. Isopropanol of 360 µL was added to the prepared lysate and vortexed. The new mixture 960 µL was transferred into the spin column, centrifuged at 10,000 xg for 30 seconds and flow through was discarded.

During column activation a spin column was placed into a 2ml collection tube, 100µl of activation buffer was added into the column and it was centrifuged at 10,000 rpm for 30 sec and the flow through was discarded. Column loading was achieved with the use of isopropanol (300 µl) was added to the prepared lysate and it was vortex for 30sec. The mixture was transferred to the activated spin column which was centrifuge at 10,000 g for 30 sec. The flow-through was discarded. In addition, primary column washing was done using primary washing buffer as 700 µl ethanol added was applied to the spin column and was centrifuged at 10000 rpm for 30 sec and flow through was discarded. Secondary column

washing was achievedsecondary washing buffer (700 µl) was apply to the spin column. It was centrifuged at 10,000 rpm for 30 sec. The flow-through was discarded. It was centrifuged at 10,000 rpm for 2 min. again to remove the residual ethanol. Elution of RNA: The spin column was placed in a DNase/RNase-free microcentrifuge tube, 45µl of Elution buffer was placed at the centre of the column membrane, it was incubated at room temperature for 1 min. it was centrifuged at 10,000 rmp for 1 minute to elute the RNA. The RNA was stored at 45 ^{0} C (Jena Bioscience, Germany).

3.2 .2 Complimentary Deoxyribonucleic Acid (cDNA) Synthesis

Each eluted RNA was used to synthesize complementary Deoxyribonucleic acid (cDNA) by reverse transcription of the extracted RNA in a 10 µl reaction mixture (Table 3.1). Each reaction tube mixture contained 5.25 µL of RNA, 2 µL of script reverse transcription buffer, 0.5 µl of Deoxyribonucleic tri-phosphate (dNTP), 0.5 µL of RNase inhibitor, 0.5 µl of Dithiothreitol (DTT) stock solution and 0.25 µL of script reverse transcriptase (Table 3.1) . Complimentary DNA primers used are AN32, AN33, AN34 and AN35 each 0.25 µL (Nix *et al*., 2006). The reaction mixture was amplified in a Veriti Thermal Cycler at 42 °C for 10 minutes and 50 °C for 60 minutes (Applied Biosystems, California, USA). The cDNA was stored at -80 0c till analysed (Nix, 2006).

The oligonucleotides of the primers are as follows: AN32: 5’ GTY TGC CA 3’; AN33: 5’ GAY TGC CA 3’; AN34: 5’ CCR TCR TA 3’; AN35: 5’ RCT YTG CCA 3’. Standard International Union of Biochemistry nucleotide ambiguity codes are used (I = Deoxyinosine N = G, A, T or C; Y = C or T; W = A or T; R = A or G) (WHO, 2004). The cDNA was stored at -80^{0}C and used for all downstream polymerase chain reaction (PCR) assays.

Table 3.1 Standard Protocol forcomplimentary deoxyribonucleic acid (cDNA)

S/N `	Component	Volume (µL) 1 Sample	Volume (µL) 10 Sample	Volume (µL) 34 Sample
1	Script RT-Buffer Complete	2	20	68
2	An32	0.25	2.5	8.5
3	AN33	0.25	2.5	8.5
4	AN34	0.25	2.5	8.5
5	AN35	0.25	2.5	8.5
6	dNTP mix	0.5	5	17
7	DTT Stock Solution	0.5	5	17
8	RNase Inhibition	0.5	5	17
9	SCRIPT RT	0.25	2.5	85
10	**Total**	**4.75 µL**	**47.5 µL**	**161.5µL**

4.7 5 µL of cDNA synthesis mix

5.25µL of extract (RNA)

10 µL of the total mix

3.2.3 Polymerase Chain Reaction (PCR) Assays

3.2.3.1 Enterovirus VP1 Gene PCR (snPCR) Assay

PCR was done in 30 μL reactions, the first-round PCR contained 0.5 µL of each of primers 224 and 222 (Nix *et al*., 2006), 10 µL of Red Load Taq, 10 µL of cDNA, and 29 µL of RNase –free water. Thermal cycling was done in a Veriti thermal cycler (Applied Biosystems, California, USA). Thermal cycling conditions were 94 ^{0}C for 3 minutes followed by 35 cycles at 94 ^{0}C for 30 seconds, 42 ^{0}C for 30 seconds, and 60 ^{0}C for 60 seconds with ramp of 40% from 42 ^{0}C to 60 ^{0}C. This was then followed by 72 ^{0}C for 7 minutes and held at 4^{0}C till being terminated (Table 3.2).

3.2.3.2 Pan-Enterovirus Polymerase Chain Reaction (PE-PCR)

The PE-PCR screen was a Panenterovirus detection PCR assay. It was done using primers PanEnt- AN89-F and PanEnt-AN88-R. Both primers target well conserved regions in the 5^I-UTR of enteroviruses amplifying an approximately 350 bp fragment (WHO, 2004). Primers were made in 25 µL concentrations. Primers of 0.3 µL each was included in 30 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of first round PCR and 20.4 µL of RNase free water. Thermal cycling condition was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30s, 52 ^{0}C for 30 s and 65 ^{0}C for 30 s. This was followed by 72 ^{0}C for 7 min and held at 4 ^{0}C till terminated (Table 3.3). Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

Table 3.2 Working Protocol VP1 Gene PCR (snPCR) Assay

Component	**Volume(µL) sample**	**Volume(µL)30 (µL)+ 4control**
Taq polymerase	10	340
Reverse Primer 224	0.5	17
Forward Primer 222	0.5	17
RNase free Water	29.0	986
Total	40µl	1360µl

Table 3.3 Showing Pan-enterovirus Polymerase Chain Reaction (PE-PCR).

Component	Volume (µl)/ sample	Volume (µl)/sample
Taq polymerase	6.0	204
Forward primer AN89	0.3	10.2
Reverse primer AN88	0.3	10.2
RNase free Water	20.4	693.6
Total	**27**	**918**

3.2.3.3 Enterovirus Species A and C PCR

This is a species specific assay. The screening was used to detect enterovirus species A and C. It was done using primers AN189 (Forward) and AN88 (Reverse). Both primers target well conserved regions of enteroviruses amplifying an approximately 350 bp fragment (WHO, 2004). Primers were made in 25 µL concentrations. For detection of the EA-PCR and EC-PCR screens 0.3 µL of each of the primers specific for the screen was added to a 27 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of the first round PCR product and 20.4 µL of RNase free water giving rise to a total 30 µ L of reaction**.** Thermal cycling condition for EC-PCR-1 was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30 s, 42 ^{0}C for 30 sec. 60 ^{0}C for 60 s with ramp of 40% from 42 ^{0}C to 60 ^{0}C. This was followed by 72 ^{0}C for 7 min and held at 4 ^{0}C till terminated. The conditions were the same for EC-PCR-2 except for the extension time which was reduced to 30 sec. (Table 3.4). All the PCR was carried out in a in a Veriti thermalcycler.Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

3.2.3.4 Enterovirus Species B Polymerase Chain Reaction (EB-PCR).

The EB-PCR screen was an assay done using primers AN187 and AN88 forward and reverse primers respectively. Both primers target well conserved regions in the enteroviruses amplifying approximately 350 bp fragment. Primers were made in 25 µL concentrations. Primers of 0.3 µL each was included in 30 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of first round PCR and 20.4 µL of RNase free water. Thermal cycling condition followed in the Veriti thermalcycler was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30 s, 52 ^{0}C for 30 s and 65 ^{0}C for 30 s. This was followed by 72 ^{0}C for 7 min and held at 4 ^{0}C till terminated. Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

TABLE 3.4 Showing the primers used in the study

	Pan Enterovirus	Enterovirus A and C	Enterovirus B
Forward Primer	AN 89	AN 189	AN 187
Reverse Primer	AN 88	AN 88	AN 88

3.2.4 Agarose Gel Electrophoresis

All PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using Ultra-Violet (UV) transilluminator. The buffer solution used was Tris Base Buric Acid (EDTA) 2 %, (2 grams of agarose was dissolved in 100 mils of TBE buffer), put in microwave for proper dissolution and electrophoresis was done for 45 minutes at 120 voltage. The TBE served as an electrolyte which allowed the flow or passage of the electricity. The loading mix was loaded in each hole of the gel (DNA of 1 µL and 5 µL of loading buffer). The fragments were visible due to Ethidium bromide dye that adhere to the DNA in the Gel, afterward it was viewed under a UV light and photographed. Five microliter (5 µL) of each reaction product was separated and visualized on two percent (2 %) agarose gel stained containing 10 ul Ethidium bromide and viewed using a Ultra-Violet (UV) transilluminator.

3.2.5 Nucleotide Sequencing and Identification of Isolates

All the resulting DNA amplicons were shipped to Macrogen Inc., Seoul, South Korea, for purification and sequencing of gel positive PCR amplicon by BigDye chemistry using the second round primers that were used for the semi-nested amplification reaction, primers AN88 and AN89.

3.2.6 Molecular Typing Isolates and Phylogenetic Analysis.

The PCR (partial VP1) amplicons generated in the snPCR were sequenced. The sequence of the Genes were edited manually.Basic Alignment Search Tool (BLAST) analyses was carried out, and the virus was assigned, named based on the virus that gave the highest VP1 identity score (identity score >75%) to the query sequence.Afterwards, the enterovirus genotyping tool (Kroneman *et al*., 2011) was used for enterovirus species and genotype determination**.**

Generated sequences were manually edited with the use of MEGA 6.06 software. Phylogenetic analysis of the sequenced viruses was done for virus identification by comparing with reference sequences from GenBank. To align the sequences described in this study (species A, B and C) with reference sequences downloaded from the GenBank, the ClustalW program in the MEGA 6.06 software (Tamura *et al*., 2011) was used with default settings. Afterwards, Maximum Likelihood (ML) neighbor-joining trees were constructed with 1,000 bootstrap replicates using the same MEGA 6.06 software (Kimura, 1980).

CHAPTER FOUR

4.0 RESULT

4.1 Sample Collection and Processing

A total of 60 samples were analyzed as 30 cases in this study. The result obtained was from six states in Southwest Nigeria. In Lagos State, 5 males and 3 females, in Oyo State, only 5 males, in Ondo State, just 3 males, in Ogun State 3 males and 3 females, in Osun State, only 1 samples from a female and finally in Ekiti State 4 male and 2 female (Table and Figure 4.1).

Only one sample of the thirty (30) samples screened yielded the expected band size for enterovirus VP1 gene detection RT-snPCR screen (Table 4.2).

4.2 Reverse transcriptase Seminested PCR result

4.2.1 Electrophoretic gel displaying band size

Samples that yielded the expected band size of approximately 350 bp for enterovirus VP1 gene detection RT-snPCR screen. Sample 27 was the only positive sample obtained in this study aside the positive controls used (Figure 4.2).

Table 4.1 Distribution of sample scollection for PCR screen according to gender

States	Total number of Samples	Gender	
		Female	Male
Lagos	8	5	3
Oyo	5	5	0
Ondo	3	3	0
Ogun	6	3	3
Osun	2	1	1
Ekiti	6	4	2
Total	30	21	9

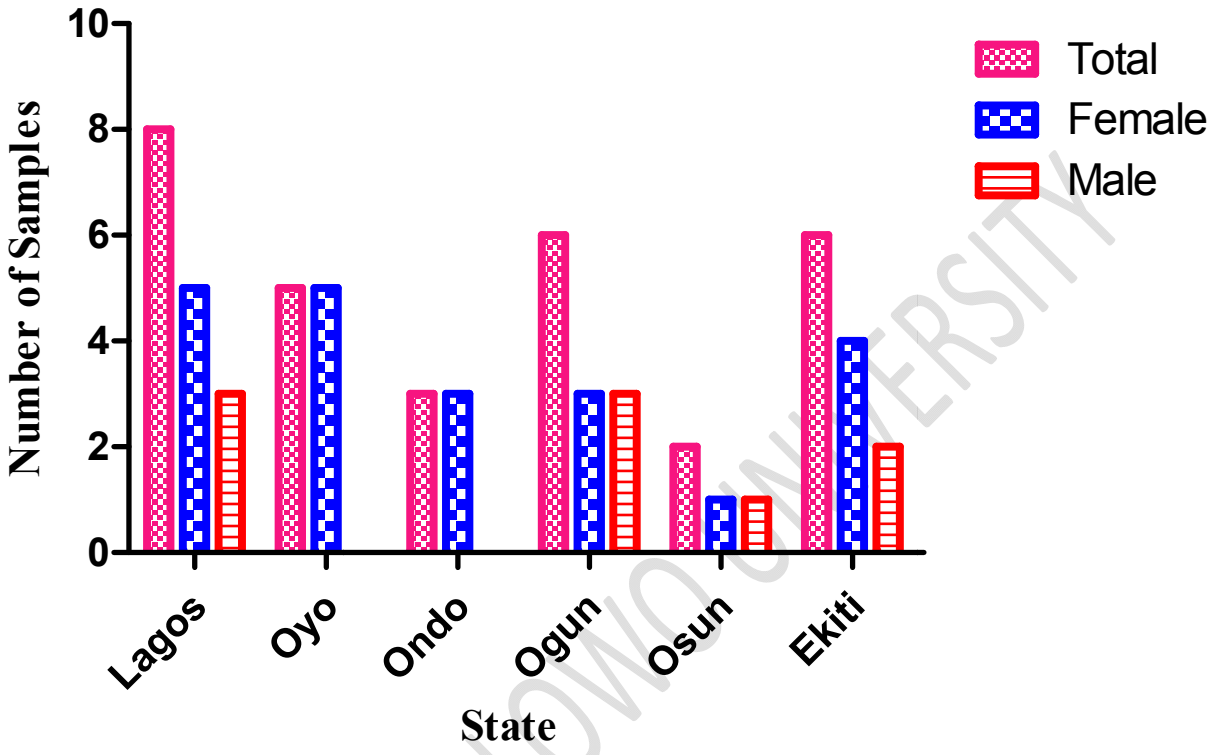


Figure 4.1 Graph of frequency of samples used with respect to the location

TABLE 4 .2Number of positive and negative samples obtained in the study

States	Positive		Negative		Total
	Female	Male	Female	Male	
Lagos	0	0	3	5	8
Oyo	0	1	0	4	5
Ondo	0	0	0	3	3
Ogun	0	0	3	3	6
Osun	0	0	1	1	2
Ekiti	0	0	4	2	6
Total	0	1	9	21	30

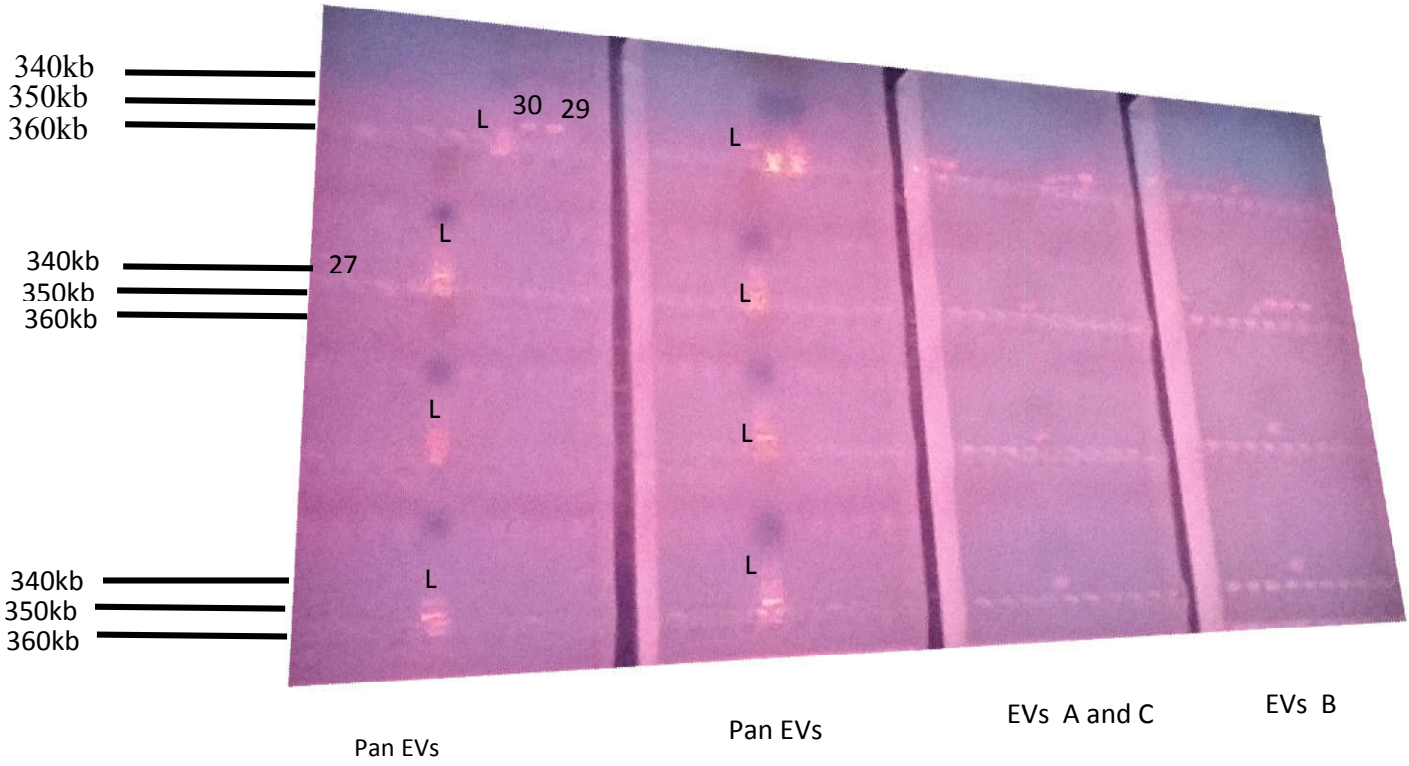


Figure 4.2 The gel electrophoresis from the right EVB, EVA and C-PCR and Repeated

Pan Enterovirus resolved on 2% agarose gel.

Key: L: MassRuler DNA ladder, each line represents amplicon from samples.

L29, L30 serves as the positive control, L27 serves as the only positive recovered sample.

TABLE 4.3 Distribution of samples according to States and gender

States	Female	Male	Total
Lagos	3	5	8
Ondo	4	2	6
Ogun	3	3	6
Oyo	0	5	5
Ekiti	0	3	3
Osun	1	1	2
Total	11	19	30

4.3 Virus Identification

From all the samples, one (1) sample that its amplicon yielded expected band size and the controls were subjected to sequencing and were typed using enterovirus genotyping tool (EGT). Afterward, sequences of the amplicon obtained from panenterovirus assay were typed as Echovirus 21, (E-21), Cosackievirus B-5 (CV-5) and Echovirus 7 (E-7) the strains from sample 27, first control and second control respectively.Furthermore the sequences revealed no member of species A and C assay (Figure 4.2). Considering the total of typed strains detected and identified from the three assays, the result obtained is 0 %, 3.3 % and 0 %, for enterovirus species A and C, Pan enterovirus and B respectively.

4.4 Phylogenetic analysis result

Considering the result obtained from this study, E 21 Echovirus (E-21) was aligned with reference sequences that were retrieved from Genbank with the aid of Cluster W program in MEGA 6.06 software with default setting (Kroneman *et al*., 2011).Subsequently, maximum likelihood trees were constructed using MEGA 6.06 (Tamura *et al*., 2011) and 1000 bootstrap replicates. The accession numbers and the sequences retrieved from the Genbank for phylogenetic analysis are indicated on the phylograms.

Table 4.4 Result of positive samples for Panenterovirus VP1 RT-snPCR and the identity of the enterovirus obtained.

Serial number	Sample Identity	Gender	Age (months)	VP1 RT-PCR	Serotype
1	27	Male	17	Positive	E-21

File: O27_187.ab1 Run Ended: 2016/7/8 19:22:54 Signal G:1671 A:2736 C:3550 T:2187

Sample: O27_187 Lane: 12 Base spacing: 16.251097 425 bases in 5183 scans FI

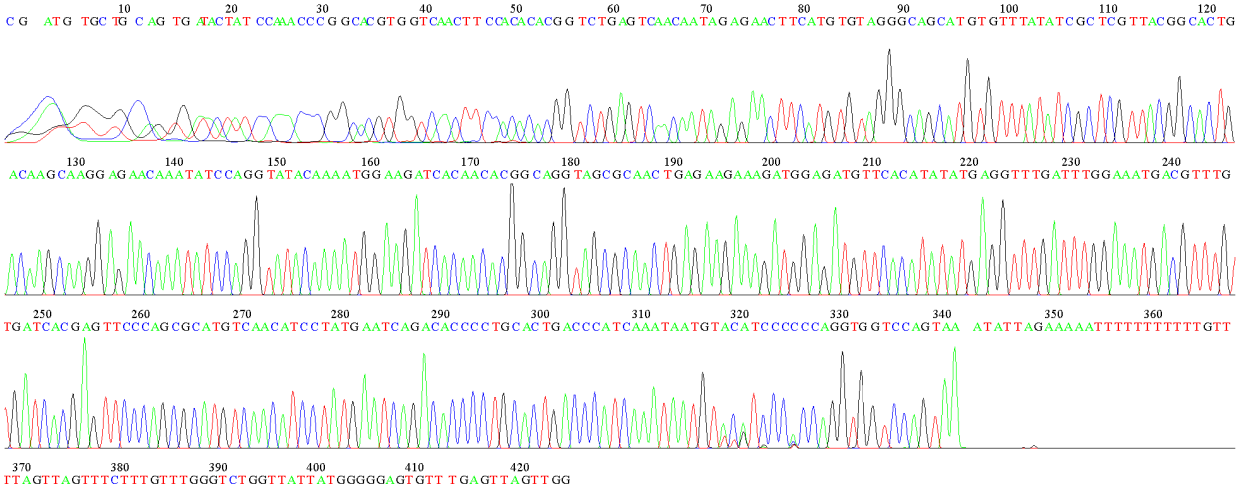


FIGURE 4.3

The electropherogram of the (E-21) sequences recovered from Southwestern Nigeria that is positive in this study is shown above.

>160707-065_E03_O27_187.ab1 425

CGATGTGCTGCAGTGATACTATCCAAACCCGGCACGTGGTCAACTTCCAC

ACACGGTCTGAGTCAACAATAGAGAACTTCATGTGTAGGGCAGCATGTGT

TTATATCGCTCGTTACGGCACTGACAAGCAAGGAGAACAAATATCCAGGT

ATACAAAATGGAAGATCACAACACGGCAGGTAGCGCAACTGAGAAGAAAG

ATGGAGATGTTCACATATATGAGGTTTGATTTGGAAATGACGTTTGTGAT

CACGAGTTCCCAGCGCATGTCAACATCCTATGAATCAGACACCCCTGCAC

TGACCCATCAAATAATGTACATCCCCCCAGGTGGTCCAGTAAATATTAGA

AAAATTTTTTTTTTTGTTTTAGTTAGTTTCTTTGTTTGGGTCTGGTTATT

ATGGGGGAGTGTTTGAGTTAGTTGG

Figure 4.4 Sequence of the Positive Sample obtained in this Study

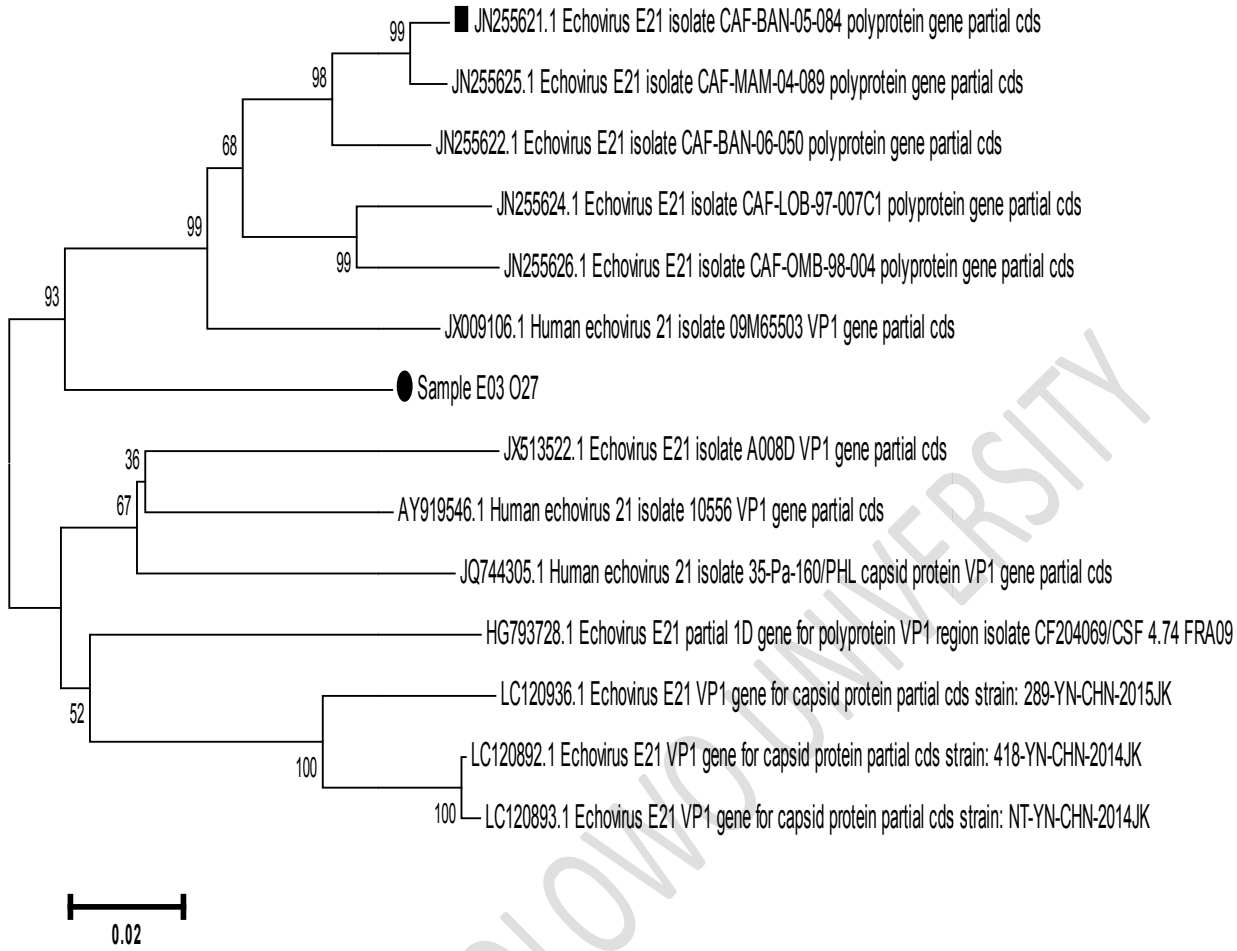


Figure 4.5 Phylogenetic of the recoveredE-21 isolate

ML Tree showing the Recovered E-27 Isolates. The newly Sequence and Reference Strains are represented. The newly strain E- 21 is highlighted with Black irregular Circle. The phylogram is based on alignment of the partial VP1 sequences. The newly sequenced strain is from Nigeria and some strains from Central Africa. The GenBank accession numbers and the name of the strains are indicated in the phylogram.

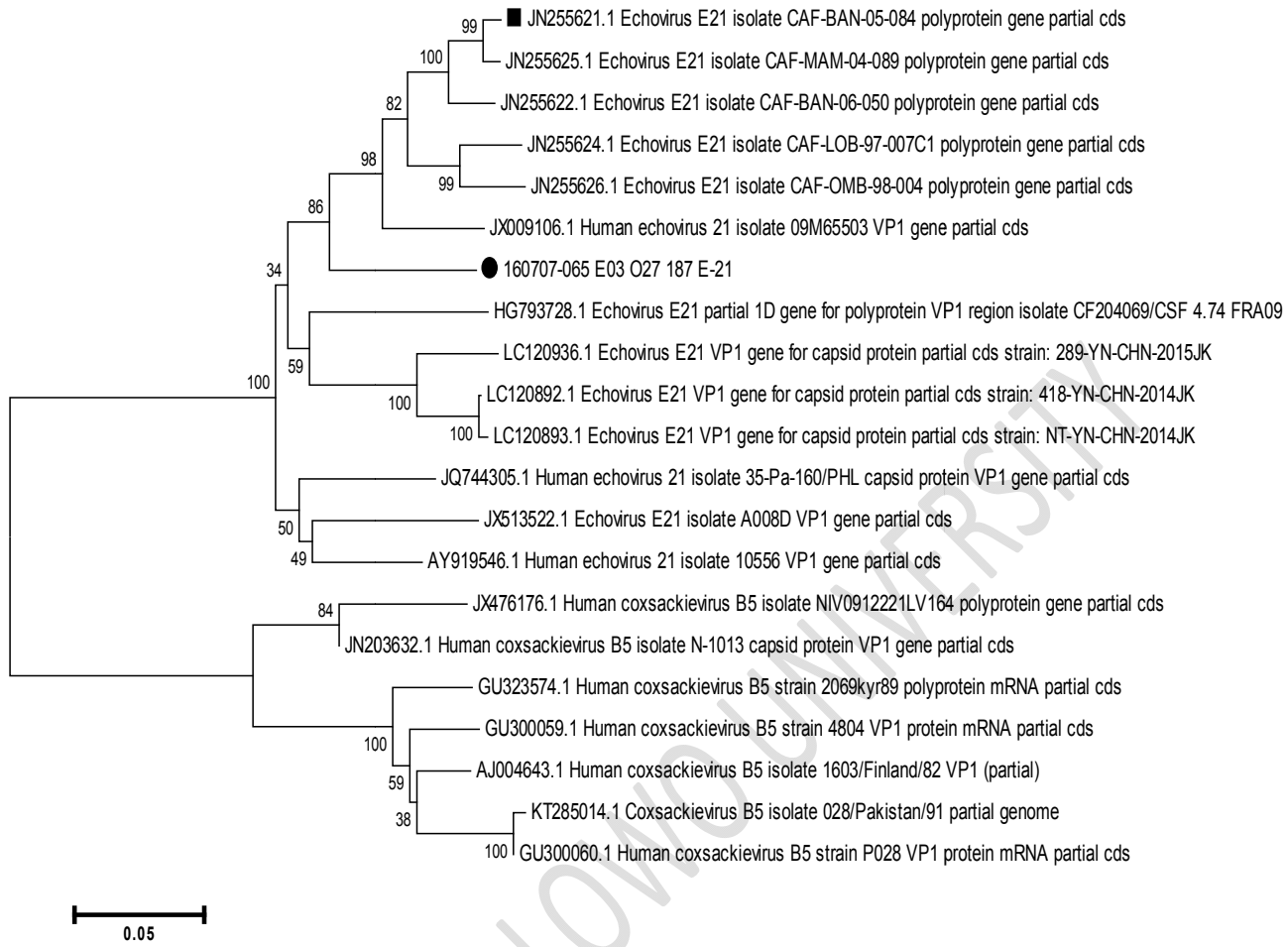


Figure 4.6 Phylogenetic relationship of recovered Echovirus 21 strain and the positive controls. The phylogram is based on alignment of the partial VP1 sequences. The recovered sample and the controls are represented and are highlighted in black irregular circle. The GenBank accession number of both the recovered strain from Nigeria and reference strains are indicated in the phylogram.

CHAPTER 5

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

This study applied phylogenetic analysis for the characterization of non-culturable enterovirus species in stool samples of patients with Acute Flaccid Paralysis in Southwestern Nigeria. The results from this study revealed the rate of detection of enterovirus in the 60 samples analyzed as 30 cases in Southwestern Nigeria in children with AFP considered in Lagos, Oyo, Ondo, Ogun, Osun and Ekiti State. Among the species of enteroviruses that exist, only species B of the enteroviruses was recovered in this study. That is, one of the 30 cases, 3.33% or (1/30) showed enterovirus species B as the only species of EVs.Faleye *et al*., (2012)reported the high prevalence of EVB EVs circulating in SW among other in his study. There was a report of ten (10) detection of EVs off which nine (9) are members of species B.

On the contrary, Faleye *et al*., (2016)revealed in his study that species A enterovirus were more commonly detected than members of other enteroviruses species but the significance of his study was not easy to determine as this may be due to the samples considered or used in the analysis.Faleye *et al*., (2006) made use of feacal samples of healthy childrenmeanwhile, in this study, feacal samples of children with AFP were considered. Also, the samples usedby Faleye *et al*. (2006) bypassed cell-culture and method of direct detection and identification of enteroviruses was employed while all the samples in this study were first subjected to cell-culture before Nix *et al* (2006).

The result of this study showed the presence of only one serotype of non-polio enteroviruses in children in six States in south-western Nigeria. This study documents the first molecular sequencedata on Echovirus 21 (E-21) in Oyo State Nigeria. Echovirus 21 in research work

has been detected in Southwest Nigeria in Lagos State from environmental sample. Echovirus 21 (E-21) isolated

from this study showed a high nucleotide similarity with the isolate from Central Africa Republic in 2012. The fact exists that the first isolation was documented to occur from thee feaces of asymptomatic children early in the 1950s, just after cell culture was developed (Yin-Murphy and Almond, 1996).

However, the virus (E-21) has not been implicated with Acute Flaccid Paralysis (Oyero *et al*., 2010) but has shown the increasing rates of genetic recombination of the species with PV in China and other part of the world (Jingjing *et al.,*2010).Echoviruses are similar to many enteroviruses and may be associated with pyrexial illness with sore throat, cough (coryzo), herpangina characterized with vesicular, oral mucosal process involving the tonsilla fossa and soft palata symptoms which include elevated temperature, pharyngitis and dysphagia commonly observed in summer outbreaks involving young children of less than 10 years and less common in young adults (Tao *et al*., 2014).

Herpagina begins abrupty with fevers as high as 104 ^{0}F and it is associated with non-persistent vomiting, myalgia and headache, dysphagia and the prominent symptoms that proceed the appearance of oral lesion. Fatal echovirus induced meningoencephalitis often associated with a dermatomyositis. The acronym Echo is for enteric cytopathic human orphan (https:www.ncbi.nlm.nih.gov, article).

5.2 CONCLUSION

In conclusion, this study documents that there is possibility the stool of children with Acute Flaccid Paralysis contain enteroviruses, also the present species of enteroviruses may not be detected by cultural method and that the genotype of the enteroviruses can characterized by phylogenetic analysis. Based on the results obtained from this study, it is apparent clear that

the prevalence ofEVs in south-western Nigeria is low. The findings of this study was able to generate result about enteroviruses species present in samples of children with AFP which were negative during culturing method. It thereby established and confirmed that enteroviruses could be present in feacal samples (stool) which may be negative when subjected to cell-culture. Furthermore, it shows that Nix *et al*., (2006) protocolis able to amplify the most prevalent genome even when mixtures are present in the samples. In addition, this study characterized the genotype of the enteroviruses by phylogenetic analysis thereby establishing the recovery of (E-21) of species B in a male child from Oyo State. Though E-21 has formally been discovered in Lagos State. Consequently, this study can serve as a baseline for other molecular studies in Polio Eradication program.

5.3 RECOMMENDATION

Based on the findings in this study, it is therefore recommended that:

Studies base on the RD cell line that have associated certain enteroviruses strains should be interpreted with care because there is likeliness that not all the enteroviruses in the samples were detected. Hence, caution should be taken before concluding that samples are free or negative of enteroviruses. Therefore, culture method should not be the only concluding method other method such as molecular technique should also be used. That is, the both cultural and molecular method of identification should be used for detection and proper isolation of non-culturable enteroviruses.

Highly sensitive and very specific method for enterovirus detection in specimens is essential especiallyin feacal samples and others which might contain viruses. As these methods will help to easily detect and identify enteroviruses anytime they are present and to resolve members of different species most especially, members that are of the same species which are present in the same specimen. Ability to do this will make it easy to better characterize

enterovirus serotypes that may be present in any sample or specimen and or concurrently circulating in a population at any time. Doing this might then shed light on the enigma presently existing in the field of enterovirology.

CHAPTER ONE

1.1 INTRODUCTION

Global Polio Eradication Initiative (GPEI), has a network of about 150 laboratories across the globe (Global Polio Laboratory Network (GPLN)) that isolate non-polio enteroviruses (NPEVs) as a by-product of poliovirus surveillance. GPEI emerged as a result of the World Health Assembly's (WHA) resolution in the year 1988 to eradicate poliomyelitis by the year 2000 and were able to achieve a decrease of cases of poliomyelitis from 350000 in 1988 to 416 in the year 2013 (WHO, 1988; Kew *et al*., 2003). With this mandate, wild Polio Virus was eliminated all over the world except from countries like Nigeria, Afghanistan and Parkistan (Kew *et al*., 2003). Oral poliovirus vaccine (OPV), a live attenuated vaccine containing Sabin polioviruses was one of the primary instruments used by the GPEI to achieve this purpose.

The epidemiological pattern of enteroviruses differ by geographical region and climate, but the occurrence of infection is higher in the summer and autumn months in temperate climates while remaining prevalent year-round in the tropical climates. Non-Polio enteroviruses are very common and distributed across the globe and go unnoticed, these viruses are also associated with occasional outbreaks in which a larger number of patients develop clinical disease, sometimes with fatal consequences (Noyce*et al*., 2011). A person gets infected with non-polio enterovirus by having close contact with an infected person, touching objects or surfaces that have the virus on them, touching mouth, nose or eyes. The virus may be excreted in the stool for many weeks (NCIRD, 2014).

Enteroviruses (EVs), being a group of more than 250 naked icosahedral virus serotypes, have a diameter of 28–30 nm, are categorized as members of the family Picornaviridae, genus Enterovirus and order Picornavirales. Within the genus are 12 species and these have been identified with human infection and disease. These include (enterovirus A–D (EV-A, EV-B, EV-C, and EV-D)) and human rhinovirus (HRV) A-C (HRv-A, HRv-B, HRv-C))

(http://www.picornaviridae, 2016). Within the naked (non-enveloped) icosahedral capsid of an enterovirus is a positive-sense, protein-linked, single-stranded, approximately 7.5 kb RNA genome, of a single open reading frame (ORF). The ORF is flanked on both sides (5^1 and 3^1 ends) by untranslated regions (UTRs) and translated into an approximately 250-kDa polyprotein. This polyprotein is auto-catalytically cleaved into P1, P2 and P3 polyproteins, which are further cleaved into VP1–VP4 (VPI, VP2, VP3, VP4), 2A–2C (2A, 2B, 2C) and 3A–3D (3A, 3B, 3C, 3D), respectively (http://www.picornaviridae 2016).

The capsid is formed as VP4 is buried (submerged) within the virion and VP1, VP2 and VP3 are exposed on the virion outer surface. That is VPI – VP4 forms the capsid. The other seven polyproteins (2A–2C and 3A–3D), are nonstructural proteins and are crucial in enterovirus replication. Enteroviruses have been associated with a host of clinical conditions. The replication of enterovirus begins right in the gastrointestinal or respiratory tract and once the virus is present in the blood stream, infection may affect various tissues and organs, causing a variety of diseases (Tassin *et al*., 2013)**.**

Enteroviruses are associated with specific syndromes for example, the viruses that are within the *Human enterovirus* speciesA, most of the time cause hand-foot-mouth disease (rash especially on the soles and palms with vesicular eruption and inflammation of the mouth). While those within the *Human enterovirus B species,* cause meningitis or myopericarditis. Besides the association of different enterovirus types with the same clinical manifestation, the same enterovirus type has also been known with different clinical manifestations.

In addition, because more than 90% of enterovirus infections are asymptomatic**,** most of the infections with clinical manifestation represent less than 10% of enterovirus infections. (Nathanson *et al*.,2010). The majority of infections are asymptomatic or mild in nature, the most common effect usually is a non-specific illness, with fever. Other manifestations include

exanthems (rashes), herpangina (vesicular eruption and inflammation of the throat), pleurodynia, gastroenteritis, acute respiratory disease, pancreatitis, hepatitis, type 1 diabetes, conjunctivitis, aseptic meningitis, encephalitis (inflammation of the brain), myopericarditis (inflammation of the heart tissue), upper and lower respiratory tract diseases and occasionally, paralysis or myelitis (Tapparel*et al*.,2013).

Moreover, anyone can get infected with non-polio enteroviruses but young children (infants) are most susceptible to infection. Infants and people with impaired immune system have a greater chance of having complications and infection of the heart or brain or paralysis. In less developed areas, children may become infected during early infancy while in more socio-economically advanced areas, first infection may not occur until adolescence (NCIRD, 2014). These groups are mostly infected because they do not have immunity. For instance, the Centre for Disease Control (CDC) reported high increase of children across the United States who developed Neurologic illness called Acute Flaccid Myelitis in 2014 and 2015. Males most of the time develop clinically-recognizable diseases than females (NCIRD, 2014).

Besides the clinical manifestation, another dimension to the enterovirus diversity landscape is in

their receptor usage. The prevailing paradigm is that a particular enterovirus serotype uses one or a defined set of receptors (and co-receptors). In human *enterovirus species* A, two receptors SCARB2 and PSGL1 are used. Studies have now recently shown that CD150 is not the only cell surface receptor for measles virus (Tatsuo*et al*., 2000)**.** When being transferred from lymphocytes to epithelial cells, measles viruses use poliovirus receptor-like 4 (PVRL4) as their receptor to enter into epithelial cellsof the host (Noyce*et al.,*2011).

In similar light, CV- A20 strains exist and can independently use either Intercellular Adhesion Molecule 1(ICAM 1) or another yet to described cell surface molecule as the receptor (Arita*et al*.,2013).

1.2 STATEMENT OF RESEARCH PROBLEM

Over the years, there has been under-reporting of non-cultural enteroviruses in Nigeria as a whole most especially Southwestern in Nigeria. Despite increasing number of cases of Acute Flaccid Paralysis detection of enterovirus in cell culture is low. It has been revealed that lack of unique cell line for all enteroviruses accounts for this hence the study**.**

1.3 RESEARCH JUSTIFICATION

Despite the declaration of WHO that Nigeria has been removed from the list of Polio endemic countries, Acute Flaccid Paralysis (AFP) caused by enteroviruses remain an important clinical presentation in the country. Most isolates recovered from the Nigeria vaccine derived poliovirus serotype 2 (VDPV2) outbreak were recombinant with nonstructural region of Non-Poliovirus enterovirus Species C (NPESC) origin. Most human Enterovirus (hEV) infections have no symptoms and consequently go unnoticed. The symptomatic infections represent approximately 1 in 100 to 1 in 1000 infections depending on the serotype being considered (Nathanson and Kew, 2010).

Reports from previous works showed that enteroviruses isolated using RD cell line were usually not the complete picture. Most of the time, other enteroviruses are present in the sample that will not grow on the Rhabdomyosarcoma (RD) cell line. Even when a clinical sample is negative for enteroviruses by the pan-enterovirus VP1 screen based on primers (Nix *et al*., 2006) usually, the species-specific screen still detects, sometimes different serotypes in the same sample. An enterovirus serotype that was not detected in the clinical

specimen even after repeated screening will show up after cell culture (http://www.picornaviridae.com, 2016).

Furthermore, studies that documented the isolation of NPESC members made use of isolation protocols slightly different from the R-L protocol. Such studies included Hep-2 cell surface expression of ICAM-1 and this has greatly improved the recovery of NPESC members, more importantly the coxsackievirus (Huang *et al*., 2000). Several studiescarried out on enteroviruses in countries like Pakistan, Afghanistan and Nigeria have immensely contributed to knowledge by throwing more light to the importanceenteroviruses which is the etiological agent of AFP. Further work and effort is required to identify the Non-Polio enterovirus species, to resolve members of different species and, more specifically, members of the same species present in the same sample. Molecular methods have not been routinely used to detect and characterize non-culturable enteroviruses in feacal samples from patients with Acute Flaccid Paralysis, therefore the study focused on detection and identification of non-culturable species of enteroviruses using a negative feacal sample of poliomyelitis which were collected from patients with Acute Flaccid Paralysis(Arita*et al*., 2005).

1.4 OBJECTIVES OF THE STUDY

The general objective of the study is to determine the presence and prevalence of non-culturable Species A, B and C enteroviruses in the feacal samples collected from patients with Acute Flaccid Paralysis in the National Polio Laboratory in Ibadan Oyo State Nigeria.

The specific objectives of this study are:

- To detect and identify enteroviruses species in patients samples with Acute Flaccid Paralysis.
- To characterize the genotype of the enteroviruses by phylogenetic analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF ENTEROVIRUSES

Enteroviruses (EVs) are human pathogens that are very important and are associated with various clinical syndromes of which their infections are common in humans across the globe. Till date, EV infections are of great importance and remain a vital public health problem. Enteroviruses belong to the family PICORNAVIRIDAE(Landsteiner *et al*., 1908). This family comprises five genera this include, rhinoviruses, hepatoviruses, cardioviruses, aphthoviruses and enteroviruses (Blomqvist and Roivainen, 2016).They cause disorders with various clinical manifestations, which include cutaneous, visceral, and neurological diseases. The study of Enteroviruses began as a result of the dreaded disease poliomyelitis. This was revealed in 1908 when enteroviruses was shown to be a "filterable agent". Afterword the concept of poliovirus (PV) started taking form.

Consequent to its isolation in tissue culture and the demonstration of its serological types (Kessel*et al*., 1949). Poliovirus started off the field of enterovirology. This occured because, in addition to polioviruses, other enteric viruses were found to be present in the faeces of children that have "paralytic disease" indistinguishable from poliomyelitis.These other viruses include coxsackievirus A (CV-A) and B (CV-B), echoviruses (Es) and the numbered enteroviruses. These viruses were isolated and identified using a combination of histopathology in newborn mice, cytopathic effect (CPE) in cell culture and serology (Dalldorf, 1949; Melnik *et al*.,1950).

For many years polioviruses were the most important Enteroviruses, since they caused great outbreaks of paralytic disease. Though known and dreaded for its paralysis-causing ability. The concept of poliovirus (PV) started taking shape after its isolation in tissue culture and the demonstration of its serological types (Kessel *et al*., 1949).

2.1.1 New Emergence of EV-D68 and other Respiratory EVS

In the year 1962 in California, USA, EV-D68 a member of the small EV-D species was first isolated in respiratory samples of 4 children that have respiratory diseases (Schieble *et al*., 1967). Enterovirus D has the characteristics of both RV and EV, such as acid lability and optimal temperature of 33^0C (Oberste *et al*., 2004). Being isolated from respiratory samples, many strains of EV-D68 were classified initially into RV genus as RV87. Later, studies based on genetics and antigenicity revealed that they were similar to EV-D68 strains. Finally, all RV87 strains were reclassified as EV-D68 type (Blomqvist *et al*., 2002) which was not common until year 2000. Some cases of EV-68 were continually reported in various parts of the world in the last decade and were accounted to mild and chronic respiratory illness (Imamura *et al*., 2011; Tokarz *et al*., 2011; Meijer *et al*., 2012). In 2014, there was a serious outbreak of EV-68 in the USA during autumn with unprecedented levels of circulation across the nation most especially, in the pediatric population. During this time, a total number of 1153 individuals in 49 states and the District of Columbia tested positive for EV-68, very common in children, some with report of wheezing or asthma (CDC, 2014; CDC, 2015).

The rapid emergence of this virus (EV-68) over the years was first believed to be as a result of new methods of detection and to former misidentification as a RV leading to an underestimated prevalence test based on retrospective findings which affirmed the increment of prevalence (Ikeda *et al*., 2012, 2016). Recently, the phylogenetic analysis revealed an increased diversity in VP1 sequences of the recently discovered EV-68 strains. These discovered strains have different genetic lineages that are obviously different from the prototype strains (Khetsuriani *et al*., 2006;Kreuter *et al*., 2011; Langereis *et al*., 2014). Aside respiratory tropism of EV-68, its infections have also been highly associated with neurologic disease and occurrence of acute flaccid paralysis (Khan, 2015; Lang *et al*., 2014; Pfeiffer *et al*., 2014, 2015) hence, reflecting the link of Viruses.

EV-D68 makes use of Sialic acids (SA) as a receptor binding molecule, this is the same with many other viruses that have great affinity for -2-6-linked SA more than -2-3-linked SA. The sialytated glycans are found on the other cell membranes of human respiratory track (Imamura *et al*., 2014). Apart from this, EV-70 and coxsackievirus A24 variant (CVA24v, member of EV-C) (Zocher *et al*., 2014) also have affinity for sialic acid and are responsible for symptoms in the upper respiratory tract and acute hemorrhagic conjunctivitis. Furthermore, they are the causative agent of neurological impairment such as acute flaccid paralysis, and are considered to have a pandemic potential. These 3 viruses (EV-67, EV-68 and CV A24v) have different pathogenesis but use the same receptor and this suggests the same mechanism that needs to be investigated the more (Nilsson *et al*., 2008).

EV-C104, EV-C105, EV-C109 , EV-C117 , and EV-C118 are the other non-RV EVs that were discovered to emerge from species C as these viruses are widely distributed across the globe they cause diseases that vary in their severity beginning without symptoms which could either be mild respiratory infections to complicated ones like pneumonia (Tokarz *et al*., 2013). Besides, another EV-C, coxsackievirus-A21 is said is to cause mild respiratory illness (Xiang *et al*., 2012) and it uses ICAM-1 as its receptor which is the main receptor used by the group of RV (Xiao *et al*., 2005).

2.1.2 ORIGIN AND CLASSIFICATION OF ENTEROVIRUS

Enteroviruses work began in 1908 when it was revealed that it is a filterable agent and has an outcome or manifestation of poliomyelitis disease which has ability to cause paralysis (Landsteiner *et al*., 1908). Then the concept of poliovirus (PV) began to take form. Before enterovirus tissue sub-culturing and application of its serological types, poliovirus made the field of enterovirology to emerge (Kessel *et al*., 1949). This occurred because aside from polioviruses found in the feces of children with paralysis, other enteroviruses like coxsackievirus A (CV-A) and B (CV-B), echoviruses (Es) and the numbered enteroviruses

were found and these are indistinguishable from poliomyelitis. Isolation and identification of these viruses was carried out using serology and cytopathic effect (CPE) in cell culture and histopathology in newborne mice (Melnik, *et al*., 1950).

Further studies in experimental animals revealed that CAVs affect skeletal and heart muscle, while CBVs do replicate in a wide range of tissues including the central nervous system, liver, exocrine pancreas, brown fat and striated muscle. The adoption of culture techniques into virus laboratories enabled the isolation of viruses which did not replicate in experimental animals. Echoviruses however were isolated from stool samples where polioviruses and coxsackieviruses are usually also found, but did not possess the pathogenic properties of these subgroups in experimental animals. The name echovirus was chosen as a result of its association with human disease which was at the time of discovery unknown ECHO enteric, cytopathogenetic, human, associated disease (Tao *et al*., 2014).

The Committee of ECHO Viruses postulated in 1955 that whenever an echovirus was affirmed as the etiological agent of a clinically specific disease, it would be removed from the group. However, it soon became evident that individual serotypes cannot be directly associated with individual illness but with a wide range of clinical manifestation. Wherefore, echoviruses are still classified together and they form the largest enterovirus subgroup which consists of several serotypes (Oyero *et al*., 2010).

Early in the year 1950, monkey kidney cell cultures was used for the growth of polioviruses and this revealed the presence of latent simian viruses. Kalter *et al*.,1980furthered the investigations that revealed that some of these polioviruses possess properties of enteroviruses and consequently 18 serotypes were described. Investigations of viruses that were infecting domesticated animals in the late 1950s revealed the presence of enteroviruses in both pigs (*Sus scrofa*) and cattle (*Bos taurus*). Afterward, about 13 porcine enterovirus

(PEV) serotypes (Honda *et al*., 1990; Auerbach *et al*., 1994) and bovine enterovirus (BEV) serotypes (Urakawa and Shingu, 1987) were described. Enteroviruses isolated from African buffalo (*Syncerus caffer*), water buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*), deer (*Cervus nippon*) and impala (*Aepyceros melampus*) have been shown to be related to BEV-1 (Urakawa and Shingu, 1987).

Implicit in the history of enterovirus classification is the existence of antigenically distinct viruses which can be said to constitute serotypes. An operational definition for a serotype includes the notion that a strain represents a new serotype if it is not neutralized to a significant extent by antisera to previously characterized viruses and if it is unable to induce significant levels of neutralizing antibodies to these viruses (Committee on Enteroviruses, 1962). Unfortunately, in practice, some virus serotypes tend to include strains occurring as an antigenic continuum which multiplex the use of reference antisera. This lead to difficulty in serotyping some isolates due to poor identification by reference antisera and the observation of extensive cross reactivity between some serotypes, identification of enterovirus was primarily based on serotypic differentiation, and these difficulties were significant for epidemiology and diagnosis. On the other hand, distinction between the three serotypes of poliovirus is clear, and inclusion of one representative of each serotype is necessary and sufficient in poliovirus vaccines.

Few years later, when enterovirology began, neutralization assay was employed for isolation and identification of isolate and this prevented the manifestation of cytopathic effect of enteroviruses (Minor *et al*., 1983; Emini *et al*.; 1983; Evans *et al*., 1983., Chow, 1985) in cell culture and was seen altered after antibodies elicited against PVI protein had neutralizing ability. Consequently, the binding sites of these antibodies which have the neutralization power were localized to certain epitopes in the protein product of the VP1 gene.

As a result of Global Polio Eradication Initiative (GPEI's) activities, annual cases of poliomyelitis decreased from 350,000 in 1988 to 416 in 2013 (www.polioeradication.org) and poliomyelitis has been eliminated globally except in countries like; Nigeria, Pakistan and Afghanistan (WHO, 2012). The method used to accomplish this goal include active poliovirus surveillance programme in addition with massive vaccination campaigns World Health Organization (2004). The inactivated poliovirus vaccine (IPV) and oral poliovirus vaccine (OPV) were the workhorses used during the vaccination campaigns. The OPV has been the vaccine of choice in developing countries because, they are not expensive and hence more affordable, as opposed to IPV confers mucosal immunity to vaccines, thereby reducing the number of susceptible individuals that can participate in poliovirus transmission (Nathanson, 2010).

Furthermore, various studies have confirmed silent circulation of enteroviruses in the environment even though in the absence of associated clinical conditions in the community. Most hEV (human enteroviruses) infections are asymptomatic, and go unnoticed. The symptomatic infection could be represented approximately 1 in 100 to 1 in 1000 infections and this will depend on the serotype that is being considered (Nathanson and Kew, 2010). As a result of this gap, symptomatic hEV infection surveillance is considered inadequate when knowledge of hEV circulation in any population is requested. Thus, environmental surveillance (ES) approach has been one of the tools used in the study of enteroviruses as it is seen to be more dependable to generate adequate information on hEV circulation in a population. Environmental surveillance for hEVs is a method for rapidly documenting circulating strains of hEV in any population by sampling sewage or sewage-contaminated water (SCW) within the community in a particular geographical area.

In 1988, great effort was made by the World Health Assembly to resolved and eradicate poliomyelitis by the year 2000 (WHO, 1988) and there was an establishment of Global Polio

Eradication Initiative (GPEI) with this mandate. Prior to molecular identification, it was difficult to type these non-polio enteroviruses (NPEVs) hence, some types were not known because the access-able panel of antisera could mainly recognize just 40 of the first 66 enteroviruses that were characterized (Oberste *et al*., 2000). As a result of this, there was an array of unidentified enteroviruses which are referred to as the 'untypables'. There has been a great improvement with the help of molecular identification and many of the previous untypable enterovirus isolates have been discovered and identified.

Hence, the use of ES has been recommended as a central part of the Global Polio Eradication Initiative (GPEI) in other to monitor the presence of poliovirus in high-risk environment most especially when gaps are suspected in the Acute flaccid paralysis (AFP) sentinel surveillance system (WHO, 2003). Aside poliovirus, which is the target pathogen of AFP, isolation, and identification of non-polio enteroviruses (NPEVs) was also done by neutralization test using pools of antisera which can only identify limited number of NPEVs (Apostol *et al*., 2012).

More recently, non-polio enteroviruses became the center of many studies, where recombination was recognized as a frequent event and was linked with the appearance of new enterovirus origins and types. The accumulation of both inter- and intra-typic recombination events could also show light on the series of successive emergences and disappearances of specific enterovirus types that could in turn explain the epidemic profile of spreading of several types (Kyriakopoulou *et al*., 2015).

2.2 CLASSIFICATION OF ENTEROVIRUSES

The human enteroviruses (EV) family consist of up to 100 immunologically and genetically distinct types. They are Polioviruses, coxsackieviruses A and B, echoviruses, and the more recently characterized 43 EV types. Constant recombinations and mutations observed in enteroviruses have been recognized as the major mechanisms for the resulted high rate of

evolution, thus enhancing them to rapidly respond and adapt to new environmental factors. The first signs of genetic exchanges between enteroviruses came from polioviruses some years back, and ever since the recombination has been observed along with mutations, as the major cause for reversion of vaccine strains to neurovirulence (Kyriakopoulou *et al*., 2015).

Before the use of molecular method for the purpose of identification, enteroviruses were classified based on historical lines as Polio viruses (PVs), Coxsackievirus A (CV-A) and Coxsackievirus B (CV-B), Es and numbered EVs. However, identification through the molecular method and phylogenetic process or analysis revealed that human enteroviruses could mainly be classified into four (4) specific species (EVA, EVB, EVC and EVD). This further proceeded in the incorporation of poliovirus into the EV-C and the reclassification of the existing CV-A15, CV-A18, HRV-87 and the swine vesicular disease virus (SVDV to CV-A11, CV-A13, EV-D68 and CV-B5 respectively (Brown *et al*., 2013).

Enterovirus species A (EV-A) contain 25 serotypes which is made up of CV-As and a few numbered enteroviruses. In Enterovirus virus species B (EV-B). There are 63 serotypes found containing CV-A and CV-B, the echovirus and some numbered enteroviruses. Enterovirus species C (EV-C) consists of 23 serotypes containing the remaining CV-As, the three poliovirus (PV1, PV2, and PV3) serotypes and some numbered enteroviruses. Enterovirus D (EV-D) contain just five serotypes which consist only of the numbered enteroviruses (http://www.picornaviridae.com 2016).

According to the International Committee on Taxonomy of Viruses (ICTV), enteroviruses (EVs) belong to the Enterovirus genus of the family Picornaviridae and have been classified into twelve main species of which only five species infect animals (EVE–J), the remaining seven species are known to infect humans. Species A–D and Rhinovirus species A–C are specifically the only species in the genus that have been reported to infect humans. Within

each species are several serotypes and the genus, as a whole, comprises of more than 200 serotypes such as polioviruses (PV), coxsackieviruses A and B (CV-A and -B), echoviruses, numbered EVs, and human rhinovirus (HRVs), among them including several important human pathogens such as PV, CV-A16, CV-B3, EV-A71, EV-D68, and HRV (http:// www picornaviridae, 2015).

2.3 ENTEROVIRUSES (EV) GENOME AND VIRAL PROTEINS (VP)

The enterovirus virion is a non-envelop, icosahedral symmetry with diameter of 27 to 30nm (27-30nm). The enterovirus genome is a positive-sense single-stranded RNA molecule having 7000–8000 nucleotides comprising a single open reading frame (ORF) with a 5^1-untranslated region (5^1-UTR) and a 3^1-UTR. The 5^1-UTR contains an internal ribosomal entry site (IRES) which is useful for the binding of the 40S ribosomal subunit to initiate cap-independent translation, while the 3^1-untranslated region (UTR) contains a pseudoknot and a poly (A) tail. The open reading frame (ORF) encodes a large polyprotein precursor, which comprises of P1, P2, and P3 regions which are further cleaved to make 11 proteins. In enterovirus (EV) infected cells, this polyprotein precursor is initially cleaved between P1 and P2 by viral 2A proteinase, while the P2-P3 point is cleaved by 3C proteinase (Racaniello, 2013).

Finally, this precursor is processed into mature viral proteins, including four structural proteins that give rise to the four viral capsid (VP1, VP2, VP3, VP4) and seven non-structural proteins (2A–2C and 3A–3D)(Figure 2.1). The 2A and 3C proteinase in particular have profound effects on host cells by modulating proteins related with translation, apoptosis, natural or innate immunity, RNA processing and polyadenylation (Weng *et al*., 2009; Lei *et al*., 2010; Wang *et al*., 2013). A link has been affirmed between VP1 nucleotide sequences and distinct serotype(Oberste *et al*., 1999;Oberste *et al*., 2005).

Moreover, VP1 nucleotide sequences have become a tool used in typing enterovirus isolates into serotypes (Oberste *et al*., 1999; Oyero *et al*., 2014; Adeniji and Faleye, 2014). Furthermore, studies have affirmed that all regions of the enterovirus genome except the 5^{I}-UTR can be used for typing enteroviruses into various species (Oberste and Pallansch, 2005;Oberste *et al*., 2006).

2.3.1 The Value of VP1 in Enterovirus Identification

At the beginning of enterovirology, identification of isolate was done by neutralization assays, and this prevented the development of the enterovirus-specific cytopathic effect in cell culture.

Later, it became clear that antibodies elicited against the VP1 protein had neutralizing activity (Chow*et al*.,1982; Wychowski*et al*.,1983)**.** Consequently, the binding sites of these neutralizing antibodies were localized (Emini *et al*., 1983; Evans *et al*., 1983; Minor *et al*., 1983; Chow*et al*., 1985) to specific epitopes in the protein product of the VP1 gene.

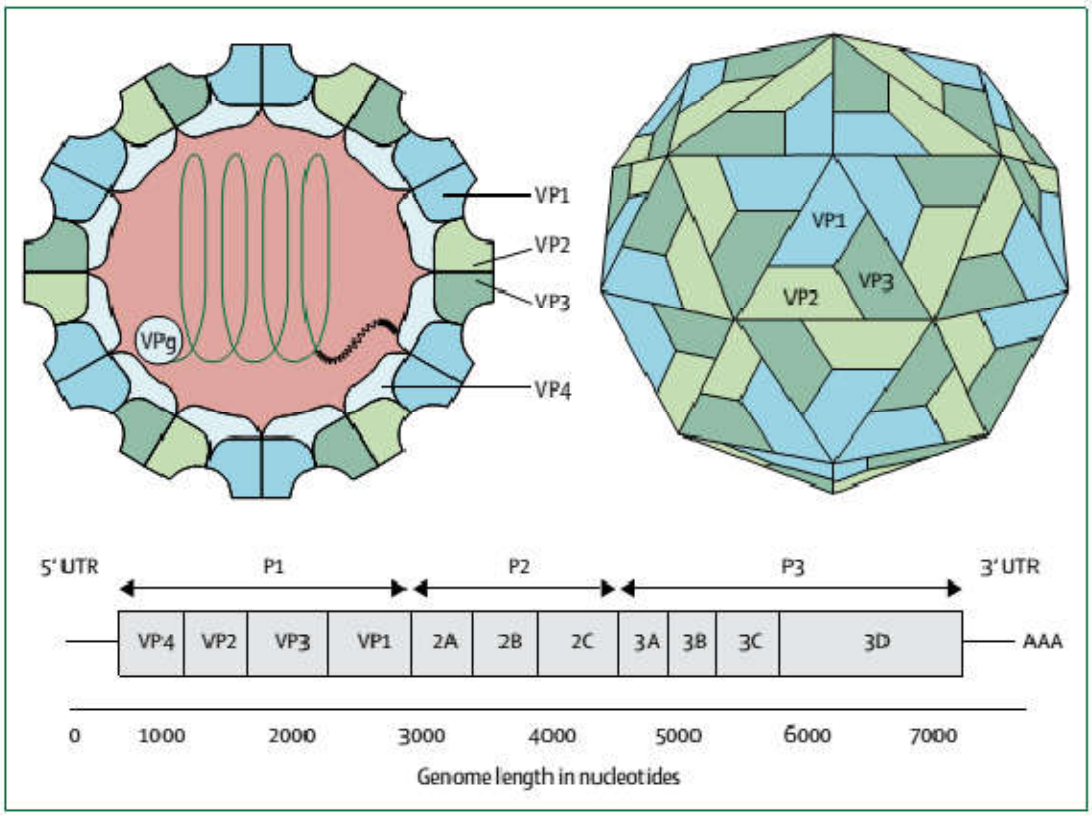
TABLE 2.1 Classification of Enteroviruses Enterovirus Species and Type

Enterovirus Species	Type
Human enterovirus A	
Human coxsackievirus	A2–8, 10, 12, 14, 16
Human enterovirus	71, 76, 89–92, 114, 119–121
Human enterovirus B	
Human coxsackievirus	A-9, B1-6
Human echovirus	1–9, 11–21, 24–27, 29–33
Human enterovirus	69, 73–75, 77–88, 93, 97–101, 106, 107, 110–113.

Human enterovirus C	
Human coxsackievirus	A1, 11, 13, 17, 19–22, 24.
Human poliovirus	1–3
Human enterovirus	95, 96, 99, 102, 104, 105, 109, 116–118.
Human enterovirus D	
Human enterovirus	68, 70, 94, 111, 120.
Rhinovirus A	
Human rhinovirus A	1, 2, 7–13, 15, 16, 18–25, 28–34, 36, 38–41, 43, 45–47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94, 96, 100–109.
Rhinovirus B	
Human rhinovirus B	3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91–93, 97, 99, 100–106
Rhinovirus C	
Human rhinovirus C	1-55

(http://www.picornaviridae, 2015).

Enterovirus genome organization



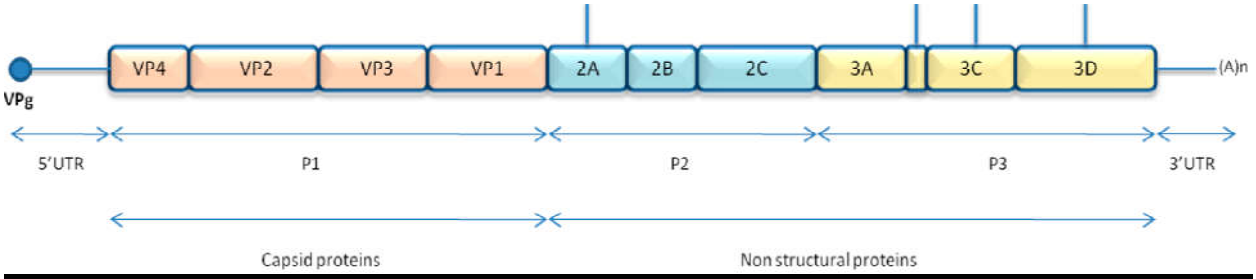


FIGURE 2.1 **Viral and genome structure of enterovirus(Brown and Pallansch, 1995).**

2.3.2 Serotypes and Genotypes of Enteroviruses

The confluence of four things encouraged enterovirus molecular identification, these include the existence of previously neutralized and identified pure cultures of enterovirus reference strains, discovery that the VP1 gene was greatly responsible for defining or identifying enterovirus serotype, the mainstreaming of primer synthesis and polymerase chain reaction (PCR) finally the automation of Sanger sequencing.

With all the above in place, Oberste *et al*. (1999) showed the relationship between VP1 sequence data and enterovirus serotypes (Casas*et al*.,2001; Caro*et al*.,2001; Norder*et al*.,2001; Oberste*et al*., 2001;Oberste*et al*., 2003; Thoelen*et al*.,2003; Blomqvist*et al*.,2008). This was independently affirmed and asserted by various investigators applying both previously-neutralized pure cultures and field strains.

Moreover, enterovirus identification became synonymous with VP1 amplification and sequencing (molecular identification). Before molecular identification, enteroviruses were

recognized and classified as PVs, CV-A and CV-B, Es and numbered EVs based on history. However, the consequent phylogenetic analysis and molecular identification revealed that "human" enteroviruses could be unequivocally classified into four (4) different species (EV-A–EV-D). This in progression resulted in the incorporation of polioviruses into the EV-C and the reclassification of former CV-A15, CV-A18, HRV-87 and Swine Vesicular Disease virus (SVDV) to CV-A11, CV-A13, EV-D68 and CV-B5, respectively (Brown *et al*., 2003).

EV-A contains 25 serotypes made up of some CV-As and some numbered enteroviruses. EV-B contains 63 serotypes consisting of CV-A and CV-B, the echoviruses and some numbered enteroviruses. EV-C contained 23 serotypes consisting of the remaining CV-As, the three poliovirus serotypes and some numbered enteroviruses. EV-D contains five serotypes consisting only of numbered enteroviruses (http://www.picornaviridae.com. 2016).

2.3.3 Poliovirus versus Non-Polio Enterovirus

There has been several reports on poliovirus as human enterovirus contains over 100 types that are classified into 4 species, EV-A to EV-D. Poliovirus is known to be associated with Acute Flaccid Paralysis (poliomyelitis). This virus has tropism for epithelial cells of the alimentary tract and the central nervous system. It causes spinal and bulbar poliomyelitis currently. Usually, paralysis is irreversible and all the three polioviruses PV1, PV2, PV3 can give rise to paralysis. Following the experience in the year 1988 when World Health Organization (WHO) recommended a standard approach for polio surveillance in investigating, discovery and detecting of acute flaccid paralysis in cases that include standardized virological analysis of feacal samples of patient and sometimes those from contacts, China, in 1994 were able to discover a lot of non-polio enteroviruses (NPEVs) asides PVs.

Furthermore, other studies affirmed the existence of non-polio enterovirus species C, which is about 20 apart from the poiloviruses (http://www.picornaviridae.com 2016). It has also been reported that there are three serological types of poliovirus (PV1, PV2, PV3) attenuated versions that were developed as oral polio vaccines (OPV) and are used for immunization campaigns. The vaccine virus can revert to wild-type virulence and transmissibility. The genomic nature of such isolates, termed circulating vaccine-derived polioviruses (cVDPVs), show them to be mostly recombinant with OPV/NPEV-C structural and non-structural region sides or region respectively (Combelas *et al*., 2011; Burns *et al*., 2013).In addition, in Nigeria 403 cases of cVDPV 2 were confirmed from 2005 to 2011 and seven (7) out of all were OPV2/NPEV-C recombinants and were resolved into (23) independent occurrences most of which took place in Northern part of Nigeria.

The global eradication of polio has been set by 2018 with strategic objectives specific to the post-eradication era. These objective have special implications for countries that are already polio free-free. Part of the objectives is to detect and interrupt transmission of wild poliovirus by 2014 and new outbreaks due to cVDPV within 120 days of confirmation of the index case. Improvement of surveillance and immunization campaigns, laboratory containment and destruction of polioviruses and ensuring rapid outbreak responses are the key activities required to maintain our polio-free status.

2.3.4 Cases of Non-Polio Enteroviruses Isolation in other Countries

The Acute Flaccid Paralysis surveillance was conducted in all Shandong Province 138 countries all together and this involved over 600 sentinel hospitals. Patients less than 15 years of age were hospitalized of which male to female ratio was 1:9:1 for all cases right from year 1990 to 2013. The data for the surveillance in the years 1988 and 1989 were unavailable but NPEV strains were isolated during the same period. Between 1990 and 2013, a total of 9263 cases of AFP were reported (Zhang *et al.,* 2011). Isolation of EV was performed on all stool

specimens and 788 NPEV strains were isolated from the stool specimens of these cases. In addition, specimens from 1059 contacts of AFP were collected during the same period of which 170 NPEVs were isolated.

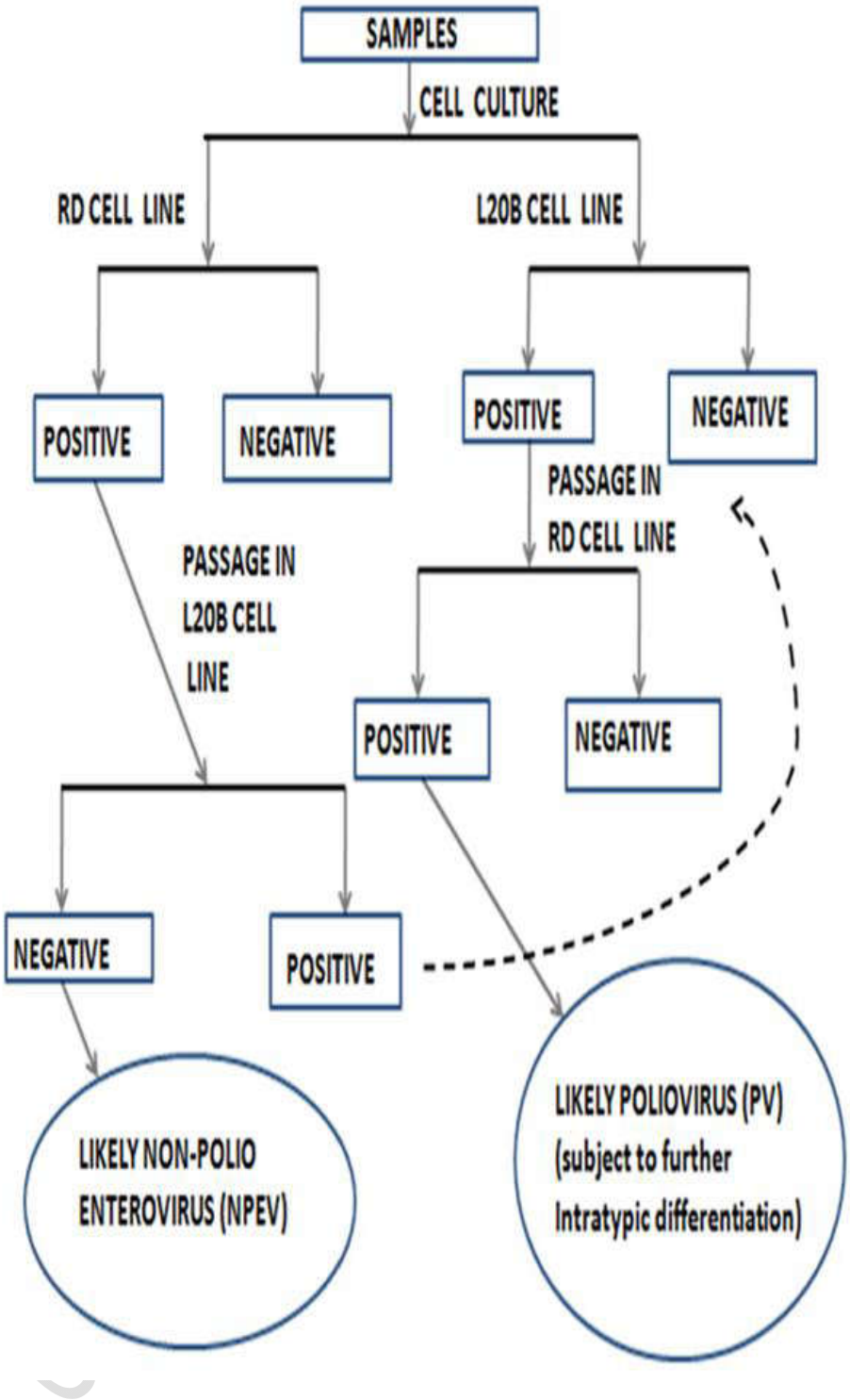


FIGURE 2.2 Poliovirus (PV) isolation algorithm as recommended by the World Health Organization (2003, 2004).

In 1990s, AFP surveillance in China was greatly increased but earlier reports before 1995 was not active and this resulted into low level of NPEV isolation before 2007. AFP reports got increased in summer and autumn months with a peak in the month of July with 1100 (11.9%) of 9255 reports with known month between 1990 and 2013. However, a more prominent seasonality of NPEV detection from AFP cases was recorded within June and October accounting for 79.3% (629 of 792) of isolation with known month of specimen collection (Tao *et al*., 2012).

2.4.0 LIFE CYCLE AND REPLICATION OF ENTEROVIRUSES

The entry of the virus in to the cell is determined by the cell surface molecule used as receptor, likewise as putative attachment receptors which is not the same in different enteroviruses. Depending on the type of virus and the type of the host cell, viral uptake can easily be mediated either by clathrin-dependent or-independent endocytosis or through macropinocytosis (Fuchs *et al*., 2010). Human beings are the only known natural hosts of human enteroviruses. The replication cycle of all species of enteroviruses is similar.Virions will then be faced by the drastic change in environment caused by the drop in PH or by receptor binding which give rise to uncovering of the hydrophobic domains, and this leads to pore-mediated release of the genome in the cytoplasm.

Afterward, a cap-independentinternal ribosomal entry site (IRES) mediated polyprotein synthesis is then mediated through the host cell ribosomal machinery, the viral polyprotein precursor acquired which is about 2000 amino acids then cleaved by the viral proteases into several viral proteins. Finally, the genome will be replicated in many folds in association with the cytoplasmic membranes (Belov *et al*., 2014).Replication of the enterovirus begins in the gastrointestinal or respiratory tract the moment the virus is present in the blood stream,

infection may affect various tissues and organs, causing a variety of diseases (Tassin *et al*., 2013).

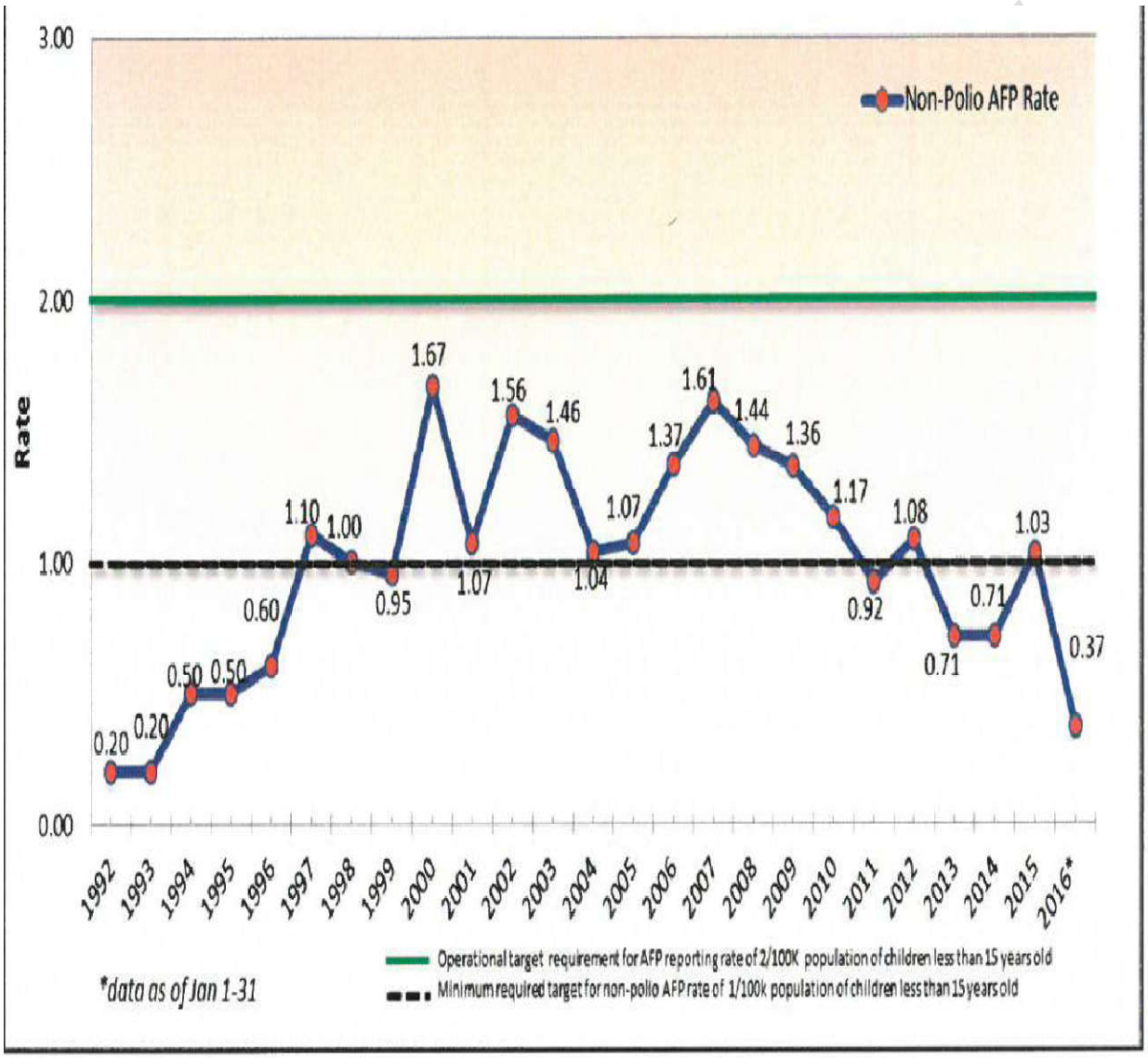


FIGURE 2.3 Trend of non-Polio AFP Rate in the Philippines 1992-2016

Enterovirus Species and Type (http:// www.picornaviridae, 2015).

It has been revealed that some enteroviruses use more than one receptor to infect a host cell. Several receptors for species A, B and C have been identified, but a ubiquitously expressed cellular receptor, scavenger receptor B2, and a functional receptor, human P-selectin glycoprotein ligand-1, found on white blood cells, are specific for EV71. Sialic-acid-linked glycan, expressed in abundance in the respiratory and gastrointestinal tracts, and dendritic-cell-specific intercellular adhesion-molecule 3-grabbing non-integrin (CD209), found exclusively in dendritic cells in lymphoid tissues, have also been identified (Yang *et al*., 2009). While some EVs are capable of utilizing multiple receptors, PV uses only one, CD155, an adhesion molecule also known as the human PV receptor (hPVR). In an in vitro blood-brain barrier (BBB) model, PV has been show to enter human brain microvascular endothelial cells (hBMECs) using hPVR in a dynamin and caveolin-dependent manner. Without any element of doubt, receptor expression on potential target cells defines the first barrier to virus entry (Yang *et al*., 2009). While the machinery of the host cellular protein synthesis is shut down by viral protease 2A, viral protein synthesis remains unaffected. An infectious virus particle is formed after the packaging of a progeny viral RNA into a virus capsid in the cytoplasm of the infected cells. Mature infectious virus particles are released when an infected cell is lysed(Tassin *et al*., 2013).

2.5 PATHOGENESIS OF ENTEROVIRUS INFECTIONS

The virus gains entry through the mouth or nose and when this happen, Enterovirus infects and replicates in the nasopharyngeal epithelium and regional lymphoid tissues, conjunctiva, intestines, mesenteric nodes, and the reticuloendothelial system.Enteroviruses can resist stomach acid and bile and are able to penetrate to the lower intestine, where they infect and

multiply in the intestinal epithelium and mesenteric lymph nodes. When viremia results; this leads to further multiplication of virus in the reticuloendothelial system. At that point, the virus can be carried by the bloodstream to target organs such as the spinal cord, brain meninges, heart, liver and skin. From the central nervous system the virus can travel via neural pathways to skeletal and heart muscles. It can be transferred by fingers and inanimate objects, such as towels and handkerchiefs to the eye, where it may replicate in the conjunctival epithelium and cornea (http:// www picornaviridae, 2009).

Individuals that are infected with Human enterovirus shed great amounts of hEV in faeces for many weeks irrespective of whether the infection is symptomatic or not (Ranta *et al*., 2001).The sensitivity of ES in detecting the circulation of different serotypes of hEVs in the absence of symptomatic infections in the community has been confirmed and documented by various studies. (El Bassioni *et al*., 2003; Sedmak *et al*., 2003; Manor *et al*., 2007;Kargar *et al*., 2009; Iwai *et al*., 2011).

Human EV infections are widespread and are the most common cause of aseptic meningitis, pericarditis, myocarditis and respiratory infections. Studies have confirmed EV infections as asymptomatic with about 50 – 80 causing clinically mild and self-limited infections. However, some species are able to cause severe and potentially fatal infections (http:// www picornaviridae, 2009).For instance, PV can invade the nervous system and cause poliomyelitis in children, the most significant disease caused by an enterovirus. CV can cause acute clinical manifestations ranging from mild febrile illness to more chronic conditions including meningo-encephalitis, pancreatitis, and fulminate sepsis in neonates (Tebruegge and Curtis, 2009). Some chronic diseases are also caused by CV, such as chronic myocarditis and type I diabetes (Whitton *et al*., 2005; Precechtelova *et al*., 2014).

2.6 IMMUNITY

2.6.1 Shut-comings of Host Protein Synthesis on the Evasion of EVs to Innate Immunity

Many species of enteroviruses tend to evade IFN responses in other to replicate or survive within the host cell. As a result, this has made the innate immune response a critical thing in controlling EV infection**.** PV infection results in the shutoff of host RNA and protein synthesis. During this process, theeukaryotic translation initiation factor 4G (eIF4G) in conjunction with the p220 subunit of the cap-binding protein complex cleaved by PV 2A proteinase (Lloyd *et al*., 1988). These revealed that PV 2A is related to diminished IFN production.

Moreover, eIF4G cleavage only partially induces translation shutoff, which implies that additional mechanisms are involved in EV-induced inhibition of translation (Kuyumcu-Martinez *et al*., 2004). Also, EV3C- mediated cleavage of poly (A)-binding protein (PABP) contribute a vital role in translation arrest.Furthermore, PV infection has the ability to hinder host cell RNA polymerase II-mediated transcription by 3C proteinase mediated cleavage of TATA-binding protein (TBP) and cyclic AMP-responsive element-consisting protein (CREB) (Weidman *et al*., 2001). These evidences demonstrated that EVs hinders the IFN signaling pathways, in some areas by the shutoff of host mRNA transcription and translation through 2A and 3C proteinase.

2.6.2 Interference by Pathogen Recognition Receptors (PRRs) Recognition

Detection of enteroviruses is done by Pathogen Recognition Receptors on cell surfaces and also in the cytosol, therefore EVs have developed mechanisms to antagonize these receptors. For instance, PV and HRV 1α infection helps the degradation of MDA5 in a proteasome and caspase-dependent manner. Moreover, degradation of this receptor is independent of 2A and 3C protease that is responsible for the inhibition of IFN production against the infection

caused by enteroviruses (Barral *et al*., 2007). On the other hand, CV-B3, EVA71 and PV 2A proteinases directly cleave in a caspase-dependent manner.Apart from MDA5, RIG-I is another special or critical target for CV-B3 and EV-A71 and their infections can cause RIG-I cleavage by 3C proteinases (Feng *et al*., 2012; 2016; Wang *et al*., 2014; 2015). These demonstrated that MAD5 and RIG-I are common targets for EVs evasion against innate immune responses.

Moreover, results obtained in 2010 revealed that 3C inhibits RIG-I and the adaptor molecule MAVS instead of cleaving RIG-I directly (Lei *et al*., 2010). When there is infection by EV-A71 NLRP3 inflammasome is activated and takes up the role of protection against EV-A71 infection (Wang *et al*., 2015). As a result of this, EV-A71 infection inhibits NLRP3 inflammasome activation by cleaving NLRP3 with 2A and 3C proteinases (Wang *et al*., 2015). With the fact that TLRs has a vital role on antiviral responses against EVs, studies is yet to confirm the ability of enteroviruses to directly target TLRs to invade innate immunity.

2.6.3 Roles of Innate Immunity Evasion in EV Pathogenesis

As natural or innate immunity is the first line of defense against viral infections, viruses have to develop mechanisms to overcome or avoid innate immune responses in other to survive. Innate immunity is important for the control of the EV infection at the early stage, as studies show that EV-induced morbidity and mortality is increased in type I IFNs- or IFNAR-knockout mice (Ida-Hosonuma *et al*., 2005; Liu *et al*., 2005).

In addition, treatment with neutralizing antibody of type I IFN tends to increase viral loads and EV-A71-induced lethality while type I IFN treatment increases the survival rate of mice (Liu *et al*., 2005).These findings revealed the fact that the innate immune responses are closely associated with EV pathogenesis. However, there are a few direct evidence demonstrating how these mechanisms to antagonize and evade innate immunity are related to

EV pathogenesis. Further works is required as to gaining further insights into these mechanisms, to verify whether recombinant EV contains mutated 2A, 3C, or 2C, which are unable to antagonize the described targets, can induce stronger IFN response compared with wild type virus.

2.7 HOST DEFENSES

Interferon and virus-specific IgA, IgM, and IgG antibodies are important in the host defense. Neutralizing antibody confers serotype-specific immunity**.** Not long after infection of the respiratory or alimentary tract occur there will be increase in amounts of interferon and subsequently virus-specific IgA-antibody are detected in the saliva and the respiratory and gut secretions. Interferon hinders virus multiplication, and IgA complexes with extracellular virus. The complexing of virus by IgA not only inhibits the spread of virus to susceptible epithelial cells but also affect by decreasing the oral and fecal shedding of infectious virus (Wang *et al*., 2014 and 2015).

The first serum antibody to appear as a result of picornavirus infection is IgM, this will occur when it is about 2 weeks, afterward IgM will be overtaken by IgG. The IgG response gets to the highest point at about 2 to 3 weeks and remains at that point for a few weeks, before it begins to fall. The IgG elicited but some enterovirus infections will remain detectable for several years. This neutralizing IgG confers serotype-specific immunity (Hosonuma *et al*., 2005). Both IgG and IgM can complex with invading virus and prevent the spread of virus through the bloodstream to a target or specific organs. Virus-antibody complexes are eliminated by phagocytosis, digestion, and excretion.

2.8.CLINICAL MANIFESTATION AND TRANSMISSION

2.8.1 Clinical Manifestation

Enteroviruses are the causative agent of many diseases, including undifferentiated febrile illnesses, upper and lower respiratory tract infections, gastrointestinal disturbances, conjunctivitis, skin and mucous membrane lesions, and diseases of the central nervous system, muscles, heart, and liver. EVs have been associated with a host of clinical conditions, which include intrauterine Enterovirus transmission with fatal outcome (Tassin *et al*., 2013) encephalitis, meningitis, pleurodynia, herpangina, conjunctivitis, gastroenteritis, myopericarditis, pancreatitis, hepatitis, type 1 diabetes, hand, foot and mouth disease, upper and lower respiratory tract diseases and paralysis or myelitis (Tapparel *et al.,* 2013).

Enterovirus infections are asymptomatic, most of the infections with clinical manifestation represent less than 10% of Enterovirus infections (Nathanson and Kew, 2013).In rear cases, enteroviruses are associated with generalized neonatal infections, diabetes mellitus, pancreatitis, orchitis, and occasionally hemolytic-uremic syndrome and intrauterine infections. (Tapparel *et al*., 2013). Prevention or treatment of EV infections is greatly affected by the diversity of species that cause the similar illness. For example, HRV infection can result in respiratory diseases including the common cold as well as more serious lower respiratory infections that exacerbate asthma (Hammond *et al*.; 2015; Stone *et al*.; 2015). However, there are three species with more than 100 HRV serotypes that can give rise to the same infections. Thus, developing a single vaccine against so many serotypes is nearly impossible (Glanville *et al*., 2015).

Also, according to the study carried out in Califonia, USA in 1962 (Oberste *et al*., 2004) it was observed that similar respiratory illnesses including mild upper respiratory infections, pneumonia, and acute flaccid myelitis can also be caused by EV-D68 (Greninger *et al*., 2015; Khan, 2015; Waghmare *et al*., 2015). Although, EV-D68 has been rarely reported in

the past 40 years, it has recently caused an epidemic in 2014 in the United States (Khan, *et al*., 2015) leading to more than one-thousand (1000) cases of severe respiratory disease.

Similarly, not less than 23 EV serotypes can cause hand, foot, and mouth disease (HFMD), a common infectious disease of infants and children.For instance, the viruses withinHuman enterovirus A (Zhang *et al*., 2010; Zhang, *et al*., 2011; Tapparel *et al*., 2013; Puenpa, *et al*., 2014). Meanwhile, EV-A71 and CV-A16 are the major causative agents of this disease, these viruses can also cause other maladies (McMinn, *et al*., 2001; Chan., *et al*., 2003; Xing, *et al*., 2014). For example, it is common of EV-A71 to cause severe central nervous system diseases, such as encephalitis and aseptic meningitis (Wang *et al*., 2008; WHO, 2011), while CV-A16 is associated with causing more mild HFMD cases. Recently, CV-A6 is becoming the major causative agent in some geographical locations (CDC, 2011; CDC, 2012).

Furthermore, the neurological syndromes associated with enterovirus 71 infection include encephalitis, especially brainstem, acute flaccid paralysis (anterior myelitis), encephalomyelitis, and aseptic meningitis occur frequently with EV A-71. Again, Cerebellar ataxia is infrequent, transverse myelitis is rare while neurological and systemic manifestations brainstem encephalitis with cardiorespiratory failure is frequent. Manifestations indicative of immune-mediated mechanisms such as guillain-Barré syndrome is infrequent, Opsoclonus-myoclonus syndrome and benign intracranial hypertension is rare (McMinn, 2002).

2.8.2 Transmission of Enteroviruses

Enteroviruses are not exempted when it comes to the issue of transmission of viruses. EVs can be found in an infected person's feces (stool), eyes, nose, and mouth secretions (such as saliva, nasal mucus, or sputum), or blister fluid. An individual can easily get exposed to the virus through maintaining a close contact, such as touching or shaking hands, with an

individual that is infected, touching objects or the surfaces that contain the virus on them, and then touching an individual's eyes, nose, or mouth before cleaning or washing the hands, changing diapers of an individual that is infected and then touching the eyes, nose, or mouth before washing of the hands, drinking water that contain the virus in it (Ranta *et al*., 2001; Noyce *et al*., 2011).

Enteroviruses are transmitted mainly via the fecal-oral route. However, transmission via the respiratory route and through conjunctival fluid have also been documented (Nathanson and Kew, 2013). For example, since its discovery in 1962, the transmission of EV68 in most cases had been described to be via the respiratory route. In the United States EV68 was found in 2 of 5 children during 2012/13 cluster of polio-like disease in California (Brown, 2003).

2.9 DIAGNOSIS OF ENTEROVIRUSES

Enteroviruses are among the most common causes of infections in humans, which are often asymptomatic. There are about 64 enterovirusserotypes that infect humans and more commonly severe disease (encephalitis, AFP and myocarditis), therefore any specimen suspected of containing them should be handled with caution and treated as pathogenic. The need for laboratory diagnosis is of great importance and antiviral therapy may be used if more virulent serotypes such as enterovirus 71 are identified, and epidemiological surveillance is important so as to manage the outbreaks (McMinn, 2001) most especially when enteroviruses are able to cause serious infections.

Laboratory diagnosis is established primarily through virus isolation or molecular detection of the virus nucleic acid in appropriate clinical specimens. This method is important as it distinguishes EV71 from other enteroviruses, such as coxsackievirus type A 16.

2.9.1 Choice of Sample

Samples for laboratory investigation should be selected according to the disease manifestations. Possibilities include throat, rectal, and ulcer swabs, and samples of serum, urine, CSF, and fluid from vesicles. The sensitivity, specificity, and usefulness of findings vary according to the sample. In particular, virus detection in samples from sterile sites, such as vesicular fluid, CSF, serum, urine, or those gathered at autopsy, is more reliable than those samples from non-sterile sites, like throat or rectum, where the present virus might merely indicate coincidental carriage. Viral shedding from the gastrointestinal tract through the throat, rectum or in stools might continue after complete resolution of the symptoms of EVs infection. A study in Taiwan showed that EV71 can be detected in the throat up to two weeks after recovery from HFMD or herpangina, and in stools it can be detected up to 11 weeks after recovery. Furthermore, when an enterovirus is detected in non-sterile sites, it is always different from that isolated in samples from sterile sites in 10% of throat swabs and 20% of rectal swabs.

However, the viral load is always very low in some of the sterile sites. As for poliomyelitis; for instance, virus is detected in 0 to 5% of CSF samples from patients with neurological disease. The yield for serum is similarly low. When vesicular fluid is present, it is preferable, but care must be taken during collection. A study revealed that the most efficient approach was to examine throat swabs for all patients, plus swabs from at least two vesicles or from the rectum for patients that has no vesicles (WHO, 2004).

2.9.2 Sample collection and transportation

Specimens are to be transported in well labelled and sealable containers. All specimens are transported in sealed plastic bags or pouches, accompanied by completed laboratory request forms. Diagnostic specimens other than stools and CSF are always transported to the laboratory in an appropriate virus transport medium. Stools and CSF specimens are collected and transported in sterile, screw-capped containers, preferably plastic rather than glass.

Specimens should be transported to the laboratory immediately after collection or within 24 hours. If delayed the specimens should be refrigerated at 4° to 8°C. If samples are stored or refrigerated, repeated freezing and thawing should be avoided and every effort should be made to ensure that specimens stored frozen remain frozen until they can be processed in the laboratory.

2.9.3 Isolation of Enterovirus

The gold standard for diagnosis of enterovirus infection is virus isolation. Several human and non-human primate cell lines can be used, including rhabdomyosarcoma, which is most efficient, human lung fibroblast cells, and African green monkey kidney cells. All specimens should be inoculated in duplicate in each cell line chosen, preferably in tube culture, or in microplate culture if cross-contamination of plate cultures can be avoided. In rhabdomyosarcoma cells, a characteristic cytopathic effect is observed typically 7–10 days after inoculation. In other to improve the yield, blind passage might be necessary before cytopathic effects become apparent (Pallansch, 2001). Once a cytopathic effect is observed, the virus is identified by neutralization tests in intersecting pools of type-specific antisera or by an indirect immunofluorescence assay with the virus specific monoclonal antibodies.

In a situation where characteristic enterovirus CPE such as rounded, refractile cells detaching from the substrate surface appears, incubation should be allowed to develop to at least 75% of the cells are affected (+++ CPE). Then they should be subjected to subsequent passages but if CPE does not occur after seven days, a blind passage can be performed for an additional seven days (WHO, 2003 and 2004). Contents of replicate cell cultures from an individual case should not be pooled for passage that is individual cell cultures should be passaged separately.

2.9.4 Enterovirus Serotyping

Recently, enterovirus laboratories use to employ antibody-based screening assays in determining the identity of viruses in CPE-positive cultures. A few of these assays include components for detecting poliovirus-positive cultures. A molecular serotyping approach developed has been towards amplification of part of the VP1gene of the cultured virus, use of PCR and panenterovirus specific primers, and sequencing of the product. Antisera have been raised in animals against many enteroviruses, but the large number of viruses make it impractical to routinely perform individual neutralization tests. To put an end to thisproblem, antisera have been pooled in an overlapping scheme that allows many viruses to be identified using about nine tests (Xiao *et al*., 2009).

Interpretation of the results is then carried out with the help of a list of the neutralization patterns of individual viruses. Type-specific monoclonal antibodies have also been used. These permit virus confirmation and differentiation by indirect immunofluorescence.Pooled horse antisera against the most frequently isolated echoviruses and coxsackieviruses have been prepared at the National Institute of Public Health and are supplied free of charge to WHO Polio Laboratory Network laboratories (WHO, 2004). Other typing reagents have been prepared and include the Lim and Benyesh-Melnick (LBM) combination neutralizing serum pools, together with commercially available monoclonal antibody reagents.

Also, several sets of primers directed at different regions of the VP1gene of enteroviruses have been developed. Enteroviruses specific primers are used to perform PCR directly on clinical samples. The advantage of this method is that it is faster than virus culture. Speed can be especially important given the explosive nature of some enteroviruses outbreaks and the need for urgent or quick public health interventions. The disadvantage is that only the virus of focus in detection can be detected and, therefore, other unexpected viruses will not be identified (Xiao *et al*., 2009). DNA microarray is a powerful, although expensive, tool

designed to detect multiple pathogen targets by hybridization of pathogen specific probes. Two groups have reported its use to distinguish EV71 and coxsackievirus type A 16 infection in primary clinical specimens, with diagnostic accuracy of about 90% (Xiao *et al*., 2009).

2.9.5 Molecular Method of Identification.

The technique was said to overcome the effect of cell culture bias as it is capable of identifying enterovirus from clinical specimen with the use of reverse transcriptase semi-nested PCR (RT-snPCR) assay (Nix *et al*., 2006). Several studies have reported the effectiveness of molecular technique in the identification of enteroviruses (WHO, 2004). As a result, the strategy has been adopted officially by WHO (WHO, 2004) in preference to the cell culture-based method for enterovirus surveillance (WHO, 2016).

During enteroviruses co-infections, the genomic concentration has an influence on the final identity on mixture of enterovirus. Studies reported that enterovirus with the highest titer in clinical samples could frequently be identified and the mixed isolates showing the identity of only one member of the mixture (Faleye and Adeniji, 2015). Application of the Nix *et al*., 2006 protocol directly to clinical samples helped in the detection of the enterovirus isolates that cannot be detected when the samples were first subjected to cell culture with the use of a susceptible and permissive cell line. The influence of genomic concentration on the enterovirus serotype identified by Nix *et al*., 2006 protocol revealed that molecular identification helps to find and identify enteroviruses that would have not been identified, hence a decision to forgo cell culture-based algorithm behind must be carefully handled to avoid being bias about enterovirus diversity landscape as confirmed by Arita *et al.*, 2015 andAdeniji 2015.

However, part of the strategies used to tackle this challenge was addition of species-specific primers screen using primers 187, 188 and 189 (Oberste *et al*., 2003; WHO, 2015) to the

second round Polymerase Chain Reaction (PCR) of the Nix *et al*. protocol. Of which instead of single second round PCR, four different second round PCR assays that use similar first round PCR product as a template were obtained (Nix *et al*., 2006). Adeniji 2015 and Faleye *et al.,*2015, used this strategy to screen feacal samples and RD cell line isolates, paired and unpaired and discovered that the Nix *et al*., 2006 protocol is very sensitive for detecting enterovirus genomes but always mask the presence of more than one enterovirus isolate per sample.

The phenomenon was said to have been inherited from primers 292 and 222 (Faleye and Adeniji, 2015) as these were upgraded to AN89 and AN88. However, primer 292 and by extention AN89 is a consensus of primers 187, 188 and 189 (Oberste *et al*., 2003) the addition of these three primers helped in the restoration of the strength of the assay (Faleye *et al*., 2015). Hence by modifying the WHO recommended Nix *et al.*protocol to singly use primers AN89, 187, 188 and 189 (forward primers) besides AN88 (reverse primer) for the second round PCR, the assay maintained its sensitivity for enterovirus detection and is presently of great value for identification due to its mixed isolate-resolving ability.

In addition, studies have reported that whatever isolate revealed on the cell line is not always the total or complete picture. Often, other enteroviruses are present in the sample that will not grow on the RD cell line (Adeniji, 2015). Hence, studies depending on the RD cell line (or others with their various biases unaccounted for) that have linked some enterovirus strains to certain clinical conditions (Tao *et al*., 2014a; 2014b) need to be interpreted with caution because there is possibility that not all of the enteroviruses in the sample were detected and identified. Therefore, considering the fact that most enteroviruses are without symptoms, go unnoticed and we hardly truly exhaustively catalogue the enterovirus diversity landscape of a sample, it is hard to conclude the type of enterovirus or combination of enterovirus types which are truly associated with the clinical manifestation(Rao *et al*., 2012).

Furthermore, studies revealed that even when a clinical sample is not positive (negative) for enteroviruses by the pan-enterovirus VP1 screen based on primers 224, 222, AN89 and AN88 (Nix *et al*., 2006), often, the species-specific screen (using the first round product of 224 and 222 as template) still detects sometimes like two different serotypes in the similar sample.

2.9.6 Effect of Cell line on Enterovirus Diversity Landscape

There has been a report about increase in the preponderance of EV-B due to the detection of former untypable Non-Polio Enterovirus (NPEV). This greatly intensified the notion that EV-B were the most diverse and further strengthened the belief on enterovirus divergence that favoured EV-B. It was later discovered that the diversity observed in EV-Bs might not be because of the fact that they are the most evolutionarily successful. On the other hand, two factors was said to have resulted to this.

To start with, some enteroviruses cannot, presently be isolated in cell culture (Brown *et al*., 2003). Although, some enteroviruses will grow in a range of mammalian cell lines which include among many others primary African green, cynomolgus or rhesus monkey kidney cells (AGMK, CMK, RMK), Madin Darby canine kidney (MDCK), human diploid cell lines (MRC-5, WI-38, SF), human embryonic kidney (HEK), human embryonic firoblast (HEF), human epithelial carcinoma (HEP-2), human rhabdomyosarcoma (RD). Enteroviruses vary in their ability to grow in different cell line. To improve the chance of successful virus isolation, several cell lines should routinely be used. Effective enterovirus laboratories usually inoculate specimens into a minimum of the three cell lines, and some may use five or six cell lines. Polioviruses are the only species C enteroviruses currently known to replicate in L20B cell line. Based on the recommendation World Health Organization (WHO) (figure 2.2), most

laboratories in the GPLN use the RD-L20B isolation protocol for poliovirus isolation (WHO 2003, 2004). The RD and L20B isolation protocol is built around two different cell lines, RD and L20B. The RD cell line is from a human rhabdomyosarcoma (a straight muscle cancer) and was shown to be quite sensitive for enteroviruses most especially poliovirus isolation (WHO, 2003).

Also, the RD cell line that GPLN use seems to enhance the replication of Enteroviruses even when the other members of enterovirus species are present (Faleye and Adeniji, 2015). There was an observable change and improvement in the detection of EV-C members when other cell lines such as Hep 2C and MCF-7 were added in the enterovirus protocols. For instance, most of the time, when the same sample is inoculated into RD and MCF-7 cell lines, the two will individually isolate EV-Bs and EV-Cs , respectively (Faleye and Adeniji, 2015). Studies revealed that in cases where both EV-B and EV-C are present in an isolate recovered on the RD cell line, direct molecular identification without first separating the mixture always selectively reveals EV-B component of the mixed isolate (Faleye and Adeniji, 2015). Behind this impression of RD cell line EV-B bias, it appears as if biology of the cell line enhances the propagation of EV-Bs. This might end up with increased titre or relative genome concentration, which is later multiplied, using molecular method of identification.

2.10 TREATMENTS OF ENTEROVIRUS INFECTIONS

2.10.1 Pleconaril

Pleconaril is an antiviral drug that inhibits the entry of several enteroviruses into cells by blocking viral attachment and uncoating. This has been used in clinical trials of aseptic meningitis (Wu *et al*., 2009). This drug is not, however, active against Enterovirus 71. Several other capsid function inhibitors have been investigated, and many have shown promising activities against EV 71 in preclinical studies. In-vitro and in-vivo studies have

shown that both ribavirin and interferons might also be useful and RNA interference approaches are being explored (Wu *et al*., 2009).

2.10.2 Intra-venous Immunoglobulin

In Asia, during the initial large outbreaks of EV71, clinicians in Sarawak and Taiwan made use of intravenous immunoglobulin on the presumptive basis with the hope that it would neutralize the virus and have non-specific anti-inflammatory properties. Retrospective comparisons of patients who did and did not receive immunoglobulin suggest a benefit from this treatment if given early (Ooi *et al*., 2005) For instance, among children with EV71 assessed in Sarawak over the period of three seasons, 204 (95%) of 215 survivors who had severe central nervous system (CNS) complications had received intravenous immunoglobulin treatment (Wu *et al*., 2010).

Intravenous immunoglobulin has become more routinely used for the treatment of severe EV71 disease and has been a standard therapy for aseptic meningitis caused by EV infection. In Taiwan (IVIg) has been introduced into the national treatment guidelines (Wang *et al.*, 2006). However, uncertainty remains, over whether this expensive human blood product treatment is really effective and randomized, placebo-controlled, phase 2 trials are needed. Such trials would be logistically and ethically challenging to establish because the treatment is widely used, and would require careful design with surrogate endpoints of disease progression, such as failure of resolution of tachycardia.

2.10.3 Serology

Serological diagnosis of an acute virus infection basically relies on a fourfold increase being revealed in the concentrations of a specific neutralizing antibody between the acute and convalescent phases. Considering the case of EV71, very high concentrations of neutralizing antibodies are frequently detectable within the first few days of the onset of illness, and thus

such a difference will not be seen (Pallansch *et al*., 2001). Furthermore, homologous antibodies are produced when young children encounter their first enterovirus infection, while heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different enterovirus serotypes. The usefulness of this test, thereby decreases with increase in age.

2.11 VACCINES AGAINST ENTEROVIRUSES

The effectiveness of immunization in protecting the host against EV infections was demonstrated by the historical Salk and Sabin vaccines against PV. Some effective vaccines have been designed and are able to stimulate both the innate and adaptive immune response in order to combat the remaining clinically relevant EVs. To combat EV infection, the adaptive immune response requires both T cells and antibodies in the clearance of the virus. Hence, the most successful vaccines activate both humoral and cell-mediated immunity and induce long viral immunity (Kew *et al*., 2003).

2.11.1 Coxsackievirus Vaccines

Several effective vaccines have been developed by some researchers against Coxsackievirus. Although, no clinically available vaccine currently exists but there has been improvement in using various vaccine techniques utilizing inactivated virus, live attenuated forms of virus or DNA plasmids expressing viral proteins.

2.11.2 Poliovirus Vaccines

The original formalin-inactivated form of the vaccine (IPV) was developed by Jonas Salk and licensed in 1955 (Salk *et al*., 1954). Both the inactivated and attenuated forms of the historical PV vaccines have been used to confer immunity to the virus. Meanwhile, the original formalin-inactivated form of the vaccine (IPV) was developed by Jonas Salk and licensed in 1955 (Salk *et al*., 1954). An orally administered live attenuated PV vaccine (OPV) was formulated in 1963, (Sabin, 1957). The occurrence of poliomyelitis drastically reduced

worldwide during the usage of these vaccines and optimized versions. Although the pathogen has yet to be eradicated worldwide. The preference for the administration of IPV over OPV was due to the discovery of circulating vaccine derived PVs (cVDPV) that is associated with the OPV, cVDPVs are virulent PVs derived from OPV that occur in a small minority of vaccine recipients. The reversion of cVDPVs from attenuation to virulence is a direct consequence of the genetic instability of the vaccine. Circulating vaccine derived PVs cVDPVs were responsible for a polio outbreak in the Dominican Republic and Haiti in 2000 (Kew *et al*., 2003). Sequencing determined that the cVDPVs were recombinant viruses and the derivation of neurovirulent polio strains from the OPV has led to the "OPV paradox," and this is based upon the idea that complete eradication of poliomyelitis is contingent upon the elimination of the attenuated form of the vaccine. Attenuation of EVs via mutations in the genome has historically led to efficient vaccine production. Perhaps the most well-known attenuated form of PV is that used for the Sabin vaccine, which has decreased neurovirulence in part controlled by two stem loops in the viral internal ribosome entry site (IRES) (Gromeier *et al*., 1999). Other mutation that can cause CNS attenuation is located between the 5′ NTR cloverleaf and IRES and reduces the binding of polypyrimidine tract-binding protein.

Studies have made recombinant viruses that use the IRES from human rhinovirus type 2 (HRV2) to attenuate neurovirulence in the Sabin vaccine strain of PV as well as in herpes simplex virus type 1. Somewhat alarmingly, a vaccine-derived PV and coxsackievirus A17 recombinant has been generated in the laboratory, hence demonstrating the possibility of such events occurring naturally.

2.12 ACUTE FLACCID PARALYSIS

Acute flaccid paralysis (AFP) is a clinical syndrome characterized by rapid or sudden onset of weakness, including (less frequently) weakness of the muscles of respiration and

swallowing, progressing to maximum severity within several days to weeks.The term "flaccid" indicates the absence of spasticity or other signs of disordered central nervous system motor tracts such as hyperreflexia, clonus, or extensor plantar responses (Alberta Government Health and Wellness, 2005).

Paralysis implies loss of contraction due to interruption of motor pathways from the cortex to the muscle fiber when applied to voluntary muscles. It is preferable to use the term "paresis" for slight loss of motor strength while "paralysis" or "plegia" is better use for chronic loss of motor strength.The differential diagnosis of AFP varies considerably with age. This abnormal condition may be caused by disease or by trauma affecting the nerves associated with the involved muscles. If the somatic nerves to a skeletal muscle are severed for instance, then the muscle will exhibit flaccid paralysis. When muscles enter this state, they become limp and cannot contract. This condition can become fatal if it affects the respiratory muscles, posing the threat of suffocation. (Saladin *et al*., 2012).

AFP is the most common sign of acute polio, and used for surveillance during polio outbreaks.

It is also caused with a number of other pathogenic agents including enteroviruses, echoviruses, West Nile virus, and adenoviruses, among others.Non-polio enteroviruses have been associated with polio-like paralytic disease, commonly followed by other clinical syndromes, such as hand-foot-and-mouth disease, aseptic meningitis and acute hemorrhagic conjunctivitis. Coxsackieviruses A and B, echovirus, enterovirus 70, and enterovirus 71 have been implicated as the causative agents of polio-like paralytic disease. (Kelly *et al*., 2006).

In some studies, outbreaks of acute hemorrhagic conjunctivitis with radiculomyelitis and paralytic illness in India, Taiwan, Thailand, and Panama were etiologically linked to enterovirus 70. Muscle weakness and wasting resulted from enterovirus 70 is always severe

and permanent. In Califonia, it was first described in 1969 to 1973 that enterovirus 71 among all other non-polio enteroviruses, as the major cause of the outbreaks of central nervous system disease and AFP. Children under 15 years of age were greatly affected. There was Global attention on enterovirus 71 as a result of severe epidemics of central nervous system disease that occurred in Japan in 1973 and in Bulgaria in 1975 (57). Of 705 patients infected with enterovirus 71 in Bulgaria, 149 (21 percent) developed paralysis, and 44 (29 percent) of those persons died. (Kelly *et al*., 2006)**.**

2.12.1 History of Acute Flaccid Paralysis.

Paralytic poliomyelitis occurred in the ancient times, it was not distinguished as a distinct disease entity with infectious or epidemic potentialities until the end of 18th century when its epidemic potential began to show up (Paul, 1954).Acute flaccid paralysis has a long history, dating back to the Egyptian eighteenth dynasty (1580-1350 BC). The historical record of polio is very fragmentary and isolated cases of poliomyelitis and acute paralysis in children have been occurring since the biblical times. Great number of epidemics were accompanied by various hysterical reactions. A striking feature of paralytic poliomyelitis has been its ever-changing epidemiology. Poliomyelitis is one of the major health problem which was first described by a physician, Michael Underwood from Britain, in 1789 (Drutz and Ligon, 2000).

Despite chronic shortage of resources of developing countries, a number of destabilizing health problems such as poliomyelitis is being experienced.Poliomyelitis is derived from two words ofGreek origin, polio which implies gray and myelon which means marrow, indicating the spinal cord. Therefore poliomyelitis, is often referred to as polio or infantile paralysis. It is an acute viral infection that influences the motor neurons within the spinal cord and brain leading to the classic manifestations of flaccid paralysis (Kumar and Taunk, 2014).Poliomyelitis being one of the major ofhealth problem is described as the debility of

the lower extremities. Poliomyelitis existed across the globe before the eradication initiative was undertaken in 1988 which marked the launch of the Global Polio Eradication Initiative (GPEI), organized by WHO, Rotary International, the US Centers for Disease Control and Prevention (CDC) and the United Nations Children's Fund (UNICEF) (WHO 2012).

2.13 EPIDEMIOLOGY OF ENTEROVIRUSES

Picornaviruses are widely prevalent.Enteroviruses are transmitted mostly by the fecal-oral route, they can also be transmitted by salivary and respiratory droplets. Some serotypes are spread by conjunctival secretions and from skin lesions exudates **(**L'Huillier *et al*., 2015**)**. Non-Polio enterovirus are widely distributed and are associated with occasional outbreak in which a larger number of patients develop clinical diseases.In temperate countries, outbreaks of enterovirus illnesses occur most frequently in summer and autumn. Enteroviruses in excreta that contaminate the soil are carried by surface waters to lakes, beaches, vegetation, and community water supplies (Noyce *et al*., 2011). These sources may serve as foci of infection. Likewise, shellfish that feed in freshwater or seawater beds contaminated by excreta become carrier of enteroviruses. Likewise, cockroaches in sewage pipelines and flies that pet on excreta may act as transient vectors. Immunity is serotype specific and the epidemiological pattern differ by climate and geographical region.

Enteroviruses contain more than 250 naked icosahedral virus serotypes categorize as members of family piconaviridae, genus enterovirus and order piconavirales and have diameter of 28-30 nm within the non-enveloped icosahedral capsid of an enterovirus is a positive-sense, protein-linked, single-stranded approximately 7.5 kb RNA genome, of a single open reading frame (ORF) flanked on both sides 5^1 and 3^1 ends by untranslated regions and translated into an approximately 250-kDa polyprotein. This polyprotein is auto catalytically cleaved into P1, P2 and P3 polyproteins which further cleaved into VP1-VP4, 2A-2C and 3A-3D, respectively (http://www.piconaviridae 2016). The replication of

enterovirus begins in the respiratory or gastrointestinal tract, and once the virus is evident or present in the blood stream, infection may affect different tissues and organs causing different types of diseases. Enteroviruses are associated with specific syndromes for instance, Human enteroviruses A cause hand-foot-mouth disease (HFMD) (Tassin*et al*., 2013).

2.13.1 Epidemiology of Enterovirus-A71

Enterovirus 71 is known to be a virulent serotype of the enteroviruses that has a wide variety of clinicalmanifestations, although CNS infection and HFMD arethe two features most frequently seen. Being one of the enterovirus serotype it was first isolated from a child of in 1969 in California, USA, with phylogenetic evidence that revealed that it was present in the Netherlands as far back as 1963 (Van der Sanden *et al*., 2008). Subsequently, there was an outbreak with neurological infections caused by EV-A71 reported in Australia, Japan, Sweden and the United State of America. Its effect in 1975 in Bulgaria during two large outbreaks and in Hungary three years later resulted into high fatality among the children.There was a report of 44 fatalities amongst 451 children with non-specific febrile illness or neurological disease in Bulgaria (Chumakov *et al*., 1979) and 47 deaths amongst 1550 children, 826 aseptic meningitis and 724 encephalitis inHungary (Nagy *et al*., 1978).

In Sarawak in the year 1997, there was an outbreak of HFMD cases as a result 34 deaths were reported. Later on, Taiwan reported the highest HFMD outbreak that involved 1.5 million cases with 78 deaths in 1998 (Ho *et al*., 1999). A great outbreak of HFMD took place in Singapore in the year 2000. This involved 3790 patients of which 5 deaths were recorded, 3 due to HFMD and 2 to non-HFMD (Chong *et al*., 2003). Since then, HFMD is recognized as an endemic mild disease in both Malaysia and Singapore. China was the next country to report a large HFMD outbreak of 490,000 infections with 126 deaths in 2008, since 2009, the number of HFMD infections in China had greatly increased and there were 2,819,581 HFMD

cases reported with 394 deaths in 2014 while Vietnam reported 4265 cases of HFMD with two deaths in 2015 (WHO , 2015). Aside the Asia Pacific region, there was an outbreaks or sporadic infections in Europe with no fatality or low fatalities (Hassel *et al*., 2015).

2.14 PREGNANCY AND NON-POLIO ENTEROVIRUS INFECTION

Pregnant women who are infected with a non-polio enterovirus shortly before delivery can pass the virus to their babies. These babies usually have only mild illness, but in rare cases they may have severe infection. Non-polio enteroviruses are very common, so a pregnant woman is likely to be exposed at some point in her pregnancy to someone who is infected, especially in the summer and fall. But most pregnant women, like other adults, have immunity (protection) from previous exposures to non-polio enteroviruses. So if she does get infected she will likely not have symptoms or will only have mild illness. Pregnant women without immunity to non-polio enteroviruses havea greater chance of getting infected and having symptoms (NCIRD, 2016).

There is no clear evidence that non-polio enterovirus infection during pregnancy increases the risk of severe complications like miscarriage, stillbirth, or congenital defects. Non-polio enteroviruses can be shed (passed from a person's body into the environment) in the stool for several weeks or longer after one has been infected. The virus can be shed from respiratory tract for 1 to 3 weeks or less. Infected people can shed the virus even if they do not have symptoms (NCIRD, 2016). Commonly, infants, children, and teenagers are most likely to get infected with enteroviruses and become ill this occur because they do not yet have immunity (protection) from previous exposures to these viruses. This is also believed to be true for EV-D68. Adults can get infected with enteroviruses but they are more likely to have no symptoms or mild symptoms.

2.15 PREVENTION AND CONTROL

Most people who are infected with non-polio enteroviruses do not have symptoms, but can still spread the virus to other people. This makes it is difficult to prevent them from spreading. But the best way to help protect oneself and others from non-polio enterovirus infections is to wash your hands often with soap and water, especially after using the toilet and changing diapers, avoid close contact, such as touching and shaking hands, with people who are sick, clean and disinfecting frequently touched surfaces.Poliomyelitis can be prevented by Salk-type (inactivated) and Sabin-type (live) attenuated poliovirus vaccines, control can be achieved via public education on transmission modes and personal hygiene, adequate sewage disposal and uncontaminated water supplies are critical for prevention of enteroviral infections, there is no specific therapy.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection and Processing

This study adopted a cross sectional design with a purposive consecutive sampling method and was carried out in the World Health Organization (WHO) Polio Laboratory, Virology Department, University College Hospital, Ibadan, Oyo State with ethical approval. Sixty stool suspensions were analyzed as thirty cases (Two stool suspensions from one person), which were selected from feacal samples from children with Acute Flaccid Paralysis (AFP) cases in South-West Nigeria.

These samples were collected from patients in the month of August, year 2015 by WHO National Polio Laboratory, Ibadan, Oyo State, within 14 -15 days ofthe onset of the paralysis as described in the World Health Organization Polio Laboratory Manual, 4th edition (WHO, 2004). The children's age involved in this study 15 years and below. The samples were previously screened using culturing method and showed no cytopathic effectand were said to be negative for Poliovirus and other enteroviruses. Also, the feacal specimen was made into 20% suspension in a balanced salt solution with antibiotics and were treated with chloroform after to which enteroviruses were resistant.

In addition to removing bacteria and fungi, this method removed potentially cytotoxic lipids and dissociates virus aggregates (Adeniji, 2014). After the treatment with chloroform and vortexing, the suspension was clarified by centrifugation at 1500xg for 10 minutes and

clarified suspensions were achieved. The samples were taken from six States in Nigeria these include Lagos, Oyo, Osun, Ekiti, Ondo and Ogun State. Considering that the samples were collected from states in Southwest Nigeria, (SW), they are referred to in this study as SW samples.

3.3 METHODS

3.3.1 Viral RNA Extraction

The extraction of viral Ribonucleic acid (RNA) was done for each stool suspension (supernatant) by using total Jena Bioscience RNA kit according to the manufactures' instruction. These include cell lysis that was done using an enzyme Protease and detergent. Column activation, with the use of buffer AW1 W2, column loading with the use of primary and secondary washing. Finally, elution of RNA that was done using elution buffer of RNA.

All reagent were mixed over leaf and label vials, 500 µL of lysis buffer 2 ME (Macfty Ethanol) already added to all approximately labelled vials. Elution buffer of 100 µL was added to each labelled vial containing the sample, vortex for 10 minutes, incubated on a bench for 10 minutes and centrifuged at 10,000 xg for 5 minutes. After this the supernatant was transferred into a new properly labelled vail. A spin column was placed into a 2 mL collection tube, 100 µL of extraction buffer was added into the spin column, centrifuge at 10,000 rmp for 30 seconds and flow through was discarded. Isopropanol of 360 µL was added to the prepared lysate and vortexed. The new mixture 960 µL was transferred into the spin column, centrifuged at 10,000 xg for 30 seconds and flow through was discarded.

During column activation a spin column was placed into a 2ml collection tube, 100µl of activation buffer was added into the column and it was centrifuged at 10,000 rpm for 30 sec and the flow through was discarded. Column loading was achieved with the use of

isopropanol (300 µl) was added to the prepared lysate and it was vortex for 30sec. The mixture was transferred to the activated spin column which was centrifuge at 10,000 g for 30 sec. The flow-through was discarded. In addition, primary column washing was done using primary washing buffer as 700 µl ethanol added was applied to the spin column and was centrifuged at 10000 rpm for 30 sec and flow through was discarded. Secondary column washing was achievedsecondary washing buffer (700 µl) was apply to the spin column. It was centrifuged at 10,000 rpm for 30 sec. The flow-through was discarded. It was centrifuged at 10,000 rpm for 2 min. again to remove the residual ethanol. Elution of RNA: The spin column was placed in a DNase/RNase-free microcentrifuge tube, 45µl of Elution buffer was placed at the centre of the column membrane, it was incubated at room temperature for 1 min. it was centrifuged at 10,000 rmp for 1 minute to elute the RNA. The RNA was stored at 45 ^{0} C (Jena Bioscience, Germany).

3.3 .2 Complimentary Deoxyribonucleic Acid (cDNA) Synthesis

Each eluted RNA was used to synthesize complementary Deoxyribonucleic acid (cDNA) by reverse transcription of the extracted RNA in a 10 µl reaction mixture (Table 3.1). Each reaction tube mixture contained 5.25 µL of RNA, 2 µL of script reverse transcription buffer, 0.5 µl of Deoxyribonucleic tri-phosphate (dNTP), 0.5 µL of RNase inhibitor, 0.5 µl of Dithiothreitol (DTT) stock solution and 0.25 µL of script reverse transcriptase (Table 3.1) . Complimentary DNA primers used are AN32, AN33, AN34 and AN35 each 0.25 µL (Nix *et al*., 2006). The reaction mixture was amplified in a Veriti Thermal Cycler at 42 °C for 10 minutes and 50 °C for 60 minutes (Applied Biosystems, California, USA). The cDNA was stored at -80 0c till analysed (Nix, 2006).

The oligonucleotides of the primers are as follows: AN32: 5’ GTY TGC CA 3’; AN33: 5’ GAY TGC CA 3’; AN34: 5’ CCR TCR TA 3’; AN35: 5’ RCT YTG CCA 3’. Standard

International Union of Biochemistry nucleotide ambiguity codes are used (I = Deoxyinosine N = G, A, T or C; Y = C or T; W = A or T; R = A or G) (WHO, 2004). The cDNA was stored at -80^{0}C and used for all downstream polymerase chain reaction (PCR) assays.

Table 3.1 Standard Protocol forcomplimentary deoxyribonucleic acid (cDNA)

S/N `	Component	Volume (µL) 1 Sample	Volume (µL) 10 Sample	Volume (µL) 34 Sample
1	Script RT-Buffer Complete	2	20	68
2	An32	0.25	2.5	8.5
3	AN33	0.25	2.5	8.5
4	AN34	0.25	2.5	8.5
5	AN35	0.25	2.5	8.5
6	dNTP mix	0.5	5	17
7	DTT Stock Solution	0.5	5	17
8	RNase Inhibition	0.5	5	17
9	SCRIPT RT	0.25	2.5	85
10	**Total**	**4.75 µL**	**47.5 µL**	**161.5µL**

4.7 5 µL of cDNA synthesis mix

5.25µL of extract (RNA)

10 µL of the total mix

3.2.3 Polymerase Chain Reaction (PCR) Assays

3.2.3.1 Enterovirus VP1 Gene PCR (snPCR) Assay

PCR was done in 30 μL reactions, the first-round PCR contained 0.5 µL of each of primers 224 and 222 (Nix *et al*., 2006), 10 µL of Red Load Taq, 10 µL of cDNA, and 29 µL of RNase –free water. Thermal cycling was done in a Veriti thermal cycler (Applied Biosystems, California, USA). Thermal cycling conditions were 94 ^{0}C for 3 minutes followed by 35 cycles at 94 ^{0}C for 30 seconds, 42 ^{0}C for 30 seconds, and 60 ^{0}C for 60 seconds with ramp of 40% from 42 ^{0}C to 60 ^{0}C. This was then followed by 72 ^{0}C for 7 minutes and held at 4^{0}C till being terminated (Table 3.2).

3.2.3.2 Pan-Enterovirus Polymerase Chain Reaction (PE-PCR)

The PE-PCR screen was a Panenterovirus detection PCR assay. It was done using primers PanEnt- AN89-F and PanEnt-AN88-R. Both primers target well conserved regions in the 5^I-UTR of enteroviruses amplifying an approximately 350 bp fragment (WHO, 2004). Primers were made in 25 µL concentrations. Primers of 0.3 µL each was included in 30 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of first round PCR and 20.4 µL of RNase free water. Thermal cycling condition was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30s, 52 ^{0}C for 30 s and 65 ^{0}C for 30 s. This was followed by 72 ^{0}C for

7 min and held at 4 ^{0}C till terminated (Table 3.3). Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

Table 3.2 Working Protocol VP1 Gene PCR (snPCR) Assay

Component	**Volume(µL) sample**	**Volume(µL)30 (µL)+ 4control**
Taq polymerase	10	340
Reverse Primer 224	0.5	17
Forward Primer 222	0.5	17
RNase free Water	29.0	986
Total	40µl	1360µl

Table 3.3 Showing Pan-enterovirus Polymerase Chain Reaction (PE-PCR).

Component	Volume (µl)/ sample	Volume (µl)/sample
Taq polymerase	6.0	204
Forward primer AN89	0.3	10.2
Reverse primer AN88	0.3	10.2
RNase free Water	20.4	693.6
Total	**27**	**918**

3.2.3.3 Enterovirus Species A and C PCR

This is a species specific assay. The screening was used to detect enterovirus species A and C. It was done using primers AN189 (Forward) and AN88 (Reverse). Both primers target well conserved regions of enteroviruses amplifying an approximately 350 bp fragment (WHO, 2004). Primers were made in 25 µL concentrations. For detection of the EA-PCR and EC-PCR screens 0.3 µL of each of the primers specific for the screen was added to a 27 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of the first round PCR product and 20.4 µL of RNase free water giving rise to a total 30 µ L of reaction**.** Thermal cycling condition for EC-PCR-1 was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30 s, 42 ^{0}C for 30 sec. 60 ^{0}C for 60 s with ramp of 40% from 42 ^{0}C to 60 ^{0}C. This was followed by 72 ^{0}C for 7 min and held at 4 ^{0}C till terminated. The conditions were the same for EC-PCR-2 except for the extension time which was reduced to 30 sec. (Table 3.4). All the PCR was carried out in a in a Veriti thermalcycler.Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

3.2.3.4 Enterovirus Species B Polymerase Chain Reaction (EB-PCR).

The EB-PCR screen was an assay done using primers AN187 and AN88 forward and reverse primers respectively. Both primers target well conserved regions in the enteroviruses amplifying approximately 350 bp fragment. Primers were made in 25 µL concentrations. Primers of 0.3 µL each was included in 30 µL reaction containing 6 µL of Red load Taq (Jena Bioscience, Jena, Germany), 3 µL of first round PCR and 20.4 µL of RNase free water. Thermal cycling condition followed in the Veriti thermalcycler was 94 ^{0}C for 3 min followed by 35 cycles of 94 ^{0}C for 30 s, 52 ^{0}C for 30 s and 65 ^{0}C for 30 s. This was followed by 72 ^{0}C

for 7 min and held at 4 ^{0}C till terminated. Subsequently, PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

TABLE 3.4 Showing the primers used in the study

	Pan Enterovirus	Enterovirus A and C	Enterovirus B
Forward Primer	AN 89	AN 189	AN 187
Reverse Primer	AN 88	AN 88	AN 88

3.2.4 Agarose Gel Electrophoresis

All PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using Ultra-Violet (UV) transilluminator. The buffer solution used was Tris Base Buric Acid (EDTA) 2 %, (2 grams of agarose was dissolved in 100 mils of TBE buffer), put in microwave for proper dissolution and electrophoresis was done for 45 minutes at 120 voltage. The TBE served as an electrolyte which allowed the flow or passage of the electricity. The loading mix was loaded in each hole of the gel (DNA of 1 µL and 5 µL of loading buffer). The fragments were visible due to Ethidium bromide dye that adhere to the DNA in the Gel, afterward it was viewed under a UV light and photographed. Five microliter (5 µL) of each reaction product was separated and visualized on two percent (2 %) agarose gel stained containing 10 ul Ethidium bromide and viewed using a Ultra-Violet (UV) transilluminator.

3.2.5 Nucleotide Sequencing and Identification of Isolates

All the resulting DNA amplicons were shipped to Macrogen Inc., Seoul, South Korea, for purification and sequencing of gel positive PCR amplicon by BigDye chemistry using the second round primers that were used for the semi-nested amplification reaction, primers AN88 and AN89.

3.2.6 Molecular Typing Isolates and Phylogenetic Analysis.

The PCR (partial VP1) amplicons generated in the snPCR were sequenced. The sequence of the Genes were edited manually.Basic Alignment Search Tool (BLAST) analyses was carried out, and the virus was assigned, named based on the virus that gave the highest VP1 identity score (identity score >75%) to the query sequence.Afterwards, the enterovirus genotyping tool (Kroneman *et al*., 2011) was used for enterovirus species and genotype determination**.**

Generated sequences were manually edited with the use of MEGA 6.06 software. Phylogenetic analysis of the sequenced viruses was done for virus identification by comparing with reference sequences from GenBank. To align the sequences described in this study (species A, B and C) with reference sequences downloaded from the GenBank, the ClustalW program in the MEGA 6.06 software (Tamura *et al*., 2011) was used with default settings. Afterwards, Maximum Likelihood (ML) neighbor-joining trees were constructed with 1,000 bootstrap replicates using the same MEGA 6.06 software (Kimura, 1980).

CHAPTER FOUR

4.0 RESULT

4.1 Sample Collection and Processing

A total of 60 samples were analyzed as 30 cases in this study. The result obtained was from six states in Southwest Nigeria. In Lagos State, 5 males and 3 females, in Oyo State, only 5 males, in Ondo State, just 3 males, in Ogun State 3 males and 3 females, in Osun State, only 1 samples from a female and finally in Ekiti State 4 male and 2 female (Table and Figure 4.1).

Only one sample of the thirty (30) samples screened yielded the expected band size for enterovirus VP1 gene detection RT-snPCR screen (Table 4.2).

4.2 Reverse transcriptase Seminested PCR result

4.2.1 Electrophoretic gel displaying band size

Samples that yielded the expected band size of approximately 350 bp for enterovirus VP1 gene detection RT-snPCR screen. Sample 27 was the only positive sample obtained in this study aside the positive controls used (Figure 4.2).

Table 4.1 Distribution of sample scollection for PCR screen according to gender

States	Total number of Samples	Gender	
		Female	Male
Lagos	8	5	3
Oyo	5	5	0
Ondo	3	3	0
Ogun	6	3	3
Osun	2	1	1
Ekiti	6	4	2
Total	30	21	9

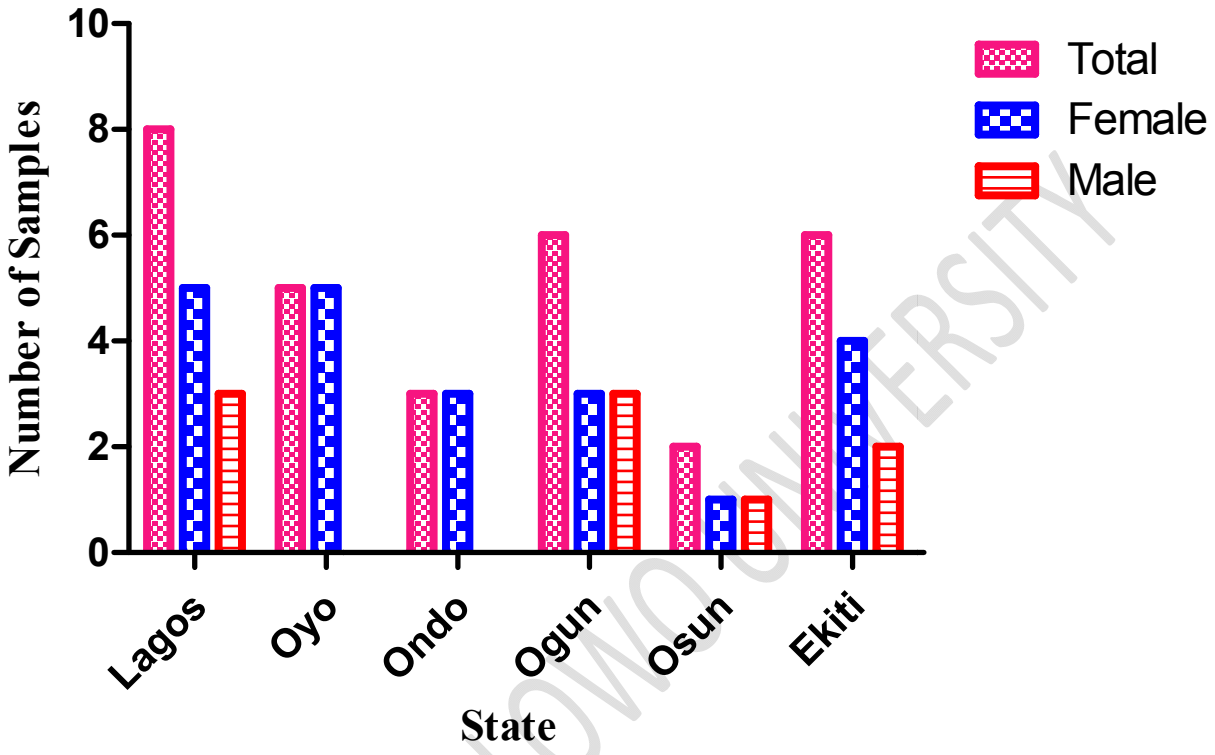


Figure 4.1 Graph of frequency of samples used with respect to the location

TABLE 4 .2Number of positive and negative samples obtained in the study

States	Positive		Negative		Total
	Female	Male	Female	Male	
Lagos	0	0	3	5	8
Oyo	0	1	0	4	5
Ondo	0	0	0	3	3
Ogun	0	0	3	3	6
Osun	0	0	1	1	2
Ekiti	0	0	4	2	6
Total	0	1	9	21	30

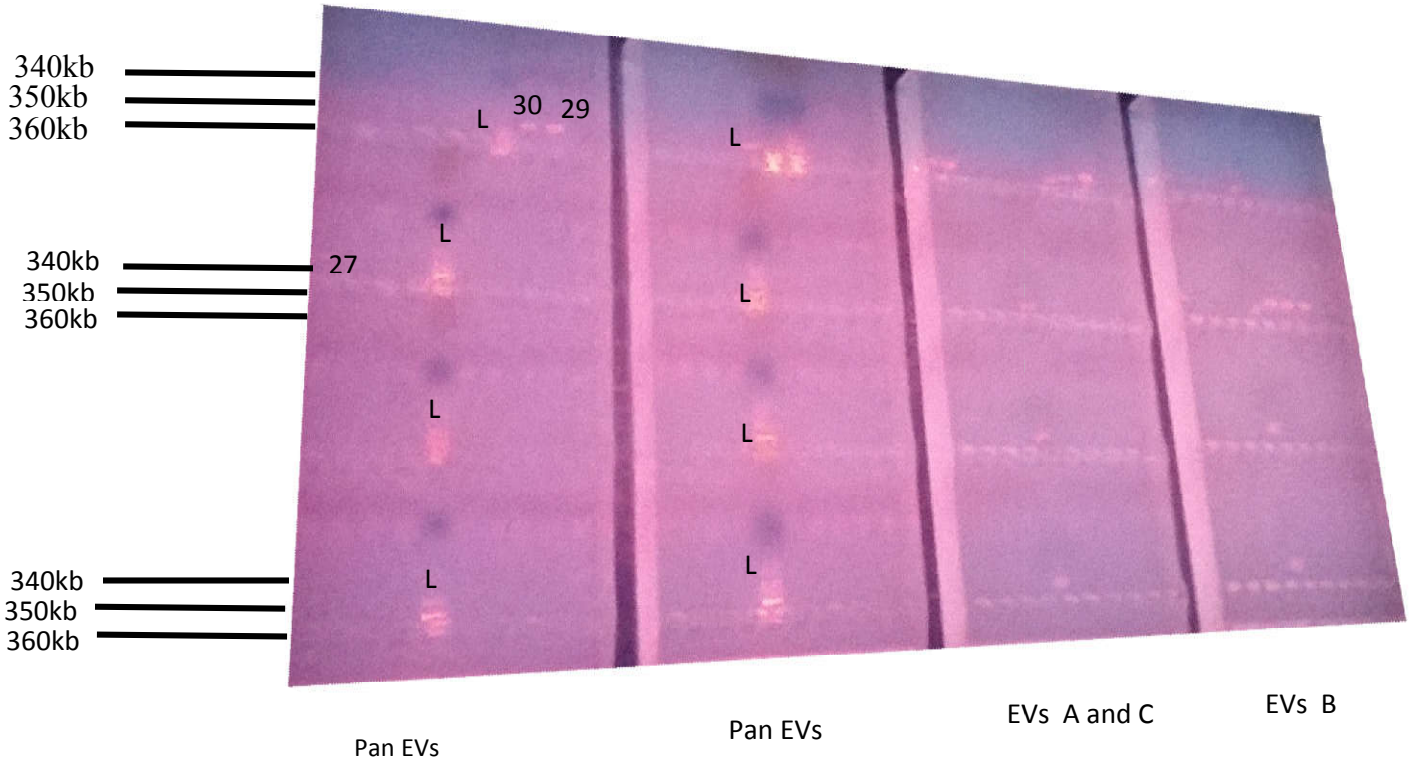


Figure 4.2 The gel electrophoresis from the right EVB, EVA and C-PCR and Repeated

Pan Enterovirus resolved on 2% agarose gel.

Key: L: MassRuler DNA ladder, each line represents amplicon from samples.

L29, L30 serves as the positive control, L27 serves as the only positive recovered sample.

TABLE 4.3 Distribution of samples according to States and gender

States	Female	Male	Total
Lagos	3	5	8
Ondo	4	2	6
Ogun	3	3	6
Oyo	0	5	5
Ekiti	0	3	3
Osun	1	1	2
Total	11	19	30

4.3 Virus Identification

From all the samples, one (1) sample that its amplicon yielded expected band size and the controls were subjected to sequencing and were typed using enterovirus genotyping tool (EGT). Afterward, sequences of the amplicon obtained from panenterovirus assay were typed as Echovirus 21, (E-21), Cosackievirus B-5 (CV-5) and Echovirus 7 (E-7) the strains from sample 27, first control and second control respectively.Furthermore the sequences revealed no member of species A and C assay (Figure 4.2). Considering the total of typed strains detected and identified from the three assays, the result obtained is 0 %, 3.3 % and 0 %, for enterovirus species A and C, Pan enterovirus and B respectively.

4.4 Phylogenetic analysis result

Considering the result obtained from this study, E 21 Echovirus (E-21) was aligned with reference sequences that were retrieved from Genbank with the aid of Cluster W program in MEGA 6.06 software with default setting (Kroneman *et al*., 2011).Subsequently, maximum likelihood trees were constructed using MEGA 6.06 (Tamura *et al*., 2011) and 1000 bootstrap replicates. The accession numbers and the sequences retrieved from the Genbank for phylogenetic analysis are indicated on the phylograms.

Table 4.4 Result of positive samples for Panenterovirus VP1 RT-snPCR and the identity of the enterovirus obtained.

Serial number	Sample Identity	Gender	Age (months)	VP1 RT-PCR	Serotype
1	27	Male	17	Positive	E-21

File: O27_187.ab1 Run Ended: 2016/7/8 19:22:54 Signal G:1671 A:2736 C:3550 T:2187

Sample: O27_187 Lane: 12 Base spacing: 16.251097 425 bases in 5183 scans FI

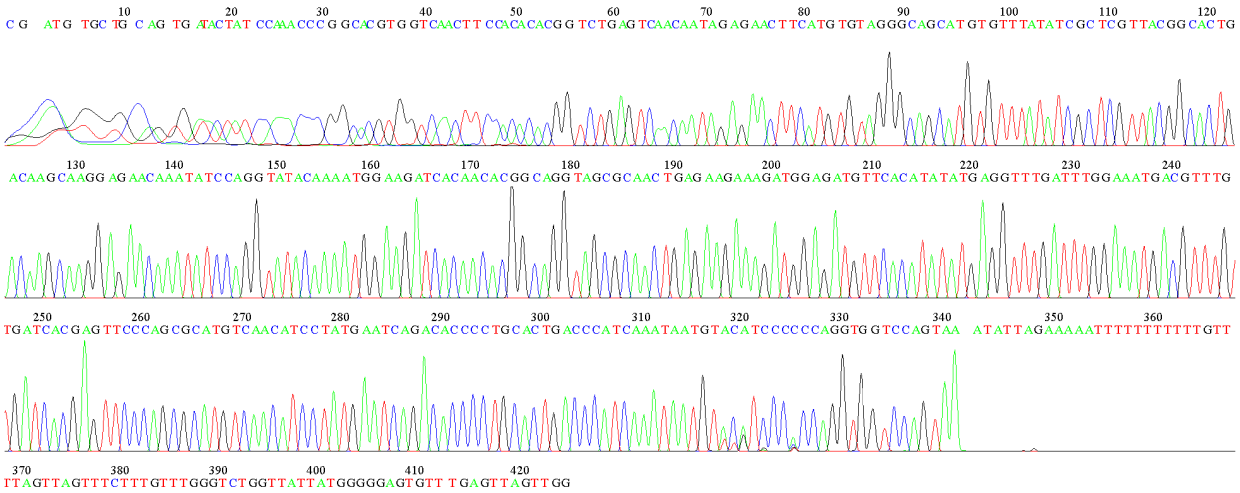


FIGURE 4.3

The electropherogram of the (E-21) sequences recovered from Southwestern Nigeria that is positive in this study is shown above.

>160707-065_E03_O27_187.ab1 425

CGATGTGCTGCAGTGATACTATCCAAACCCGGCACGTGGTCAACTTCCAC

ACACGGTCTGAGTCAACAATAGAGAACTTCATGTGTAGGGCAGCATGTGT

TTATATCGCTCGTTACGGCACTGACAAGCAAGGAGAACAAATATCCAGGT

ATACAAAATGGAAGATCACAACACGGCAGGTAGCGCAACTGAGAAGAAAG

ATGGAGATGTTCACATATATGAGGTTTGATTTGGAAATGACGTTTGTGAT

CACGAGTTCCCAGCGCATGTCAACATCCTATGAATCAGACACCCCTGCAC

TGACCCATCAAATAATGTACATCCCCCCAGGTGGTCCAGTAAATATTAGA

AAAATTTTTTTTTTTGTTTTAGTTAGTTTCTTTGTTTGGGTCTGGTTATT

ATGGGGGAGTGTTTGAGTTAGTTGG

Figure 4.4 Sequence of the Positive Sample obtained in this Study

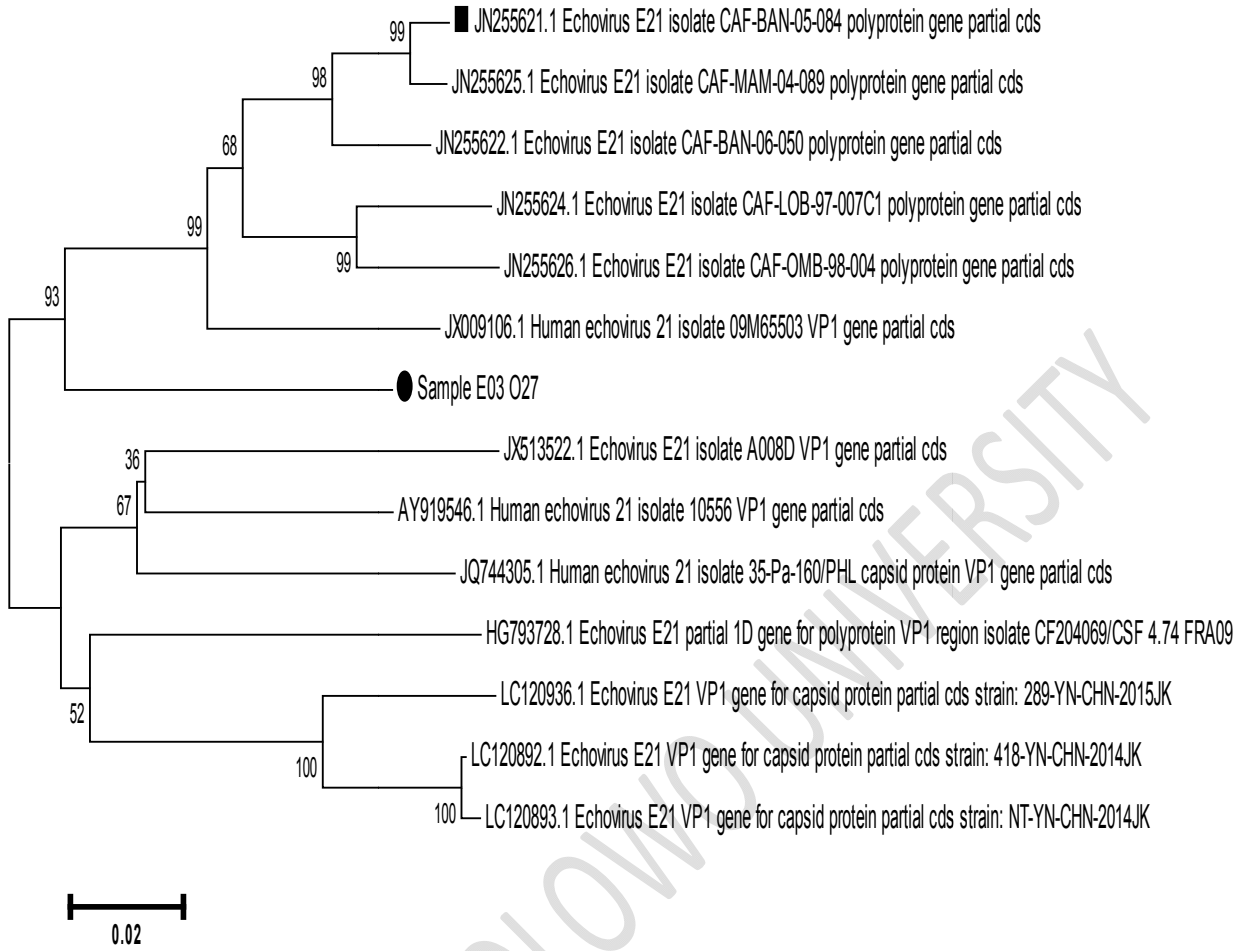


Figure 4.5 Phylogenetic of the recoveredE-21 isolate

ML Tree showing the Recovered E-27 Isolates. The newly Sequence and Reference Strains are represented. The newly strain E- 21 is highlighted with Black irregular Circle. The phylogram is based on alignment of the partial VP1 sequences. The newly sequenced strain is from Nigeria and some strains from Central Africa. The GenBank accession numbers and the name of the strains are indicated in the phylogram.

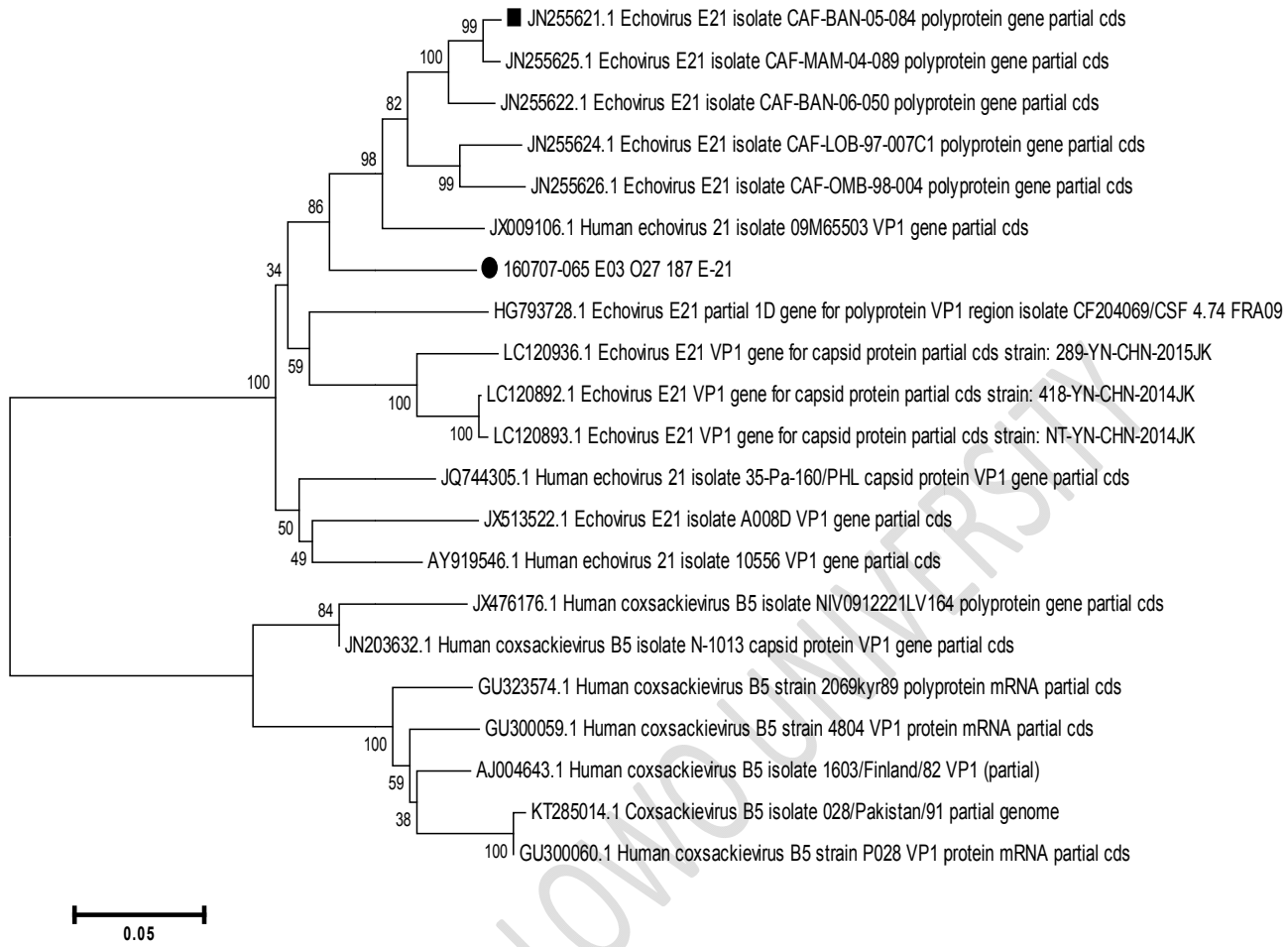


Figure 4.6 Phylogenetic relationship of recovered Echovirus 21 strain and the positive controls. The phylogram is based on alignment of the partial VP1 sequences. The recovered sample and the controls are represented and are highlighted in black irregular circle. The GenBank accession number of both the recovered strain from Nigeria and reference strains are indicated in the phylogram.

CHAPTER 5

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

This study applied phylogenetic analysis for the characterization of non-culturable enterovirus species in stool samples of patients with Acute Flaccid Paralysis in Southwestern Nigeria. The results from this study revealed the rate of detection of enterovirus in the 60 samples analyzed as 30 cases in Southwestern Nigeria in children with AFP considered in Lagos, Oyo, Ondo, Ogun, Osun and Ekiti State. Among the species of enteroviruses that exist, only species B of the enteroviruses was recovered in this study. That is, one of the 30 cases, 3.33% or (1/30) showed enterovirus species B as the only species of EVs.Faleye *et al*., (2012)reported the high prevalence of EVB EVs circulating in SW among other in his study. There was a report of ten (10) detection of EVs off which nine (9) are members of species B.

On the contrary, Faleye *et al*., (2016)revealed in his study that species A enterovirus were more commonly detected than members of other enteroviruses species but the significance of his study was not easy to determine as this may be due to the samples considered or used in the analysis.Faleye *et al*., (2006) made use of feacal samples of healthy childrenmeanwhile, in this study, feacal samples of children with AFP were considered. Also, the samples usedby Faleye *et al*. (2006) bypassed cell-culture and method of direct detection and identification of enteroviruses was employed while all the samples in this study were first subjected to cell-culture before Nix *et al* (2006).

The result of this study showed the presence of only one serotype of non-polio enteroviruses in children in six States in south-western Nigeria. This study documents the first molecular sequencedata on Echovirus 21 (E-21) in Oyo State Nigeria. Echovirus 21 in research work has been detected in Southwest Nigeria in Lagos State from environmental sample. Echovirus 21 (E-21) isolated

from this study showed a high nucleotide similarity with the isolate from Central Africa Republic in 2012. The fact exists that the first isolation was documented to occur from thee feaces of asymptomatic children early in the 1950s, just after cell culture was developed (Yin-Murphy and Almond, 1996).

However, the virus (E-21) has not been implicated with Acute Flaccid Paralysis (Oyero *et al*., 2010) but has shown the increasing rates of genetic recombination of the species with PV in China and other part of the world (Jingjing *et al.,*2010).Echoviruses are similar to many enteroviruses and may be associated with pyrexial illness with sore throat, cough (coryzo), herpangina characterized with vesicular, oral mucosal process involving the tonsilla fossa and soft palata symptoms which include elevated temperature, pharyngitis and dysphagia commonly observed in summer outbreaks involving young children of less than 10 years and less common in young adults (Tao *et al*., 2014).

Herpagina begins abrupty with fevers as high as 104 ^{0}F and it is associated with non-persistent vomiting, myalgia and headache, dysphagia and the prominent symptoms that proceed the appearance of oral lesion. Fatal echovirus induced meningoencephalitis often associated with a dermatomyositis. The acronym Echo is for enteric cytopathic human orphan (https:www.ncbi.nlm.nih.gov, article).

5.2 CONCLUSION

In conclusion, this study documents that there is possibility the stool of children with Acute Flaccid Paralysis contain enteroviruses, also the present species of enteroviruses may not be detected by cultural method and that the genotype of the enteroviruses can characterized by phylogenetic analysis. Based on the results obtained from this study, it is apparent clear that the prevalence ofEVs in south-western Nigeria is low. The findings of this study was able to generate result about enteroviruses species present in samples of children with AFP which

were negative during culturing method. It thereby established and confirmed that enteroviruses could be present in feacal samples (stool) which may be negative when subjected to cell-culture. Furthermore, it shows that Nix *et al*., (2006) protocolis able to amplify the most prevalent genome even when mixtures are present in the samples. In addition, this study characterized the genotype of the enteroviruses by phylogenetic analysis thereby establishing the recovery of (E-21) of species B in a male child from Oyo State. Though E-21 has formally been discovered in Lagos State. Consequently, this study can serve as a baseline for other molecular studies in Polio Eradication program.

5.3 RECOMMENDATION

Based on the findings in this study, it is therefore recommended that:

Studies base on the RD cell line that have associated certain enteroviruses strains should be interpreted with care because there is likeliness that not all the enteroviruses in the samples were detected. Hence, caution should be taken before concluding that samples are free or negative of enteroviruses. Therefore, culture method should not be the only concluding method other method such as molecular technique should also be used. That is, the both cultural and molecular method of identification should be used for detection and proper isolation of non-culturable enteroviruses.

Highly sensitive and very specific method for enterovirus detection in specimens is essential especiallyin feacal samples and others which might contain viruses. As these methods will help to easily detect and identify enteroviruses anytime they are present and to resolve members of different species most especially, members that are of the same species which are present in the same specimen. Ability to do this will make it easy to better characterize enterovirus serotypes that may be present in any sample or specimen and or concurrently

circulating in a population at any time. Doing this might then shed light on the enigma presently existing in the field of enterovirology.

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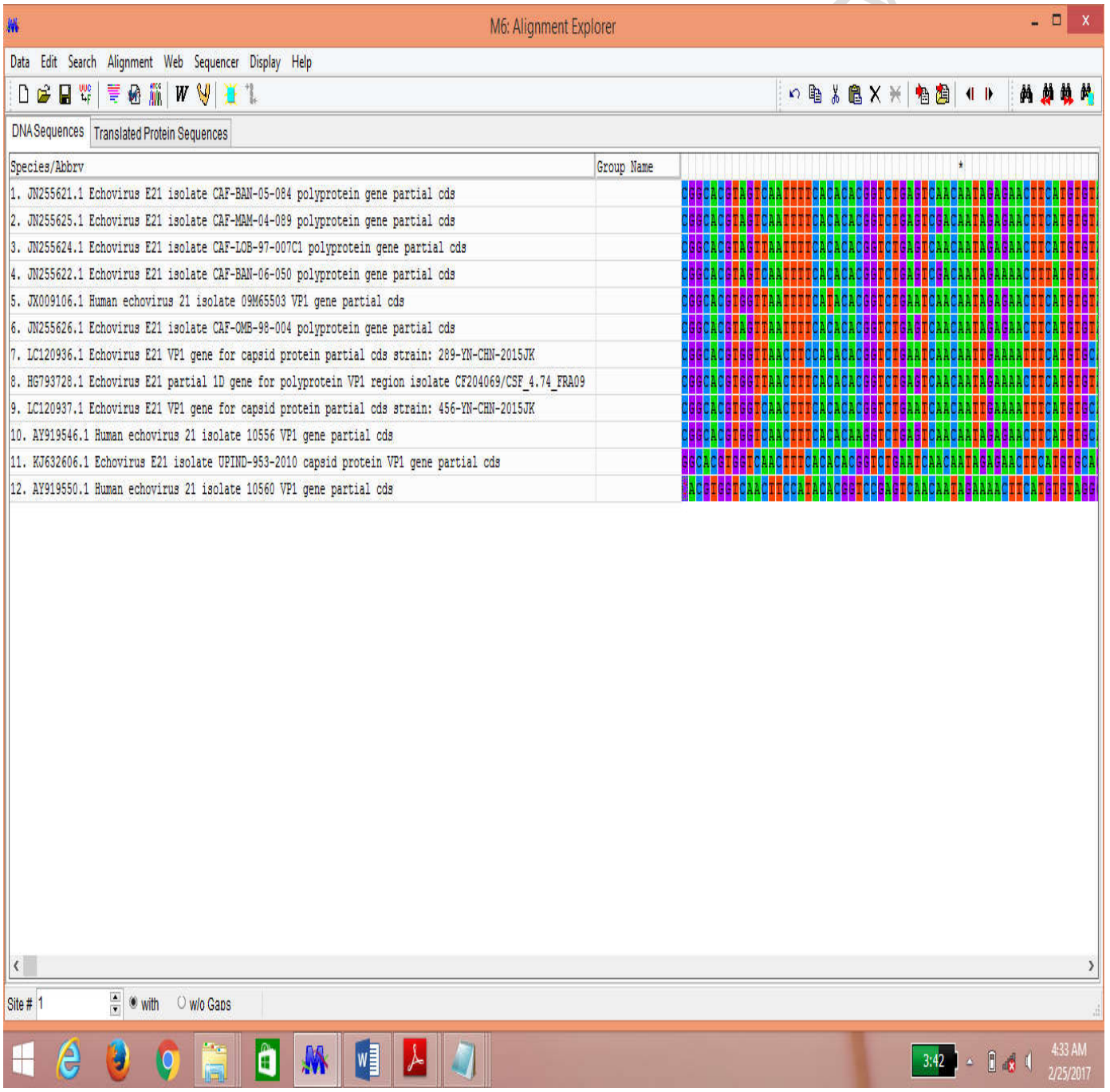
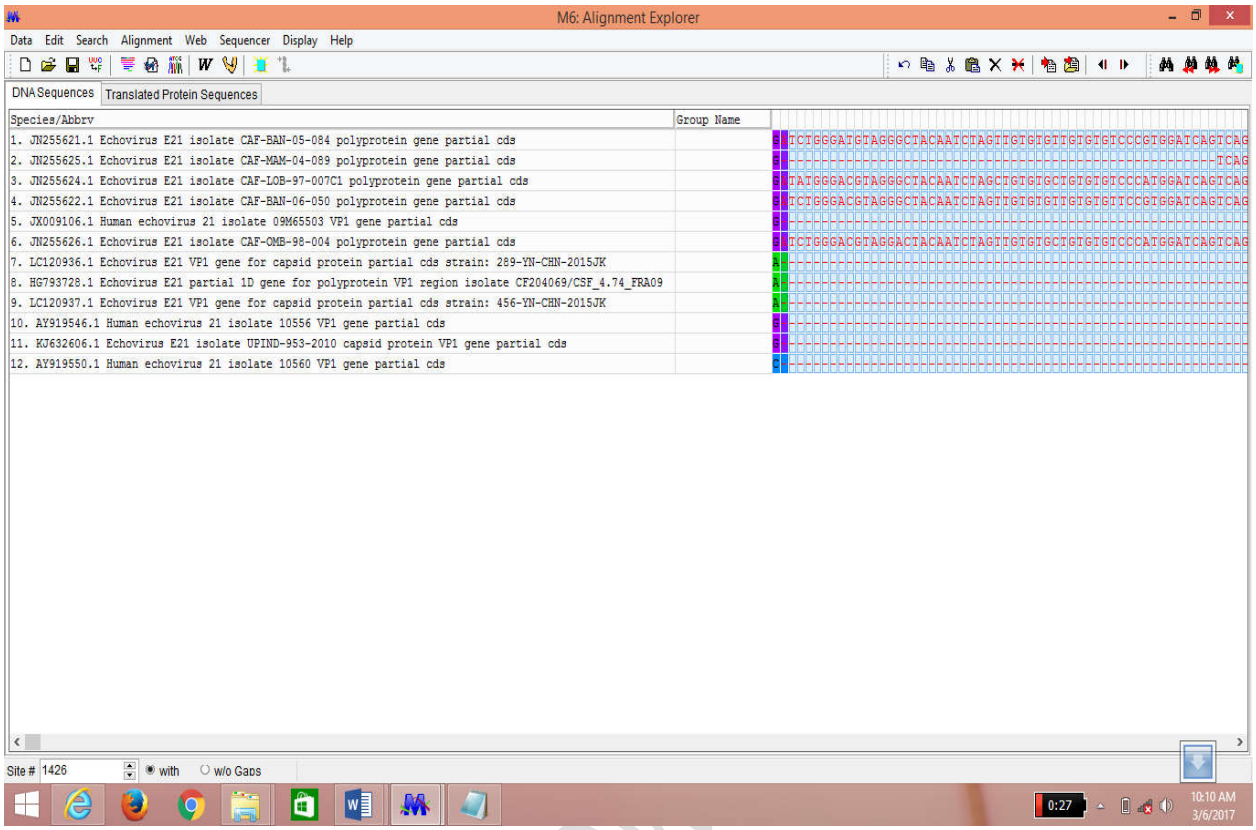
APPENDIX

Appendix 1: Alignment of the sequence for Echovirus-21 with the use of cluster W



Appendix 2: **Initial state of the Alignment of the sequence for the recovered Isolate**



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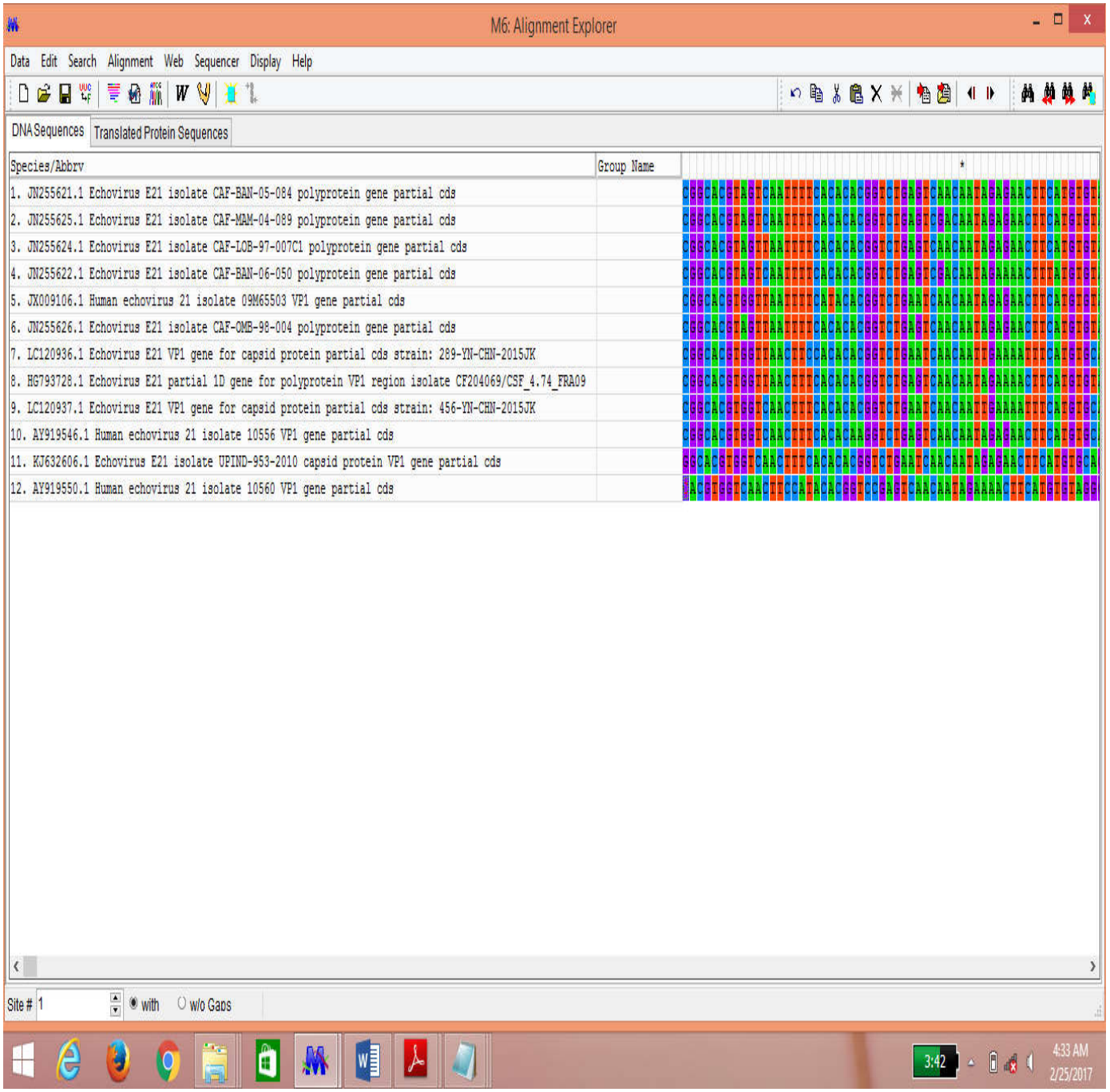
APPENDIX

Appendix 1: Alignment of the sequence for Echovirus-21 with the use of cluster W



Appendix 2: **Initial state of the Alignment of the sequence for the recovered Isolate**