UNIVERSITY OF CAPE COAST

ENVIRONMENTAL SURVEILLANCE OF POLIOVIRUS IN FOUR DISTRICTS WITHIN TWO REGIONS OF GHANA

BY

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Thesis submitted to the department of Microbiology and Immunology, School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast in partial fulfillment of the requirement for the award of Master of Philosophy degree in Infection and Immunity degree

OCTOBER 2020

DECLARATION

Candidate's declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature…… Date……

Name: ERNEST OBESE-DJOMOAH

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date…….

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ABSTRACT

The eradication of poliovirus is at its last phase through the efforts and strategies of Global Polio Eradication Initiation (GPEI). There are very few countries that are still endemic with wild poliovirus (WPV) and others with circulating vaccine derived poliovirus (cVDPV). The aim of the study was to detect silent circulation of WPV and VDPV in four districts within the Eastern and Volta region of Ghana. A systematic longitudinal design was used for the study. The open sewage systems were located in New Juabeng and Ho districts while the close sewage systems were located in Asuogyaman and Ketu South districts. A total of 35 sewage samples were collected from September 2018 to May 2019. L20B and RD cell lines were used for isolation poliovirus (PV) while real-time reverse transcriptase polymerase chain reaction (rRT-PCR) was use to characterize the serotypes of the PVs. The findings of the study showed that the prevalence of non-polio enterovirus (NPEV) and Sabin were 65.71% and 14.29% respectively. The characterized Sabins were serotype 1 and serotype 3 which were circulating in the two districts within the Eastern Region. The open sewage systems were located in New Juabeng and Ho districts while the close sewage systems were located in Asuogyaman and Ketu South districts. The study did not detect any WPV and VDPV but isolated Sabin strains of the poliovirus. This necessitates the need for continuous environmental surveillance for poliovirus nationwide.

ACKNOWLEDGEMENTS

First and foremost, I express my profound gratitude to my supervisors, Prof. Samuel Victor Nuvor and Dr. John Kofi Odoom for their patience, constructive criticisms and supervision. Words cannot express my heartfelt gratitude for your encouragement and inspiration. God bless you.

Secondly, I am grateful to Dr. Mrs. Evangeline Obodai, for her generous contribution to make this work better.

Again, many thanks to the Polio Project team, staff of Virology Department, Noguchi Memorial Institute for Medical Research for their support

Finally, I am grateful to all my cherished friends and loved ones for their assistance in diverse ways in course of my programme.

DEDICATION

To my wife, Doreen Asantewa Abeasi.

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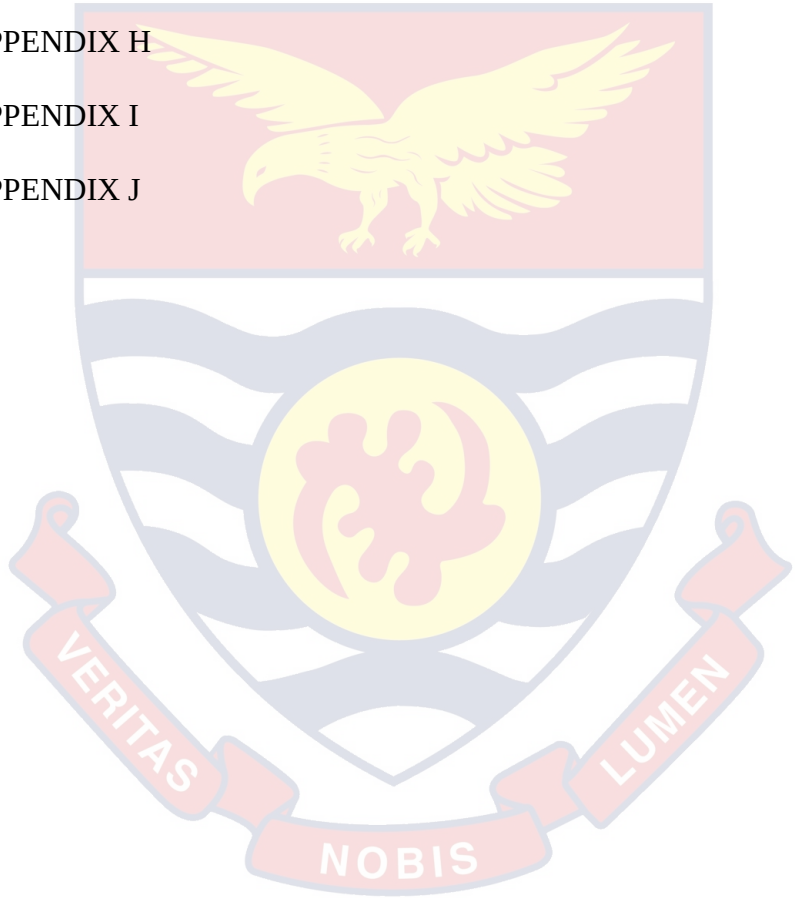

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LIST OF ACRONYMS

AFP	Acute Flaccid Paralysis
bOPV	Bivalent Polio Vaccine
CNS	Central Nervous System
CDC	Centre for Disease Control and Prevention
cVDPV	Circulating Vaccine Derived Poliovirus
cDNA	Complementary DNA
CV	Coxsackievirus
CPE	Cytopathic Effect
EV	Enterovirus
ES	Environmental Surveillance
ELISA	Enzyme-Linked Immunosorbent Assay
EPI	Expanded Programme on Immunization
GCC	Global Commission for Certification
GPEI	Global Polio Eradication Initiation
GPLN	Global Polio Laboratory Network
HEV	Human Enterovirus
Hep-2	Human Epidermoid Carcinoma
RD	Human Rhabdomyosarcoma
IPV	Inactivated Polio Vaccine
ITD	Intratypic differentiation
mRNA	Messenger Ribonucleic Acid
NIDs	National Immunization Days

NMIMR	Noguchi Memorial Institute for Medical Research
NPEV	Non-Polio Enterovirus
ORF	Open Reading Frame
OPV	Oral Polio Vaccine
PV	Poliovirus
PCR	Polymerase Chain Reaction
rRT-PCR	Real-Time Reverse Transcriptase Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RV	Rhinovirus
RNA	Ribonucleic Acid
SL	Sabin-Like
SIAs	Supplementary Immunization Activities
tOPV	Trivalent Oral Polio Vaccine
UTR	Untranslated Region
VDPV	Vaccine-Derived Poliovirus
WPV	Wild Poliovirus
WHA	World Health Assembly
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

Summarizing the current concept, understanding and the background information about the topic, stating the purpose of the work in the form of the hypothesis, question, or research problem, briefly explaining the rationale of the study, methodological approach and highlighting the potential outcomes of the study. Finally, operational definition of terms used in this study was duly explained.

Background to the Study

Poliovirus (PV) is a single-stranded positive-sense RNA virus which is a member of Human enterovirus C (HEV-C) and belongs to the family *Picornaviridae*, with the order of Picornavirales. It is the causative agent of poliomyelitis (commonly known as polio) which mainly affects children under the age of 5 years. There are three serotypes of PVs: PV1, PV2 and PV3 (Pallansch & Roos, 2007) and the disease can be caused by anyone of the three serotypes.

Polioviruses are transmitted by the fecal-oral route; they multiply in the gut whether or not the infected persons are symptomatic. Virus infects sensitive cells of lymphoid tissue in the mouth, nose and throat. The incubation period lasts from 2 to 35 days. It leads to a transient viremia and the virus spreads to the reticuloendothelial system without causing clinical symptoms. In very rare cases of about 1–2% of infected individuals, the virus enters the central nervous system (CNS) and replicates in motor neurons within the spinal cord, brain stem, or motor cortex causing irreversible paralysis (Sabin, 1956). The infected individuals

usually do not show symptoms of paralysis after few days of infection. The replication of the virus may progress to affect the skeletal and respiratory muscles which results in spinal poliomyelitis and bulbar poliomyelitis respectively. The infected person can also develop bulbo-spinal poliomyelitis (that is the effect of the PV replication in both the respiratory and skeletal muscles) (Jones et al., 2017).

The wild poliovirus type 2 (WPV2) was declared eradicated worldwide in September 2015 by the Global Commission for the Certification of Poliomyelitis Eradication (GCC). WPV2 was last detected in the year 1999 at Aligarh, Northern India (GPEI, 2017), while the wild poliovirus type 3 (WPV3) has not been detected globally since November 2012 and was declared eradicated on 17th October 2019. The wild poliovirus type 1 (WPV1) is currently the only serotype in circulation and restricted to Pakistan and Afghanistan (WHO, 2018).

The World Health Assembly (WHA) resolved to put strategic efforts to eradicate poliovirus in 1988 (Pallansch, Oberste, Whitton, 2013). The strategic plan and implementation of high level of routine immunization, acute flaccid paralysis (AFP) surveillance, national immunization days (NIDs) and mop-up campaigns by the Global Polio Eradication initiative (GPEI) has interrupted the indigenous circulation of wild strain of the PV globally except in two countries (Afghanistan, and Pakistan) where ongoing wars have made it difficult to eliminate the virus (WHO, 2019). The presence of PV transmission in these endemic countries make all countries prone to the virus especially those with weak public health immunization services. Travelling and trading in these

endemic countries poses a risk of virus importation to polio-free countries. There have also been instances of reintroduction of PV in countries like Syria and Somalia which has resulted in outbreak due to insecurity in the quality of immunization service for the mass population movement (Taylor, 2009).

Polio is a preventable disease when susceptible individuals are immunized with any of the two types of polio vaccines – Inactivated polio vaccine (IPV) and oral polio vaccine (OPV). The OPV consists of a mixture of live attenuated PV strains of each of the 3 serotypes. This is also referred to as Sabin strains 1, 2, and 3 (WHO, 2015). However, in April 2016, the whole world switched from the use of the trivalent polio vaccine (tOPV) to a bivalent polio vaccine (bOPV). The mutation in the 5'NCR at position 480 of the capsid protein make the live attenuated vaccine revert to neurovirulence of OPV strains which is transmitted from person to person. The virulent form of this strain from the OPV is termed as vaccine-derived poliovirus (VDPV) which is able to circulate in districts with low immunization coverage and may cause paralysis (Dowdle, De Gourville, Kew, Pallansch & Wood, 2003). VDPVs display 1–15% nucleotide difference within VP1 from parental vaccine strain (CDC, 2007).

Acute flaccid paralysis surveillance has been the gold standard for the detection of PV but paralytic poliomyelitis occurs in small factions of individuals infected with the PV. This faction is further reduced as vaccination currently covers a large portion of the population globally (Nathanson, Kew, 2010), therefore rendering AFP surveillance insensitive to detect an outbreak that requires an immediate response. Stools samples taken within 14 days from people

that have experienced acute flaccid paralysis is a strategic effort by the WHO to detect WPV and mutated vaccine PV. Contact tracing of vulnerable children within the geographical area of the affected patient is sampled as supplementary to AFP surveillance. Environmental surveillance (ES) serves as an additional method to monitor the transmission of poliovirus by testing sewage samples, which may contain polioviruses in human faeces (WHO, 2003). Environmental surveillance has been useful in detecting the presence of PVs circulating in districts where there have not been any detection of AFP from infected persons (Deshpande et al., 2003). Combination of ES and AFP surveillances could be more sensitive to detect low circulation of WPV and circulating vaccine derived poliovirus (cVDPV), in order to sustain poliovirus eradication.

The role of ES can be summarized as identifying the transmission of residual WPV in an endemic country; prompt detection of PV importation into polio-free country and confirmation of Sabin-related virus as a result of the oral polio vaccine (OPV). Environmental surveillance also serves as monitoring tool around containment facilities to check the biosecurity of the facility before it becomes means of transmission of the PV to the community. Environmental surveillance also provides evidence for global certification when no PV or VDPV is detected in any country (Ndiaye, Mbathio & Diop, 2014; Gumede et al., 2015). Studies conducted in some African countries including Nigeria and Egypt using ES isolated WPVs from sewage samples with no isolation of WPVs in AFP patients (Theodore et al, 2013; WHO, 2013).

Statement of the Problem

AFP surveillance has been the gold standard for polio eradication (Odoom et al., 2017;WHO, 1998) but it is limited in detecting PVs. This is attributed to the asymptomatic nature of the disease in which most people are infected with the PVs but only a small fraction present acute flaccid paralysis. In Ghana, the last case of wild poliovirus was recorded in November 2008, since then, all samples from AFP have tested negative for polio (Odoom et al., 2014).

Surveillance indicators for acute flaccid paralysis for Eastern and Volta regions were not optimum in 2017 and 2018 according to the Disease Surveillance Department, Ghana Health Service. These indicators include; notification of all AFP as prospective polio case, collection of two stool samples within 24-48 hours apart. The stool samples should be adequate and all these stool samples should be collected within 14 days of the onset of paralysis. However, the Eastern and Volta regions reported very low case of AFP as a result of delay in notifying disease control officers of any prospective AFP and collecting one sample instead of two samples to be transported to the laboratory for investigation. Another important surveillance indicator was the non-polio AFP rate of 2 AFP cases per 100,000 population of children under 15 years which was also not achieved (CDC, 2012a).

In addition, the sanitation in most districts within the Eastern region is poor and open defecation is widespread. Human excreta contaminated with PV may have been shed in the environment predisposing children who play around in such poor environmental conditions (Bandyopadhyay et al., 2019).

The Volta region which has communities which share border with Togo on the east part of Ghana is faced with security porosity and there are no measures to check for migrants that have been vaccinated against PV. Those who might be infected with the PV but are asymptomatic escape into these communities thereby shedding the virus in the environment which can infect and paralyze susceptible host.

Environmental surveillance plays an important role in boosting the sensitivity of detecting silent transmission of the PV without any record of clinical evidence from AFP cases after laboratory investigation (Battistone et al, 2014). Whereas AFP detects 0.5-1% of all polio cases, ES detects 100% of all polio cases. ES is to supplement AFP surveillance to detect all circulating polioviruses among those who are asymptomatically infected and not showing clinical signs of paralysis.

The inability to meet all the indicators of AFP, whereas there is challenge in assessing the immunity of influx migrants as well as poor sanitation means there is the need to carry out ES to help in early detection of PV that might have been imported or emerged within the districts.

Purpose of the Study

The purpose of the study is to determine silent circulation of poliovirus from the sewage system and waste water that were running from the large catchment area of New Juabeng, Asuogyaman, Ketu South and Ho districts before entry into any treatment plant.

Research Objectives

Aim

To determine the silent circulation of wild type and vaccine-derived poliovirus in four districts within the Eastern and Volta Regions of Ghana.

Objectives

1. To determine the presence of poliovirus and non-polio enterovirus (NPEVs) in the New Juabeng, Asuogyaman, Ketu South and Ho districts.
2. To characterize the poliovirus isolates.
3. To determine the genotypes of the poliovirus strains isolated.

Hypothesis

There is no silent circulation of wild and vaccine-derived poliovirus in the selected districts of the Eastern and Volta regions.

Significance of the Study

Acute flaccid paralysis surveillance identifies and examines stool samples from suspected AFP patients in order to isolate PV from those stool specimens. This gives the basis to further identify and investigate the risk level of the community or district where such patients with poliomyelitis are shedding the PV in the environment (WHO, 2004b). Unlike ES, the human sewage from the drainage system in the community is examined and PV isolates are linked from unknown individuals to the population.

The rationale for ES is based on the fact that infected individuals, whether they present with the symptoms or not, excrete poliovirus in faeces up to several weeks. Environmental surveillance is used for monitoring PV transmission in

human populations by examining environmental samples contaminated by human faeces. The large proportions of infectious poliovirus remain in the environment depending on the favourable conditions of the wastewater. ES provide valuable information, particularly in high-density urban populations where AFP surveillance is absent or does not achieve its optimum indicators but there is suspicion of silent circulation of PV or frequent virus reintroduction.

Delimitations

The study was confined to sewage system identified in four districts in only two regions in Ghana. The sewage was running from a large catchment area of the total population within the selected communities.

Isolation from the sewage samples that showed cytopathic effect (CPE) on both L20B and RD cell lines were further characterized and sequenced but those that show CPE in only RD cell line or none of the cell lines were not further investigated.

Limitation

The sensitivity of the procedure used to concentrate the sewage samples before inoculation on the cell lines are not well known to detect low levels of poliovirus circulation. This makes it challenging to interpret a negative finding from this project since the sensitivity is less known.

The small sample size due to the time constrain may affect the generalization of the findings of the study.

Organization of Study

The study is organized into five chapters. Chapter one describes the background to the study, problem statement, purpose of the study and the significance of the study. Chapter two reviews literature relevant to the study. Chapter three describes the materials and methods employed in the study. Chapter four provides the results for the study and furthers discusses the results in relation to existing relevant literature. Chapter five provides summary, conclusion, recommendation and future perspectives that can be considered.

Definition of Terms

Circulating Vaccine Derived Poliovirus: mutated vaccine polioviruses, usually 1–15% sequence differences from the parental oral polio vaccine PV1 and PV3 0.6-15% for PV2.

Environmental Surveillance: It is detection of the presence of poliovirus in sewage samples by a variety of laboratory methods for concentration, separation and identification.

Poliovirus: It is the agent responsible for paralytic poliomyelitis, is a member of the *Picornaviridae* family, a group of non-enveloped positive-strand RNA viruses.

Sabin strains: They are composed of live-attenuated strains of the three poliovirus serotypes that are replicated in the guts of immunocompetent persons for a limited period after vaccination.

Sewage: This is a type of waste water that is produced by a community of people. It carries bodily wastes (primarily feces and urine), washing water, food preparation wastes, laundry wastes, and other domestic waste.

Wild Poliovirus: They are naturally occurring isolates known or believed to have circulated persistently in the community.

Chapter Summary

This chapter began by providing insight to the background of the study and also examined the gap in literature that needs to be addressed in relation to environmental surveillance of poliovirus detection. In Ghana available studies for the detection of PV have been on AFP surveillance where there has been isolation of different serotypes of PV. ES on PV in Ghana are scarce and very few districts have been explored to examine the sewage to detect PV. The study therefore hypothesized that there is silent circulation of wild type and vaccine-derived poliovirus in four districts within the Eastern and Volta Regions of Ghana. Again, the chapter described the delimitations and limitations of the study and ended with operational definition of terms as applied to the study.

CHAPTER TWO

LITERATURE REVIEW

Introduction

This chapter reviews the literature related to environmental surveillance of poliovirus. It identifies some of the gaps in existing knowledge on the problem and it provides fundamental understanding of the theoretical concepts from similar research to direct future research. A computer search for related literature was done using databases such as Pubmed, Hinari, Google, Web of Science. The key terms used to review the work were environmental surveillance, poliovirus, and non poliovirus. For chronology, the review was organized based on the research objectives which were;

- To determine the presence of poliovirus and non-polio enterovirus (NPEVs) in the New Juabeng, Asuogyaman, Ketu South and Ho districts.
- To characterize of the poliovirus isolates.
- To determine the genotypes of the poliovirus strains isolates.

Overview of Poliovirus

Poliovirus (PV) is the causative agent of poliomyelitis, an acute paralytic disease which can be life threatening among children under five years. PV is a ribonucleic acid (RNA) virus which belongs to the genus Enterovirus C and in the family, Picornaviridae. There are three serotypes of PV (PV1, PV2 and PV3) (Figas, Wieczorek, & Żuk-wasek, 2018). This virus can exist in a form of mixtures called quasispecies. The PV is prone to high mutation during its replication process in human. The RNA polymerase enzyme of the virus lack

proof reading activity that would have aided in correcting any nucleotide changes in the genetic lineage (Figas et al., 2018). Another unique property of the PV is its ability to form combination strain of the same serotypes of PV (intratypic recombinants) or other serotypes to enterovirus (intertypic recombinants) (Figas, Wieczorek, & Żuk-wasek, 2018).

Classification of Enterovirus

The International Committee on Taxonomy of Viruses published the updated version of the classification of enteroviruses in the 9th issue of Virus Taxonomy in 2012 (Adams et al., 2016). The genus *Enterovirus* of the family Picornaviridae has nine enterovirus species (EV-A to EV-J) and three rhinovirus species (RV-A to RV-C). In 2015, a new species of enterovirus was discovered in camels called Enterovirus I (Nikonov, Chernykh, Garber, & Nikonova, 2017).

The species *Enterovirus A* includes 25 serotypes: coxsackievirus A2 (CVA2), CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, CVA16, enterovirus A71 (EVA71), EVA76, EVA89, EVA90, EVA91, EVA92, EVA114, EVA119, EV A120, EVA121, simian enteroviruses SV19, SV43, SV46, and baboon enterovirus A13 (BA13) (Nikonov et al., 2017).

The species *Enterovirus B* include 63 serotypes: coxsackievirus B1, CV B2 – B6, CVA9, echovirus 1 (E1), E2 – E7, E9, E11 – E21, E24 – E27, E29 – E33, enterovirus B69 (EVB69), EVB73 – EVB75, EVB77 – EVB88, EVB93, EVB97, EVB98, EVB100, EVB101, EVB106, EVB107, EVB110 (from chimpanzee), EVB111, EVB112 (from chimpanzee), EVB113 (from mandrill), and simian enterovirus SA5 (Nikonov et al., 2017).

The species *Enterovirus C* includes 23 serotypes: poliovirus (PV) 1, PV2, PV3, coxsackievirus A1 (CVA1), CVA11, CVA13, CVA17, CVA19, CVA20, CVA21, CVA22, CVA24, EVC95, EVC96, EVC99, EVC102, EVC104, EVC105, EVC109, EVC113, EVC116, EVC117, and EVC118 (Harvala et al., 2017).

The species *Enterovirus D* is relatively uncommon; it includes five serotypes: EVD68, EVD70, EVD94, EVD111 (from human and chimpanzee), and EVD120 (from gorilla). The species *Enterovirus E* includes bovine enterovirus group A: from EVE1 to EVE4. The species *Enterovirus F* includes bovine enterovirus group B (at present, six types are described): from EVF1 to EVF6. The species *Enterovirus G* consists of 16 serotypes*:* from EVG1 to EVG16 (Harvala et al., 2017).

The species *Enterovirus H* includes three monkey viruses isolated in 1950 (SV4, SV28, and SA4) and A2 plaque virus. However, these four viruses were joined into a single serotype enterovirus H1 (EVH1) due to their strong similarity at the molecular level. The species *Enterovirus J* contains six simian enterovirus species: SV6, EVJ103, EVJ108, EVJ112, EVJ115, and EVJ121 (Nikonov et al., 2017).

EVs cause a wide spectrum of infections in humans, including non-specific febrile illness and viral exanthema, respiratory infections, hand, foot and mouth disease (HFMD), myocarditis, meningitis, encephalitis and, rarely, acute flaccid paralysis (AFP) (Harvala et al., 2017).

History of Poliovirus

Polio has been in existence during the olden days but associated with myth and destiny of children who suffer from the life threaten acute paralysis as they are been infected. There are Egyptians creative designs that indicate the deform nature of the limbs of children and the use of walking sticks during the period of 1403 to 1365. The name Polio was first given in 1789 by Dr. Michael Underwood, an English physician which means "debility of the lower extremities" (Mehndiratta, Mehndiratta, & Pande, 2014).

In the 18th century, many of the localized paralytic polio epidemics occurred in the Europe infecting about 27000 patients and more than 6000 of the patients died. The mystery of the polio made crescendo to associate the poliovirus infection as a curse from God unto the infected children between the 1940 and 1950. Parents of these infected children were anxious because most of the exposed children were quarantine (Schaulies, Schaulies, & Meulen, 2005).

Landsteirner and Popper (1909) contributed remarkably to identify the cause of the "debility of the lower extremities". They transmitted the PV to monkeys by the intracerebral inoculation of the human brain tissue homogenates. Enders, Weller and Robbins (1949) cultured the virus in non-neural tissue, thereby eliminating animals as the reservoir or the transmission of the PV to the human race. These discoveries made by these great scientists marked significant contribution to further study the structure, mode of transmission, clinical presentations and prevention of the poliomyelitis. The fight against poliovirus cannot be complete without the acknowledging efforts of Salk and Sabin who

developed polio vaccines to prevent the transmission of the poliovirus (Sabin & Boulger, 1973; Figas, Wieczorek, & Żuk-wasek, 2018).

PV was the first RNA virus genome to have its VP1 gene region to be molecularly cloned and sequenced in 1981. These efforts made it possible to detect the three different serotype of the PV (La Monica, Mariam & Raccaniello, 1986). The World Health Assembly (WHA) launched the Global Polio Eradication Initiative (GPEI) in 1979 after they came to admit the need to put in strategies to stop the transmission of the PV when they had successfully eradicated small pox globally (WHA, 1988). The strategies and efforts of GPEI implemented in three decades' have reduced the incidence of wild poliovirus (WPV) transmission by 99%. Only two out of the six regions of the World Health Organization (WHO), South Asia Region (Afghanistan, Pakistan) and Africa Region (Nigeria) are still endemic (Yusof et al., 2018). The WPV2 was declared eradicated globally in 2015 by WHO when it recorded its last incidence in India in 1999 when WPV1 and WPV3 were still in circulation. WPV1 has been isolated in Afghanistan while WPV3 has not been detected since 2012 (GPEI, 2019).

Pathogenesis of Poliomyelitis

The PV is able to survive in only human host. Most infected people develop asymptomatic to the infection with the PV but only 1-2% of the cases results in acute flaccid paralysis. The PV is transmitted through fecal-oral route via droplets from the pharynx. The PV infects the oropharynx and the gut which most often does not develop any symptoms in most infected people exposed to it. The PV is incubated in the oropharynx and gut for 2 to 35 days and shed in stools

or throat swaps after 2 to 5 days (Mehndiratta et al., 2014). PV is excreted in stools for 4–6 weeks. PV multiplies extensively in the tonsils, lymph nodes of the neck, and then subsequently in Peyer patches and small intestine, but the target cell where initial multiplication occurs is still unknown (Mehndiratta et al., 2014). There is a strong affinity for the poliovirus receptor, CD155, which is expressed in the intestinal epithelium, including the follicle-associated epithelium and microfold M cells of Peyer's patches, and in follicular dendritic cells and B cells of germinal centers within Peyer's patches when the PV attached to the target cells. It has been demonstrated that PV particles adhere specifically to and are endocytosed by human intestinal M cells, suggesting that M cells in humans are the site of PV penetration of the intestinal epithelial barrier (Gonzalez, Khademi, Borg, & Olssonet, 2012).

The PV causes extensive damage to cells that it infects due to its cytopathic characteristics it has as one of its properties. It destroys the anterior horn cells of the spinal cord which results in the limb paralysis. The PV can spread widely into the posterior horn cell of the spinal cord, the motor neuron of the thalamus and hypothalamus. The brain cells can also be affected which could be fatal in the bulbar form of poliomyelitis. PV is also released from lymphoid tissues into the bloodstream (viremia) and this spread to other tissues (Mehndiratta et al., 2014). Cells that are infected by PV are engulfed by mast cells and macrophages which end up in degeneration of axons. There is a severe atrophy leading to flaccid paralysis when the destruction is to the extreme. The infection can be fetal when the respiratory paralysis is affected to the extreme.

There is the possibility of post-polio syndrome (PPS) after the individual has been exposed to the PV and suffer some initial clinical symptoms twenty-five to thirty years ago (Gonzalez, Khademi, Borg, & Olssonet, 2012). According to Schaulies, Schaulies & Meulen, (2005), PPS is seen to steadily deteriorating the motor neuron thereby its effect is progressively affecting the muscles. Other school of thought also hypothesized that the presence of abnormal cytokines possibly due to the persistence of the PV in the brain and spinal cord. Figure 1 illustrate the pathogenesis of poliomyelitis in affected individual.

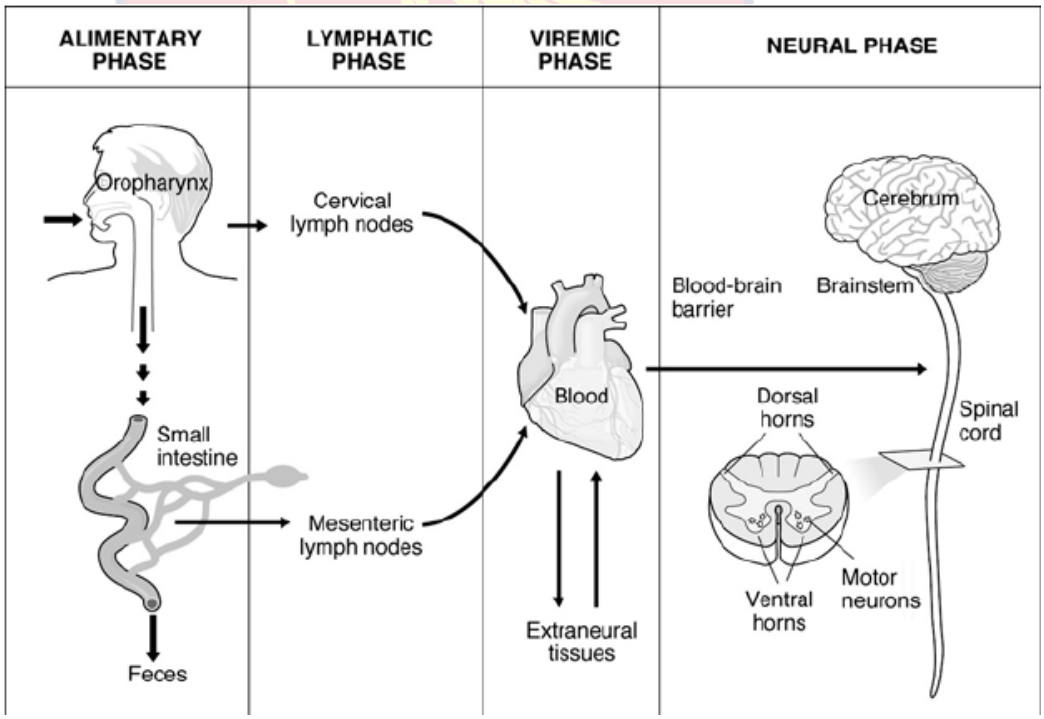

Figure 1: Poliomyelitis pathogenesis Source: (Blondel, Colbère-Garapin, Couderc, Wirotius, & Guivel-Benhassine, 2005)

Modes of Transmission

The manifestation of the clinical symptoms of poliomyelitis is seen when maximum of the PV is excreted two to three days after a week of infection in a susceptible host. The PV is highly communicable and spread through fecal-oral routes when the sanitation of the environment is poor and the immunization coverage in the community is low. This put non-immunized children at risk of being infected when they are exposed to the virus. The spread of the PV records high incidence in the summer month around temperate regions while tropical regions do not have significant difference from their epidemiological seasons.

Nursing mothers give some level of protection to their susceptible neonates and infants as the mothers introduce PV antibodies of all the three serotypes of the virus. The protective effect of the mother is as a result of her being exposed to any form of the virus or vaccine, put the child also at risk of being infected and protected simultaneously without any residual effects. McQuillen and McQuillen (2005) reported that poor sanitation, lack of personal hygiene, densely populated community water quality and sewage handling facilities were the contributory factors that expose children that have lived beyond the maternal antibodies protection.

According to Kretsinger et al. (2014) areas of good sanitation and safe drinking water can also have children exposed to the PV through secretions from the upper respiratory tract as they associate closely in contact with infected persons when they release large particles of aerosols contaminated with the virus

through vomitus and cough. The oral-oral route may account for some of the cases for PV infection.

Clinical manifestations of poliovirus infections

The response to PV infection is variable and has been categorized based on the severity of clinical presentation (Jones, Balalla, Theadom, Jackman, & Feigin, 2017; CDC, 2002a). More than ninety percent of the different serotypes of WPV infections do not show any form of symptoms in infected people (Gumede et al., 2018). There are 3 major clinical syndromes that are related to PV infection, namely abortive poliomyelitis, aseptic meningitis and paralytic poliomyelitis (Jones et al., 2017). As the infection progresses, a minor illness may be followed by a major, severe illness. However, infants and children usually experience biphasic disease course than adults (Mehndiratta et al., 2014).

Approximately 4% - 8% of PV infections are characterised by a minor, non-specific illness without clinical or laboratory evidence of CNS invasion (CDC, 2002a). This syndrome is known as abortive poliomyelitis and results in upper respiratory infection (sore throat and fever), gastrointestinal disturbances (nausea, vomiting, abdominal pain, constipation or diarrhoea) as well as influenza-like illness (CDC, 2002a). Nearly 10% of patients with abortive poliomyelitis will develop aseptic meningitis (non-paralytic poliomyelitis) indistinguishable from the minor illness due to non-polio enteroviruses (NPEVs) (CDC, 2002a). Typically, symptoms (stiffness of the neck, back and legs) will last from 2 to 10 days, followed by a complete recovery (CDC, 2002a).

Less than 2% of all polio infections result in flaccid paralysis, which begins with a minor febrile illness followed by a short asymptomatic period of 2 to 3 days (Gumede et al., 2018) . A sudden onset of asymmetric flaccid paralysis with no significant sensory loss is the characteristic finding of paralytic disease (Gumede et al., 2018) . Paralysis is presented by severe myalgia in the involved limb resulting from involvement of single or multiple muscle groups (Rotbart, 1997). Motor and sensory disturbances may be observed in the same affected muscle groups (Gumede et al., 2018). Paralytic polio is classified into three types (spinal, bulbar and bulbospinal polio) depending on the level of involvement. Spinal polio is characterised by asymmetric paralysis mostly of the legs (Gumede et al., 2018; CDC, 2002a). Cranial nerve involvement may result in bulbar paralysis, which leads to difficulties in breathing, speech, swallowing, eye movement and facial muscle involvement (Gumede et al., 2018). Bulbospinal polio accounted for 19% of cases in the United States during the period of 1969 to 1979 and was due to a combination of bulbar and spinal paralysis (Gumede et al., 2018; CDC, 2002a).

Characterization of Poliovirus genome

The size of the PV ranges from 27 to 30nm in diameter, it is non-enveloped and consist of a single stranded positive sense RNA and a protein capsid, which forms about 30% of the mass of the whole virion. The capsid has been arranged in sixty repeating protomeric units an icosahedron form composing of four proteins (VP1, VP2, VP3 and VP4) (Adekunle, Oladapo, Cephas, & Olubusuyi, 2017). Each protomer carries a single attachment site for the PV

The polyprotein cleaves as it is translated by virus encoded protease (P2A, P3C and uncleaved P3CD), producing the active proteins that are involved in virus replication (Blondel et al., 2005). The structural proteins (VP1, VP2, VP3 and VP4) that form the shell of the virus particle are encoded before the non-structural proteins (P2A, P2B, P2C, P3A, P3B, P3C and P3D), which are involved in the replication of the PV genome and alter the host cell's synthetic machinery to produce viral proteins (Blondel et al., 2005).

The first 500 nucleotides of the 5'UTR play an important role in viral replication (internal initiation of translation rather than the ribosome-scanning model proposed for cellular mRNAs) Point mutations in this region are known to affect virulence, temperature sensitivity and plaque morphology (Blondel et al., 2005; Adekunle, Oladapo, Cephas, & Olubusuyi, 2017).

The ORF following the 5'UTR is translated into a polyprotein, which is co- and post translationally cleaved to give four structural proteins (VP4, VP3, VP2 and VP1) and seven non-structural proteins (P2A, P2B, P2C, P3A, P3B, P3C and P3D) as illustrated in figure 3 (Hogle, 2006). Four neutralisation determinants have been identified and have been mapped to VP1, VP2 and VP3. Determinants of attenuation of virulence, virion thermostability, altered host range, *in vitro* cell tropism, persistent infection and plaque morphology have been mapped to the capsid-encoding region (Hogle, 2006).

The coding region in the PV genome is followed by a 70 to 100 nucleotide 3'UTR. This region is important in the initiation of the complimentary strand of the positive RNA synthesis, but no specific sequences have been identified for

polymerase binding. A genomic polyadenylate (A) tail with an average length of 75 nucleotides follows the 3'UTR (Blondel et al., 2005).

A number of PV genomes have been sequenced completely and the extensive knowledge concerning the molecular biology of PV, suggests that it may be possible to understand virulence and attenuation in molecular terms (Minor, 2001).

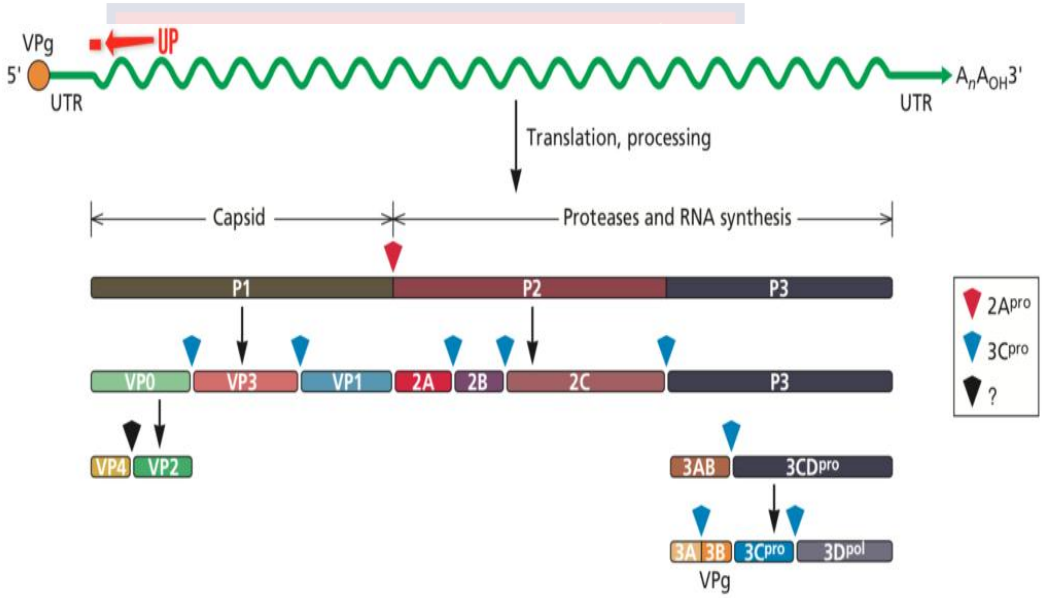

Figure 3 Genomic organisation of poliovirus (Blondel et al., 2005)

Poliovirus vaccines

Protection against poliomyelitis is achieved through immunization or natural exposure to the PV thereby developing immunity. Immunity is serotype-specific therefore protection against one type of the PV does not protect the susceptible host against the other serotypes. Antibodies are produce in the blood, secretory gut and the upper respiratory tract to prevent PV from invading the CNS (CDC, 2002a).

PV is the only enterovirus that has vaccine to protect susceptible host from poliomyelitis disease. In 1955, Jonas Salk developed the first successful vaccine against poliomyelitis known as inactivated poliovirus vaccine (IPV) (Singh, Amit, & Monga., 2013). Albert Sabin developed oral, live attenuated poliovirus vaccine (OPV) that contained all the three PV serotype in the year 1963. OPV was readily accepted as a global recommended vaccine for immunization by virologist because it has a long-lasting protection against the PV but the IPV only had sufficient immunogenicity to provide immune protection against PV. However, both the OPV and the IPV do not provide 100% immunity against PV infection (Singh, Amit, & Monga., 2013).

Inactivated poliovaccine

Since the year 2000, the United State used IPOL®, Poliovirus Vaccine Inactivated, produced by, Pasteur Merieux Connaught and its shot is given by the leg or arm, depending on the patient's age. The vaccine is produced by culturing the PV in a Vero cell line from the kidney tissue of monkey while the inactivation is achieved with formaldehyde. The constituent of IPOL are 2-phenoxyethanol, neomycin, streptomycin, polymyxin B which help prevent bacterial and fungal growth, and all three serotypes of PV (CDC, 2002a).

Countries in Europe have been able to achieve polio-free status by eliminating all the wild type of PV with the use of IPV. The IPV is very effective in inducing PV antibodies in the blood, thus preventing PV in the gut from entering and replicating in the CNS. Nevertheless, the serum antibodies do not impede the initial replication of PV in the gut and also hinder PV in the blood

from invading the spinal cord which will result in paralysis. The immunity protection from IPV is not certain but it gives protection for a quite number of years after taking all the four doses from 2 months through to 6 years (CDC, 2002a; Figas et al., 2018).

Oral poliovirus vaccine

The production of Oral Poliovaccine (OPV) is of live attenuated PV strains which began from a virulent PV isolate gotten from human. Some of the attenuated strain derived from Mahoney strain PV type 1 which lacked neurovirulence but useful for vaccine production include Sabin 1, CHAT and Cox (Martin, & Minor, 2002). Sabin 1 was developed from passage in monkey tissues, whereas the CHA and Cox strains involved passages in monkey, mouse and chicken embryo cell substrates (Martin, & Minor, 2002).

The OPV induce mucosal immunity by inhibiting PV replication in the gastrointestinal tract when the attenuated strain multiplies in the human gut. The OPV produces long-lasting mucosal immunity by stimulating the formation of secretory IgA antibodies in the intestine and also serum antibodies in the bloodstream. A single dose of trivalent OPV generate immunity coverage for all the PV strains. Studies have shown that the attenuated PV strains can be excreted for two to three months after vaccination in healthy children and faecal oral transmission from these vaccinated healthy children is capable of protecting other children who are susceptible to the PV infection (Njile et al., 2019; Hovi, Lindholm, Savolainen, Stenvik, & Burns, 2004).

The support from Global Polio Eradication Initiative (GPEI) to many countries have made them have access to OPV which has help achieved elimination of WPV (Kew, et al., 2005). The maintenance of routine administration of OPV is important to sustain the high immunization coverage of countries that have attain WPV elimination and prevent any importation of PV and person-to-person transmission (Buttinelli, et al., 2003).

Both OPV and IPV containing different strains of the PV enhance individual's immunity against the polio virus. While the OPV contain live attenuated viral particles which is administered orally to enhance mucosal immunity to patients, the IPV contains inactivated viral particles which is administered by subcutaneous or intramuscular routes to stimulate the production of antibodies against the PV. OPV provide better immunity relative to IPV but OPV can get reactivated to cause vaccine-derived poliomyelitis while IPV does not cause vaccine-induced poliomyelitis (Levinson, 2014).

Vaccine-derived poliovirus

Research conducted in different part of the globe have shown that the OPV could lead to paralytic poliomyelitis known as vaccine-associated paralytic poliomyelitis (VAPP) (Figas et al., 2018; Sartori, et al., 2015). There is genetic variation that differentiate the natural WPV type from the live attenuated OPV strains. This is due to mutations or reversion of the vaccine virus that has undergone prolonged replication in human body. The PV vaccine strain that could result in neurovirulence as a result of reversion of attenuation or mutations is

called vaccine-derived poliovirus (VDPV). VDPV could occur in immunodeficient patients (Njile et al., 2019).

The isolations of the VDPV serotypes from VAPP cases and the sabin strains serotypes do not have any distinquish properties. There is >1% of nucleotide sequence divergence of the VP1 capsid coding gene for type 1 and 3 VDPV strains while there is >0.6% divergent for the VDPV type 2 when they are compared with the parental OPV strain. The mode of transmission of VAPP is similar to WPV which could result in premant paralysis (Kew, & Pallansch, 2018). It is crucial to note that secretions from the nasopharyngeal and stool may contain VDPV after it had undergone prolong replication. In 2017, there was an out break of VDPV in Democratic Republic of Congo and Syria and they detected 22 and 74 cases respectively (Alleman, et al., 2018). Ghana has not detected any VDPV from ongoing AFP and environmental surveillance.

There are three categories of VDPV isolates. These are immunodeficiency-associated VDPV (iVDPV), circulating VDPV (cVDPV) and ambigous VDPV (aVDPV) (Burns, Diop, Sutter, & Kew, 2014). The iVDPV has defect in the B-cell thereby affecting production of antibodies which prolongs replication of Sabin strains. PV1 and PV3 isolates with ≤ 99% VP1 region sequence identity to the parental Sabin strain while PV2 isolates with < 99.4% VP1 region sequence identity are considered as VDPV. The iVDPV could have chronic PV infection which can persist for more than 10 years. When there is evidence of person-to-person transmission of Sabin strains from an immunised individual to an unimmunised individual with sufficient mutation to cause

neurovirulence, it is termed cVDPV. The ambiguous VDPVs (aVDPVs) category are either clinical isolates from persons with no known immunodeficiency or sewage isolates whose ultimate source is unknown (Burns et al., 2014).

The outbreaks begin when a VDPV infects inadequately vaccinated individuals, leading to the spread of the pathogenic virus. There have been sporadic outbreaks of cVDPV in South Sudan, Afghanistan, Pakistan, Nigeria, Burma, Guinea (cVDPV2), Madagascar, Ukraine and Laos (cVDPV3) from January 2015 to May 2016 (Jorba et al., 2016).

AFP Surveillance in Ghana

The WHO recommend AFP surveillance to be conducted in countries including Ghana as an effective way of identifying new cases or importation of PV. AFP can also be caused by Guillian Barre syndrome, transverse myelitis, traumatic neuritis and transient paralysis which are all associated with non-polio enterovirus (NPEVs) (Odoom et al., 2014). AFP among children less than 15 years are quickly reported to Clinicians and Disease Surveillance Officers within the 260 districts in the country by health care providers.

Two stool samples are collected within 24 - 48 hours apart within fourteen days of the onset of paralysis (CDC, 2012; Odoom et al., 2014). The AFP core indicators that is achieved by most regions in the countries are non-polio AFP rate and stool inadequacy. The target set by WHO for non-AFP rate is 2 per 100000 children under 15 years of age and two adequate stool specimens for AFP cases. Ashanti, Eastern and Western regions are few of the regions that consistently meet the target set by WHO but the Greater Accra region has not been consistent

in its achievement of the target over the past few years (Odoom et al., 2014). Residual paralysis in at least 80% cases is examined by following up 60 days of onset of paralysis.

The Expanded Programme on Immunization (EPI) was introduced in Ghana in the late 1970s where childhood vaccination to prevent poliomyelitis are administered four doses of live-attenuated oral polio vaccine at birth, six, ten and fourteen months of age. The GPEI program strengthened Ghana in 1996 to intensify routine polio immunization, introduced supplementary immunization activities (SIAs) and also enforced active AFP surveillance for PV with laboratory support (WHO, 2013). The immunization coverage of the nation is greater than 80% which is good as the world is preparing to eradicate PV.

Ghana recorded its last case of WPV outbreak in November 2008 as a result of importation from Nigeria. The sensitive nature of the AFP surveillance helped to identify the imported PV and the effective OPV immunization help to interrupt the transmission of the virus. Since the last recorded case, the country has not detected any PV examining all reported cases sent to the Regional Reference Polio Laboratory (Odoom et al., 2014). Ghana is still at risk of re-importation of PV since Afghanistan, Pakistan and Nigeria are still considered endemic countries for PV.

According to Odoom et al., (2014), health professional in the clinical facilities should be sensitized on the importance of detecting and reporting AFP case so that it increase the sensitivity of the AFP surveillance system. There is the

need to add environmental surveillance to supplement the AFP surveillance to promptly detect any silent circulation of PV.

Environmental Surveillance of Poliovirus

According to the guidelines of WHO for environmental surveillance (ES) of poliovirus circulation, ES is serving as additional method to monitor and access the duration of the transmission of PV which might be contained in human faeces from tested sewage samples in specific population (Gumede et al., 2018). Individuals infected with the PV shed the virus for several weeks into the environment but they appear asymptomatic. Shed PV virus remains infectious to susceptible individual as far as the virus has favourable conditions to survive for a length of time (Gumede et al., 2018).

The importance of implementing ES as a supplementary method for AFP to detect PV cases include; identifying residual WPV transmission in endemic areas, providing an early indication of newly imported PV into polio-free area and confirmation of the presence of vaccine-related virus preceded by the use of OPV either by routine vaccination or national immunization day or supplementary immunization day. The crucial role played by ES in detecting PV will provide evidence for global certification (Gumede et al., 2018; Ndiaye, Mbathio, & Diop, 2014).

The African Region of WHO started ES in July 2011 in Nigeria. In 2013 Kenya had a pilot study to implement ES as a supplement to AFP surveillance. More African countries have implemented the ES and from 2011-2016, there have been 126 cVDPV2, 10 WPV1 and 3 WPV3 detections in the African Region

(Gumede et al., 2018). There was also isolation of 68 Sabins and 339 Non-polio enterovirus (Gumede et al., 2018). According to Odoom et al., (2017) a pilot study in the year 2016 on ES carried out in two regions (Greater Accra and Eastern Region) of Ghana reported that, out of the 36 sewage samples collected from 6 sampling sites there were no WPV transmission but 7 non-polio enteroviruses were detected. A research study conducted in Yaounde and Douala in Cameroon from 2016-2017 on ES also revealed that there were no WPVs and VDPVs from the 325 specimens sent for laboratory diagnosis (Njile et al., 2019). However, earlier specimens' analyses detected Sabin type 1 and 3 isolates whereas Sabin 2 was part of the isolates detected from January to May 2016 from the sewage samples. The isolation of Sabin 2 was a result of all countries using OPV switching from tOPV to bOPV after the certification of the eradication of WPV type 2 in 2015 (Njile et al., 2019). The combination of two or three serotypes of the PV strain to form OPV create the optimal conditions for recombination between viral genomes. The PV strains therefore undergo genetic variation during the replication process in the human host (Figas et al., 2018).

The GPEI program has advocated the implementation of ES as supplementary to the gold standard AFP surveillance for detection of PV. Notwithstanding, the ES is a useful tool to detect circulation of virus pathogenic to human which may include adenoviruses, caliciviruse, rotaviruses, noroviruses, Echovirus, and Coxsackie virus (Odoom et al., 2017; Gumede et al., 2018). Virologist with expertise in the detection of PV have also indicated that ES is more sensitive than AFP surveillance in monitoring circulation of PV and the

efficacy of immunization intervention to stop the transmission of PV (Gumede et al., 2018).

Environmental Surveillance in Ghana

Addition of ES to supplement AFP surveillance as the world is almost eradicating polio has been established in many countries in the AFRO regions such as Nigeria, Cameroon, Kenya, Madagascar, Senegal, and South Africa (Gumede et al., 2018). Ghana has adopted and is currently conducting ES after its pilot study in six high risk districts within the Eastern and Greater Accra regions of Ghana. These regions were selected because Greater Accra could not meet the 80% stool adequacy and non-polio AFP rate of two per one hundred thousand population of children < 15 years from 2010 – 2015 while some district in Eastern region could also not meet the AFP core indicators. The aim of the pilot study was to test the nations capacity and preparedness to undertake the ES as part of the routine national surveillance activity for PV (Odoom et al., 2017).

Thirty-six sewage samples were collected from September to December 2016, and sent to the Regional Reference Polio Laboratory for processing. The sewage samples were collected from sample sites that met four important criteria recommended by WHO as a guideline in sample site selections. The sewer lines selected for the studies were observed to carry waste water from a considerable proportion of the population catchment area; the sewer lines did not receive any industrial waste that could impede the survival of the virus; the sewage system were located in districts that are at high risk of PV transmission and also had poor AFP performance indicators (Odoom et al., 2017; Yusof et al., 2018).

Three out of the six district sites detected NPEVs which indicated that the standard operating procedure applied for the study was sensitive enough to detect WPV and VDPV if they were present in the sewage. Although no PV were detected, a variety of enteroviruses belonging to the human enterovirus (Echovirus 6, Echovirus 13, Echovirus 29, Coxsackie virus A 16 and Coxsackie virus 24) were isolated from the sewage samples (Odoom et al., 2017; Zeng et al., 2015). Odoom et al., (2017) concluded that Ghana has the capacity to carry out ES as a routine activity even though the study could not detect any Sabin, WPV and VDPV.

Isolation and Identification of Poliovirus

Sample Collection

PV are shed in human faeces that are contain in wastewater or sewage that flow to the downstream of the population catchment area. The process of collection of the raw sewage water is very important in the isolation of the PV. There are two sampling methods: the trap method and the grab method (Matrajt, Naughton, Bandyopadhyay, & Meschke, 2018).

The trap method uses a bag of nonspecific absorbing material that is hung in the sewage stream for several days. The absorbed material bag soaked with the running sewage is removed and transported to the laboratory where the absorbed material is eluted and analyzed for the presence of PVs. The common adsorbing material are gauze pads, cotton-made fabrics and macroporous glass in permeable bags (Matrajt et al., 2018). The trap method is qualitative because it is difficult to determine the specific volume of sewage water that pass through the pads or

glass. In addition, the method has poor recovery efficiency and sensitivity (Matrajt et al., 2018).

The grab method is specific on the amount of raw sewage sample collected at the selected sample site. Collection of the sewage can be done during the peak hours in the morning and there can also be twenty-four hourly collections at a regular interval. Approximately, 1litre of the sewage is preferred for processing in the laboratory (Matrajt et al., 2018). The Global Polio Laboratory Network (GPLN) and WHO prefer the grab sampling method to the trap sampling method because it is more quantitative and sensitive (WHO, 2015).

Concentration Method of PV

There are two methods used to concentrate viruses from ES sewage samples. These are precipitation and filtration methods. The filtration methods are used to concentrate viruses based on the principle that viruses can be adsorbed to or retained in a filter medium and then be eluted with an organic solution. These methods are based on charge adsorption and are widely used for PV and other enteric viruses (Francy, Stelzer, Brady, 2013; Matrajt et al., 2018). The filters come in two different formats: flat rates that will determine the charge, particle size and amount of sample that can be filtered (Matrajt et al., 2018).

Precipitation methods used to concentrate viruses from ES sewage samples. These methods can be used as a primary concentration method when the sample is first processed or they can be used as a secondary concentration method when the virus are recovered from the filter or membrane (Matrajt et al., 2018).

The polyethylene glycol and dextran mixture interacts with sewage water and after intense stirring, the mixture separates on the basis of their weight, carrying along viruses that have similar weight. Viruses can be recovered from the denser phase for further characterization. The WHO recommended concentration and separation of environmental sewage is the 2-phase separation method whereby PEG 6000 and dextran are used to perform this method (Matrajt et al., 2018).

The 2-phase method has been extensively used during PV ES campaign and has been successful at detecting WPV. Sabin-like PV strains, VDPV and other PV strains. This method is only effective for 0.5 to 1L volume of sewage which has an effect on the sensitivity (WHO, 2015; Matrajt et al., 2018).

Isolation Cell Lines for Poliovirus

Cell culture is the gold standard for the diagnosis and surveillance of PV. The cell lines for the isolation of PV are L20B cells, Human rhabdomyosarcoma (RD) cells and Human epidermoid carcinoma (HEp-2) cells. L20B cell is genetically engineered recombinant mouse cell line that express the human receptor for PV, CD 155 (Ivanova et al., 2019; Kaundal, Sarkate, Prakash, & Rishi, 2017). Research studies have shown that L20B cells are more sensitive and specific in the detection of PV by showing cytopathic effect (CPE) in samples suspected to contain other enterovirus. Other human enterovirus also replicate in L20B cells (Chapel et al., 2018). The RD cell line, human rhabdomyosarcoma tumor cell, allows the growth of several human enteroviruses including PV. WHO

recommends L20B and RD cell lines for isolation of enteroviruses (Figas et al., 2018; Pellegrinelli et al., 2017).

Molecular techniques for the detection of PV

The poliovirus RNA in a clinical or environmental sample can be used for reverse transcription PCR assay without necessarily extracting the RNA genome from the samples. Degenerated primers have been designed to target the conserved sequences within the 5'UTR or the capsid protein-coding region of the PV genome (Veiga, Campos, & Tavares, 2012). The PCR primers and probe that have been designed to sequence PV target within the capsid region only. Any sequence out of the capsid region are not considered as PV because PV undergo frequent recombination with other enterovirus during circulation (Kilpatrick, Chen, & Services, 2011).

The differentiation of the PV serotypes are established using PCR test because the technique is highly sensitive and specific (WHO, 2003). The cytopathic effect produced on L20B and RD cell lines cannot distinguish between PV serotypes and non-polio enterovirus. The molecular technique is able to differentiate between Sabin strains, WPV and NPEVs due to the sensitivity and specificity of the technique which is a limitation in cell culture (WHO, 2003).

Intratypic differentiation (ITD) plays a crucial part in laboratory surveillance as the molecular detection method that can identify and distinguish wild and vaccine-like poliovirus isolated from AFP cases or ES sources (Gerloff et al., 2017). WHO recommend the following methods for ITD for PVs. These are

enzyme-linked immunosorbent assay (ELISA), Nucleic acid probe hybridization, Restriction fragment length polymorphism (RFLP) and diagnostic polymerase chain reaction (PCR), using specific approved procedures. Two independent methods should be used, or one method may be used with partial genomic sequencing to confirm the results (Lukacs & Bhadra, 2012). All L20B cell cultures exhibiting viral cytopathic effect (CPE) are tested to identify WPV, VDPV and PV2 through the ITD process (Gerloff et al., 2017).

Nucleic acid hybridization with specific probes is a rapid, reliable and sensitive method for detection of specific viral sequences as compared with ELISA and RFLP methods which gives epidemiologically relevant information. ELISA does not discriminate between vaccine and wild-type poliovirus-induced antibodies. The limitation of the PCR for the ITD is the inability to identify the positive WPV from the Sabin virus genotype (Lukacs & Bhadra, 2012). The nucleic acid hybridization detection and characterization uses PV synthetic oligonucleotides probes as well as subgenomic riboprobes. The probes are complementary to the conserved sequences of related strains near the 5' terminus of the VP1 gene (Gerloff et al., 2017).

The nucleic acid probe hybridization applies the principles of PV diagnostic real-time reverse transcription-PCR (rRT-PCR). The rRT-PCR is performed on L20B positive cell culture that are suspected to contain PV. The viral RNA (vRNA) is converted to complementary DNA (cDNA) using reverse transcriptase. The cDNA is amplified in a PCR reaction using Taq polymerase. The PCR products are detected and identified by hybridization with specific

Taqman® probes. Both the cDNA synthesis and the PCR reaction use multiple sets of oligonucleotide primers that are tagged with probes with different specificities. This combination of primers and probes result in the serotype identification and intratypic differentiation of PV isolates.

The rRT-PCR ITD 5.0 uses a set of six rRT-PCR assay that serve as accurate diagnostic tools to easily detect and differentiate PV serotypes and genotypes. The primers and probes use are Quadruplex EV+Sabin, PanPV, WPV1, PV Type 2, WPV3-I, and WPV3-II. Each of the six rRT-PCR assay identifies between one and four different targets and follows a specific algorithm. The quadruplex EV-Sabin assay includes primers and probes for the detection of any enterovirus (EV) and the oral vaccine strains (Sabin 1, 2, and 3). The other five assay detect any PV (PanPV), WPV1, PV2 and WPV3. Both the ITD and VDPV assays estimated run time is 132 minutes in the Applied Biosystems 7500 system (Kilpatrick, et al., 2011).

The design of the rRT-ITD 5.0 algorithm incorporates redundancies to detect all polioviruses with multiple assays to protect against genetic drift or user error. Any virus discordant results (e.g., PanPV positive but negative for all other polio assays) should be referred for VP1 sequencing (Kilpatrick et al., 2011).

The molecular epidemiologic information for poliovirus isolates are further characterized by sequencing the ~900-nucleotide region encoding the major capsid protein, VP1. Sequence analysis of VP1 gives information to determine the genetic relationships of WPV isolates or VDPVs chains of

transmission, sources of imported viruses causing outbreaks, and estimates of the duration of virus circulation (Kilpatrick, Chen, et al., 2011).

Chapter Summary

This chapter reviewed the structural characteristics, pathogenicity and the serotypes of PV as well as studies that have been carried out on AFP surveillance and ES to detect circulating PV. Literature revealed that most of the isolates identified from AFP stool samples and ES sewage samples were sabin-like PV, NPEV and cVDPV. It was noted that sabin isolates were note sequenced except those that were discordant in their ITD investigations.

Most of the characterised isolates identified were serotype 1 and 3 with few circulating VDPV serotype 2 in endemic countries. The few recorded wild type of PVs were identified in endemic countries like Pakistan and Afghanistan that have challenges in immunization strategies to prevent the transmission of the PV.

The various methods sensitivity for the concentration of sewage samples were reviewed as spelt out in the WHO guidelines for ES. The two main cell lines, RD and L20 cells were used by most literature in cell culture whiles others applied HEp-2 cell lines which had its limitations.

rRT-PCR were applied in performing ITD by other studies which was fast and more sensitive than ELISA methods that was laborious and its sensitivity and specificity was relatively lesser than rRT-PCR.

CHAPTER THREE

RESEARCH METHODS

Introduction

This chapter describes the research methodology that was used in the collection of sewage samples, the isolation procedures and molecular identification of the poliovirus. It also describes the research design, sampling sites, research instruments, analysis of data, ethical consideration, validity and reliability of the study.

Study Design

The study was an environmental surveillance within four districts in two regions of Ghana identified to be at risk of polio and have a proper sewer network and waste water canals in the communities that drains a large number of people living in the catchment area.

Sampling Site

Volta and Eastern regions were the selected regions purposely for the study. Ho is the capital of the Volta Region with an estimated population of about 2,118,252 inhabitants while Koforidua is the capital of the Eastern Region with an estimate population of about 3,171,740 inhabitants as estimated from the 2010 population census (Ghana Statistical Service, 2012).

There were three criteria that were taken into consideration for the selection of the two regions for the studies.

i. The districts were classified as high risk for PV transmission, based on existing data (i.e, population density, high-risk population, sanitation, living conditions, routine immunization, and supplementary immunization activities coverage);

ii. The sewer lines received waste from a considerable proportion of the population in the catchment area, with a minimum amount of waste coming from other areas;

iii. There was absence of industrial waste in the proposed site.

The districts considered were Ho district with an estimated population of 214,028 and Ketu South with an estimated population of 193,619 in the Volta region as at 2018 with reference from 2010 population and housing census (Ghana Statistical Service, 2012). The Asuogyaman district had an estimated population of 117,597 and the New Juabeng district had an estimated population of 222,459 as at 2018 with reference from 2010 population and housing census (Ghana Statistical Service, 2012). The Eastern and Volta regions were selected because there are a lot of trading activities that bring people from other regions into these regions which expose the region to the likelihood of PV importation among the regions. These selected regions have continually met the required non-polio AFP rate; an important surveillance indicator. The Ketu South is a border town and there is the possibility of importation of PV from the immigrants into the country. All these four districts satisfied the site sample selection criteria and two sites from each selected region had open sewage lines that can pose a high risk of exposure of the PV to the population in the region.

Inclusion Criteria

Districts that are found to be at risk of poliovirus outbreak due to the poor performance of AFP indicators and also availability of proper sewer lines merging from the districts.

Exclusion Criteria

The study did not consider known sewage that contain industrial waste that can be toxic to cell cultures and interfere with poliovirus replication.

Sample Size

A total of 36 sewage samples were collected from the four districts sewage sites between September 2018 to May 2019. Every four weeks' raw sewage or waste water samples from a running sewage channel or canals were collected for processing.

Sampling Method

A convenient systematic sampling method was applied to select the sewage sites for the study after these sites satisfied the inclusion criteria for the study.

Training of Collectors and Supervisor

Two research assistances were trained on how to collect the sewage samples at the Noguchi Memorial Institute for Medical Research (NMIMR) Legon. The focus of the training was to teach the collectors how to wear the personal protective equipment (PPE) and the skill they need to apply in collecting the waste water. The training was in the form of presentation, hands on training

and discussions. The supervisor was trained on how to ensure that the collectors effectively use the PPE and assemble all the logistics that are needed to collect the sewage sample and transport to the lab. The field investigation forms were introduced to the supervisor and collectors for them to familiarize themselves with what the forms entails especially the specific time range in the morning they are to collect the sample and how to use the digital thermometer to take the atmospheric temperature. The training also focused on how to disinfect the logistics that were sent to the sampling site. Hand hygiene was practiced and disinfectants that were needed to perform disinfections were made available.

Tools and Techniques for Sample and Data Collection

Onsite Data

A structured field investigation form as referenced in Appendix VII was filled by the sample collectors to obtain the time, atmospheric temperature of the sites and other demographic characteristics that makes the site suitable for yielding viable results.

Sample collection/Transportation /Storage

Raw sewage samples were collected from the selected sites in the morning between the hours of 8am to 10am. The running sewage which effectively carries enteroviruses in human feaces were collected into 1.5 L plain plastic containers. The cup or bucket used for the collection was lowered gently into the sewage for the water to run into the container. The preferred spot for collection was where the velocity of the running waste water was high. The sewage was carefully transferred into the 1.5 L plastic bottle using a funnel to avoid spillage on the

bottle. One litre of the sewage was collected into the bottle, closely tight and labelled with appropriate site identification names and epidemiological number. The samples were placed on a cold box with ice and sent immediately to the National Polio Reference Laboratory, Virology unit of the Noguchi Memorial Institute of Medical Research (NMIMR) where they were worked on the same day or kept at 4^0C and processed the next day according to the standard protocols (WHO, 2003; Odoom et al., 2017).

Laboratory Investigation/ Procedures

Sewage Concentration

The sewage samples were taken out of the cold chain and allow to stand on the bench for 5 minutes for sedimentation of large solid material. A volume of 500 ml of the sewage sample were centrifuged for 20 min at 1500 g (minimum) at 4°C. The supernatants and the dry pellet were stored in 1 litre flask and 250ml centrifuge tube at 4°C respectively. The pH of the supernatant was adjusted to neutral (pH 7-7.4) using NaOH or HCl. If the supernatant was found to be acidic, approximately 0.2 ml 1N NaOH was added sequentially to it till the required pH was attained. A volume of 500 ml of the supernatant was poured into an Erlenmeyer flask for precipitation of the sewage. The rest of the corresponding raw waste water sample was kept at 4°C which served as backup until microscopy of the cell cultures inoculated with the concentrate shown absence of toxicity. After that it was stored at -20°C till all results were ready.

A concentration of 0.07M PEG6000, 0.56M dextran and 5.85M NaCl was added to 500ml of the supernatant. It was mixed thoroughly and kept in a constant

agitation for 1 hour at room temperature using a magnetic stir plate at a speed sufficient to form a vortex. This continuous agitation was done at 4°C. One litre sterile conical separation funnel per sample was elevated and attached to a retort stand. Grease was spread on the gliding glass surfaces of the valves. Water tightness with a small volume of sterile water was checked. The valves were also checked if they were closed tightly. The mixture of the supernatant sewage sample with the reagents was poured into the separation funnels. The mixture was left to stand overnight at 4°C.

The lower phase and the fuzzy interphase in the separation funnel were harvested carefully and slowly by drop-wise into a sterile tube. The dry pellet was resuspended from the centrifuge tube with a few millilitres of the harvested concentrate and added to the rest of the concentrate (WHO, 2003; Odoom et al., 2017).

Treatment of Sewage Concentrate

Two millilitres of 20% chloroform stabilized with ethanol was added to 10ml of the suspension sample, 1 to 6g of sterile glass beads was added and vortexed briefly. The tube was later shaken vigorously for 20 minutes with Heidolph tube shaker. The tube was centrifuged according to the WHO Polio Laboratory Manual faecal suspension procedure (1500 g at minimum for 20 minutes at 4°C). The supernatant phase in the sterile 50ml centrifuge tube was aliquoted while the interphase and the deposits were discarded.

Penicillin G concentration of 100 IU/ml and 50µg/ml of gentamycin were added to the sample before inoculated on the cell culture medium. Two to four

millilitres (ml) of the extracted concentrate was aliquoted and frozen at -20°C till the final results were determined. An approximate volume of 0.5 ml was inoculated on to the fresh monolayer cultures of L20B and RD cell lines. Five T-25 (25 cm^2) flasks contained L20B cell lines while one T-25 flask contained RD cell lines per sample concentrate. The cultures in the flask were observed and marked for 5 days using the Poliovirus Investigation Worksheet (Appendix VI) (WHO, 2003; Odoom et al., 2017).

Virus isolation

Virus growth was monitored daily by microscopy for cytopathic effect (CPE) for 5 days using the Poliovirus Investigation Worksheet. Samples that did not show CPE after 5 days were frozen and thawed twice, vortexed and re-inoculated and observed for CPE for another 5 days. Virus isolates were harvested after attaining 80-85% CPE and the infected cells frozen at -20ºC. Virus isolate showing CPE on L20B cells were subjected to intratypic differentiation (ITD) using real-time reverse transcriptase polymerase chain reaction (rRT-PCR PCR) kit version 5.0 (provided by Centers for Disease Control and Prevention, Atlanta, USA). The ITD for poliovirus determined the serotype and the origin of the poliovirus while virus isolated on RD cells only were considered as non-polio enteroviruses (WHO, 2004a).

Identification and characterization

Poliovirus Real Time Reverse Transcriptase Polymerase Chain Reactions (Polio rRT-PCR ITD 5.0 Kit)

The rRT-PCR worksheet with name, date, primers, samples and sample order as designed in appendix IV was filled out. Wells were named using thermocycler software for samples and controls. The Positive control constituted non-infectious control RNA supplied with Polio rRT-PCR kit while the reagent control constituted Quanta ToughMix + primer/probe with no template. 1.5 ml Eppendorf tubes were labelled with primer/probe names for each primer/probe target. Virus isolated were thawed. PCR reagents were placed on ice and centrifuged. For each of the primers, 10µl Quanta ToughMix reagent was dispensed into each tube in the biosafety cabinet (BSC) designated for Master mix. Approximated volume of 8 µl nuclease free water was added. A volume of 1µ primer was added to the appropriate labelled eppendorf tube. A volume of 19µl of Master mix was dispense into appropriate PCR tube strips. A volume of 1µl of samples (cell culture supernatant) and controls were added to the appropriate PCR tube strips in the designated BSC.

The PCR tube strips were centrifuged briefly at 5000 rpm and placed in rRT-PCR ABI 7500 machine. rRT-PCR conditions version 5.0; 40 cycles in each run:

- 50°C for 30 min
- 95°C for 1 min
- 95°C for 15 seconds
- 50°C for 45 seconds

- 25% ramp
- 72°C for 5 seconds

Highlight annealing step was performed to collect product. The rRT-PCR assay probes and filters that needs to be selected and the results of ITD Screen and Reporting has been indicated in appendix IV. Figure 3.1 gives a graphical presentation of how the results for the ITD was read.

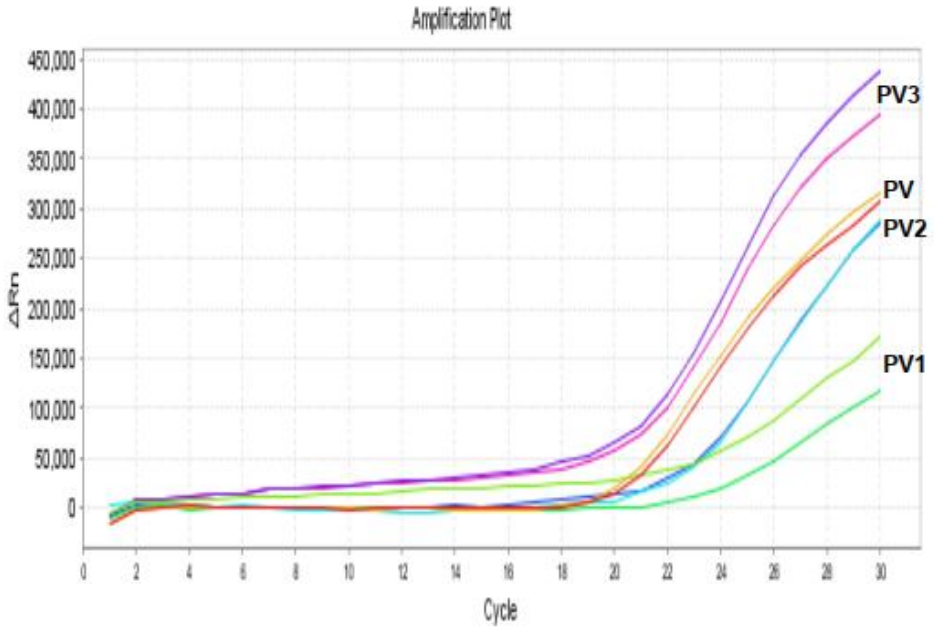

Figure 4: ITD rRT-PCR graphical presentation on the ABI 7500

Real time Reverse Transcriptase Polymerase Chain Reactions (Polio rRT-PCR VDPV 5.0 Kit)

The rRT-PCR worksheet with name, date, primers, samples and sample order as indicated in appendix IV was filled out. Wells were named using thermocycler software for samples and controls. The positive control constituted

non-infectious control RNA supplied with Polio VDPV rRT-PCR kit and the reagent control constituted Quanta ToughMix + primer/probe with no template. The Eppendorf tubes (1.5 ml) for each primer target was labelled. The suspected virus isolates and PCR reagents were thawed on ice and centrifuged at 5000 rpm. For each primer set, 10µl Quanta ToughMix reagent was dispensed into each tube in the Master mix in the designated BSC. An approximate volume of 8µl nuclease free water was added. One microlitre (1µl) of primer/probe was added to the appropriate tube. Nineteen microlitres (19µl) of Master mix was added into the appropriate PCR tube strips. A volume of 1 µl of sample (cell culture supernatant) or control were added to the appropriate tubes in the designated BSC. The tubes were centrifuged briefly and placed in rRT-PCR ABI 7500.

rRT-PCR conditions for version 5.0; 40 cycles in each run:

- 50°C for 30 mins
- 95°C for 1 min
- 95°C for 15 seconds
- 50°C for 45 seconds 25% ramp
- 72°C for 5 seconds

Highlight annealing step was performed to collect product. The rRT-PCR assay probes and filters that needs to be selected and the results of ITD Screen and Reporting has been indicated in appendix III. Figure 3.2 illustrate the graphical representation of the positive serotypes of PV.

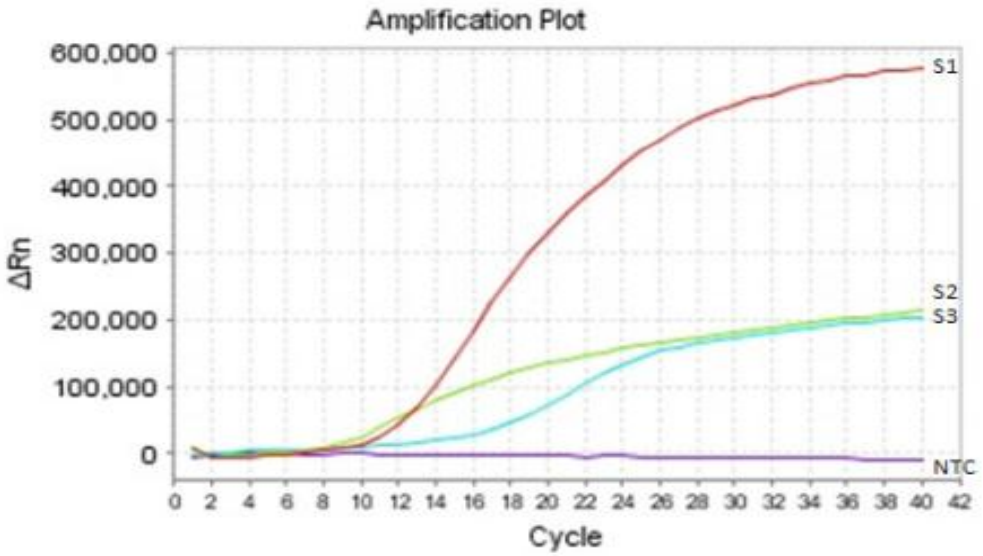

Figure 5: VDPV rRT-PCR graphical presentation on the ABI 7500

RNA Extraction

The QIAamp Viral RNA Mini Kit from QIAGEN (User Manual, QIAamp viral RNA mini kit, 2014) was used to extract genomic RNA from the tissue culture supernatant. The tissue cultures were freeze-thaw twice to release intracellular virus. The supernatant was centrifuged at 13,000 x g for 1 minute to pellet cell debris. The supernatant was aliquoted into a sterile, RNase-free microcentrifuge tube. Five hundred and sixty microliters (560 µl) of the AVL buffer was pipetted into a 1.5-ml Eppendorf tube. Briefly, approximately 140µl of the respiratory specimens were lysed under highly denatured conditions, in a volume of 560µl prepared buffer AVL (AVL+RNA carrier) which inactivate RNases and ensured intact viral isolation. This mix was pulse vortexed for 15 seconds and incubated at room temperature (15-25^{0}C) for 10 minutes. An

absolute ethanol of about 560µl was added to the mix and pulse-votexed for another 15 seconds. Then, the mixture solution of about 630µl was carefully aliquoted and transferred to the QIAamp Mini column provided in the kit. This was centrifuged at 7000 g for 1minute and this procedure was repeated twice with the remaining mix in a new collection tube. The filtrate was discarded and a new column placed and a pipetted 500µl AW1 buffer added. This was again span at 7000 g for another 1 minute. With a new column placed under a collection tube, 500µl of buffer AW2 added, and span at 18000 g for 3 minutes. The filtrate again was discarded and span again for 1 minute. RNA was eluted with 60µl of Buffer AE in a clean 1.5ml microcentrifuge tube. The eluted RNA was stored in minus 30°C freezer until further analyses. A negative extraction control (sterile molecular grade water) was used in parallel to all procedures.

Reverse Transcription (RT) Of Viral RNA

The tubes containing the RNA extract were kept on ice throughout the procedure. The reagents for the master mix were removed from freezer and thawed on ice 4^0C. The required number of PCR tubes needed were taken out plus two control tubes. The tubes were labelled appropriately and placed on ice. A Master mix was prepared for the PCR reactions that gave a final volume of 47µl per PCR tube. The constituent for the master mix is shown in Table 1

Table 1: Preparation of Master Mix for Conventional PCR

Reagent	Amt/PCR tube (µl)	X10 reactions (µl)
PCR water (DNase-RNase free)	37	370
10X PCR buffer	5	50
dNTPs (10mM each)	2	20
RNase-Inhibitor (40U/µl)	0.25	2.5
Reverse Transcriptase (20U/µl)	0.25	2.5
Y7R primer (40pmol/µl)	1	10
Q8 primer (10pmol/µl)	1	10
Taq Polymerase (5U/µl)	0.5	5
TOTAL	47µl	470µl

Table 2: Degenerate Primers (Primer Reference Virus = Sabin 1 Genebank AY184219)

Primer	Gene	Location (nt)	Primer Sequence
Y7R	VP3	2399 – 2421	5'–GGTTTTGTGTCAGCITGYAAYGA- 3'
Q8	2A	3504 – 3485	5' –AAGAGGTCTCRTTCCACAT- 3'

Forty-seven microliters of the master mix were pipetted into each PCR reaction tube on ice. Three microliters of the RNA extract were aliquoted into each of the tube and mixed by gently pipetting the reaction up and down several times. The excess polio RNA extract was stored at -20^{0}C. The PCR reactions were placed in the PCR thermocycler and the following PCR running conditions were set as shown in Table 3.

Table 3: Conventional PCR amplification protocol running conditions

RT reaction:	42^{0}C 45 minutes	
	94^{0}C 3 minutes	
Immediately followed by		
	94^{0}C 30 seconds 42^{0}C 45 seconds 0.4^{0}C/sec to 60^{0}C 60^{0}C 2 minutes	X 35 cycles
HOLD:	60^{0}C 5 minutes	
	4^{0}C to hold	

After the completion of the PCR run, reaction products were examined by agarose gel electrophoresis.

Gel Electrophoresis

Gel electrophoresis was employed to determine the yield and size fractions of the PCR products. Five microlitres (5µl) each of the PCR products were subjected to gel electrophoreses on 1.0% agarose gel in 1X Tris-borate EDTA

(TBE) buffer (Life technologies, Ambion®, USA) with ethidium bromide (Life technologies, Invitrogen™, USA) staining. Samples were diluted in 1.5µl of 6X DNA loading buffer and 5µl were applied to the respective gel wells. A Molecular weight marker of 1000bp was co-electrophoresed together with the samples. Running of gels in 1X TBE buffer was done at 70V for 45 minutes. The PCR products were then visualized under ultraviolet trans-illumination using the Benchtop 3UVTM Transilluminator Imaging system (Cambridge, UK). Positive samples were expected to have an average product size of 906bp for VP1 region of the PV.

Data Analysis

The laboratory data was compiled in MS excel which were imported into SPSS version 17 and analyzed. Univariable analysis of case investigation and isolations of viruses in the districts and the period of isolations were expressed as frequency distributions, percentages and charts.

Ethical Consideration

The study ethical approval was obtained from the Scientific and Technical Committee of the Noguchi Memorial Institute, University of Ghana. The approval number was STC paper 3 (3) 2018-19 as indicated in Appendix II.

Chapter Summary

Sewage water were collected from four districts within the Eastern and Volta regions of Ghana between September 2018 and May 2019. The grab method was used to collect sewage samples every four weeks. The two-phase

separation method was used to concentrate the virus in the sewage sample and antibiotics applied to get rid of any bacterial contamination. Meanwhile, L20B and RD cell lines were used for the virus isolation. The isolated viruses were characterized by performing ITD using rRT-PCR. The viral capsid VP1 gene was determined using the conventional PCR and the amplifications were run on gel electrophoresis.

CHAPTER FOUR

RESULTS AND DISCUSSION

The purpose of this study was to detect circulating PV within the Eastern and Volta regions of Ghana using environmental surveillance. Raw sewages were sampled from four district sample collection sites and they were processed using two-phase separation method, virus isolation, intratypic differentiation to determine the various serotypes of the virus isolated and genotypic methods to detect the presence of VP1 gene. The results in this chapter are presented in tables and charts.

Results

General characteristics and demography of study

A total of 36 sewage water samples from four sewage sampling sites situated in four districts within two regions were collected from September 2018 to May 2019 for the study. Some of the sewage water samples appeared cloudy while others appeared clear with suspended organic particles. None of the sewage samples were turbid or dark. The sewage systems that were reported in the study were open sewage system (50%) and close sewage system (50%). In each of the regions, at least there was a closed sewage system and an open sewage system where sampling took place. The four sites (New Juabeng, Asuogyaman, Ho and Ketu South districts) had 9 sewage samples collected shown in table 4.

Table 4: Sewage samples from Collection sites

COLLECTION SITE	No. of Sewage Sampled
New Juabeng	9
Asuogyaman	9
Ho	9
Ketu South	9
Total	36

Isolation from Cell Culture

Sewage samples collected from the Eastern region were inoculated in two cell lines; RD and L20B cell lines and observed for the first five days for cytopathic effect (CPE). It was observed that most of the monolayer cell cultures from the concentrated sewage samples showed CPE within 3-8 days' incubation. It was observed that all the sewage concentrates from the New Juabeng collection sites showed CPE in the RD cell lines forming 25% of the total number of sewage that showed CPE in RD cell lines in the Eastern region (Table 5). Sewage samples from Asuogyaman collection sites showed that most of the concentrate inoculated in the RD cell lines showed CPE (7, 19.44%) and only two samples never show CPE in the RD cell lines.

Table 5: Collection Sites with virus isolates from Eastern and Volta Regions using RD cell line

Regions	Collection Sites	Isolates	Frequency (n=36)	Percentage
EASTERN	New Juabeng	Positive	9	25.00%
	Asuogyaman	Positive	7	19.44%
		Negative	2	5.56%
VOLTA	Ho	Positive	6	16.67%
		Negative	3	8.33%
	Ketu South	Positive	3	8.33%
		Negative	6	16.67%

There were also CPE in RD cell lines from sewages that were sampled from collection sites within the Volta region (Table 6). It was observed that Ho sewage concentrates showed twice CPE in the RD cell lines (16.67%) as compared with the sewage samples collected from Ketu South (8.33%) as displayed in table 5. It was also observed that most of the sewage from Ketu South did not show CPE in the RD cell lines (16.67%) as compared with the sewages concentrates from the Ho (8.33%).

CPE in L20B cell lines showed more specific to PV as compared with other enteroviruses (Table 6). The number of L20B cell lines that showed CPE for the concentrated sewage samples from the two collection sites (19.44%) were less than those that could not show CPE in the L20B (30.56%) for the two district sites under the Eastern region. The negative CPE in the L20B cell line was almost

twice the sewage that showed CPE in the L20B cell lines. Sewage concentrates from the New Juabeng showed approximately twice of CPE in L20B (13.89%) more than the CPE in L20B from Asuogyaman sewage concentrates (5.56%).

Table 6: Collection Sites with virus isolates from Eastern and Volta Regions using L20B cell line

Region	Collection Sites	Isolates	Frequency (n=36)	Percentage
	New Juabeng	Positive	5	13.89%
		Negative	4	11.11%
EASTERN	Asuogyaman	Positive	2	5.56%
		Negative	7	19.44%
	Ho	Negative	9	25.00%
VOLTA	Ketu South	Positive	2	5.56%
		Negative	7	19.44%

Table 6 shows only two (5.56%) isolates from L20B in the Ketu South sewage collection sites but none was isolated from the Ho collection sites. The percentage of negative isolates in the L20B cell line was 44.44% which gives the indication of low probability of isolating PV from the Volta region.

Isolation of Enterovirus

Enteroviruses were also isolated from the cell culture in the RD cell lines and L20B cell lines since these enteroviruses also replicates in these cells lines. Replication of enterovirus from tissue culture flasks containing the L20B cells and the RD cell flasks showed that there was approximately higher enterovirus isolation from the New Juabeng collection sites (25.00%) as compared with the Asougyaman collection site (19.44%) (Table 7). There were 2 (5.56%) non-enterovirus from the Asougyaman collection sites but none was recorded in the New Juabeng collection sites.

Region	Collection Sites	Virus	Frequency (n=36)	Percentage (%)
EASTERN	New Juabeng	Enterovirus	9	25.00
	Asuogyamang	Enterovirus	7	19.44
		Non-Enterovirus	2	5.56
VOLTA	Ho	Enterovirus	6	16.67
		Non-Enterovirus	3	8.33
	Ketu South	Enterovirus	3	8.33
		Non-Enterovirus	6	16.67

Table 7: Positive Isolates in sewage from Eastern and Volta Regions

Table 7 shows that Volta region sites isolated 25.00% enterovirus and approximately 25.00% of non-enterovirus. The Ho sewage collection sites

isolated twice the enterovirus (16.67%) as compared with the Ketu South sewage collection sites (8.33%).

Isolation of Poliovirus and Non-polio enterovirus

Table 8 shows the distribution of PV and NPEV isolated from the Eastern and Volta regions. Approximately quarter of the positive isolates from the cell culture were PV and these PV isolates make up approximately one fourth of the total isolates of the NPEV detected in the Eastern region. The mean difference in NPEV isolation from New Juabeng and Asougyaman sewage sites was not significant ($p>0.05$). The PV isolated in New Juabeng (14.29%) is four times more than the isolates of PV in Asuogyaman (3.57%) sewage sites.

Table 8: Distribution of PV and NPEV in relation to Eastern and Volta regions

Region	Collection Sites	Enterovirus	Frequency (n=28)	Percentage (%)
	New Juabeng	PV	4	14.29
		NPEV	8	28.57
EASTERN	Asuogyaman	PV	1	3.57
		NPEV	6	21.43
VOLTA	Ho	NPEV	6	21.43
	Ketu South	NPEV	3	10.71

The Volta region sewage sites might not be contaminated with PV isolates (0.00%) because none of the two districts that were sampled had any PV isolates

(Table 8). The isolation of NPEV from the Ho sewage collection sites (21.43%) was as twice (6) as the isolation of the NPEV from the Ketu South (3) sewage collection sites (10.71%).

Intratypic differentiation of Poliovirus

The PV isolates were subjected to rRT-PCR intratypic differentiation where the serotypes for the PV were determined. There were PV Sabin-like 1 serotype (SL1) and Sabin-like 3 serotype (SL3) strains that were identified (Table 13). Three of the PV isolates were single isolates (one SL1 and two SL3) while two of the PV isolates were mixtures (SL1 & SL3). All the single Sabins were isolated from Koforidua while the mixtures Sabin were isolated from both Koforidua and Akosombo.

Table 9: Molecular Characterization of PV Isolates in Eastern Region

Collection Sites	Sabin 1	Sabin 3	Sabin 1 & 3	Total
New Juabeng	1	2	1	4
Asuogyaman	0	0	1	1
Total	1	2	2	5

Environmental conditions for the isolation of NPEV and PV

The collection of the sewage samples in the Eastern region began in the 37th week of 2018 and ended in the 21st week of 2019. The atmospheric temperature during the sample collection at New Juabeng ranged from 26.0^{0}C to 37.1^{0}C at the hours of 10:34 GMT to hot afternoon 13:06 GMT. Asuogyaman

sewage collection sites recorded atmospheric temperature range of 25^{0}C to 36.6^{0}C at the hours between 8:00 GMT to 10:09 GMT as shown in table 10.

Table 10: Time and Temperature readings per epidemiological week for isolation of NPEV in the Eastern Region

		2018 Epidemiological Week					2019 Epidemiological Week			
Collection Sites	Time/Temp	wk 37	wk 41	wk 45	wk 49	wk 2	wk 7	wk 12	wk 17	wk 21
New Juabeng	Time(GMT)	11:30	13:06	12:18	12:06	12:11	11:32	12:00	11:15	10:34
	Temp(^{0}C)	26.0	33.1	37.1	35.7	34.1	28.6	35.7	32	29.1
	NPEV	1	1	1	0	1	2	1	1	1
Asuogyaman	Time(GMT)	9:20	10:09	9:14	9:42	9:27	9:08	9:00	8:25	8:00
	Temp(^{0}C)	25	30.6	34.9	22.8	30.9	28.6	36.6	30	27.4
	NPEV	1	0	2	1	1	0	1	1	0

Table 11: Time and Temperature readings per epidemiological week for isolation of NPEV in the Volta Region

		2018 Epidemiological Week					2019 Epidemiological Week			
Collection Sites	Time/Temp	wk 38	wk 42	wk 46	wk 50	wk 3	wk 8	wk 13	wk 18	wk 22
Ho	Time(GMT)	11:17	14:14	13:45	13:25	13:15	12:00	12:50	10:15	10:20
	Temp(^{0}C)	28	23.8	40.3	36.7	37.9	29	35.5	29.7	30.3
	NPEV	1	0	1	0	1	1	0	1	1
Ketu South	Time(GMT)	NS	11:39	11:16	10:50	10:51	9:30	10:25	8:00	7:30
	Temp(^{0}C)	NS	34.8	22.6	31.7	35.2	25.8	30.4	27.9	28.4
	NPEV	NS	1	0	0	0	1	0	1	0

**NS means No Sample collected

Table 12**: Distribution of PV in relation to epidemiological week from the Eastern region, 2018-2019**

		2018 Epidemiological Week				2019 Epidemiological Week					
Collection Sites	PV	wk 37	wk 41	wk 45	wk 49	wk 2	wk 7	wk 12	wk 17	wk 21	Total
New Juabeng	Sabin	0	0	0	SL1+SL3	SL1&SL3	0	SL3	0	0	4
Asuogyaman	Sabin	0	SL1&SL3	0	0	0	0	0	0	0	1
	Total	0	1	0	2	1	0	1	0	0	5

"&" represent mixtures

"+" represent single Isolates

"SL" represent Sabin-like virus

The isolation of NPEV from the sewage samples had been consistent throughout the study period in the Eastern region (Table 10). There was no isolation of NPEV in the 49th epidemiological week (2018) at New Juabeng but there was isolation of NPEV in Asuogyaman sewage collection site during that week. Asuogyaman on the other hand had no isolation of NPEV in three different epidemiological weeks, 41st week (2018), 7th week and 21st week (2019). There was approximately a single isolation of NPEV every epidemiological week with rare occasions where the NPEV isolation increased to two in a particular collection sites at an epidemiological period. The epidemiological week 45 (2018) and 7 (2019) had an increase in the NPEV isolations from the Asuogyaman and New Juabeng collection sites respectively.

Table 11 displays the time and temperature per epidemiological week in the isolation of NPEV from the Volta region. The samples were collected in the late morning through the afternoon at the Ho from 10:15 – 14:14 (GMT) at an atmospheric temperature range of 23.8- 40.3^{0}C. There was occasional isolation of NPEV from the Ho collection sites. There was no isolation in epidemiological week 42, 50 (2018) and 13 (2019). In the Volta region sewage samples were collected from Ketu South was between the hours of 7:30 – 11:40 GMT while sewage from Ho was collected between the hours of 10:15 – 14:15 GMT. There was sporadic isolation of NPEV in Ketu South collection sites. On the epidemiological week 50 (2018) and week 13 (2019) there were no NPEV in both collection sites in the Volta region.

Variables	Collection sites	Region	PV

The poliovirus that was isolated from the sewages collected in the Eastern region was confirmed using rRT-PCR. All the PV were vaccine strain virus (Sabin-like virus) (Table 12). There was sporadic isolation of these SL strain from 2018 to 2019. The vaccine strain was circulating from epidemiological week 41 (2018) through to epidemiological week 12 (2019) where there were isolation of singles and mixtures of SL from both districts in the Eastern region. There were single isolates of the Sabin-like (SL3) from the sewage collected from the New Juabeng sample sites at wk 12 (2019) while the mixtures of the vaccine strain serotypes in both New Juabeng were SL1+SL3 at wk 49 (2018) and SL1&SL3 on wk 2 (2019). The SL1&SL3 isolates from Asuogyaman were on week 41 (2018). Table 13 shows strong positive correlation between the regions and collection sites [$r = 0.89$, $\rho < 0.01$]. Indicating the collection sites are likely to be among the major drains in the region. It also shows that a negative relationship exists between PV and collection sites [$r = -0.39$, $\rho < 0.05$]. This means as the collection sites may not be convenient for PV isolation. Again, PV was negatively correlated with the regions [$r = -0.35$, $\rho < 0.05$] where the study was carried out. This also support the above with decreasing PV isolation in the regions and that the regions may not harbour PV in the sewages.

Collection sites	-		
Region	0.894**	-	
PV	-0.397*	-0.349*	-

Table 13: Correlation among Collection sites, Region and Poliovirus

For CPE of RD cell lines, there is a significant difference among the sites of collection (p=0.020) (Table 14). However, there was no differences among the sites of collection with regards to CPE on L20B (p=0.343). Also for no CPE there was a significant difference among the collection sites (p=0.039). The viruses detected on RD may vary among the collection sites whereas there was no difference in sites where PV were not detected.

Table 14: Crosstabulation of Collection Sites and Cytopathic effect in cell culture

CELL LINES	New Juabeng	Asuogyaman	Ho	Ketu South	X^2	P-value
CPE_RD	9	9	9	9	15.045	0.020
CPE_L20B	9	9	9	9	6.770	0.343
No_CPE	9	9	9	9	8.390	0.039

Discussion

Processing of Sewage sample

Circulation of PV still remain a significant public health problem worldwide. The implementation of ES in countries that have not recorded WPV

and VDPV over a long period through the AFP surveillance has become crucial. This is to monitor the importation of PV and immunization coverage in a catchment habitats or districts.

The current study applied the two-phase aqueous separation method as recommended by WHO in processing raw sewage samples (WHO, 2003). This processing aided to increase the virus concentration in the raw sewage which is free from other bacterial and fungal contaminations. This process was applied in ES studies conducted in Ghana (Odoom et al., 2017), Malaysia (Yusof et al., 2018), Senegal (Ndiaye, Diop & Diop, 2014), Cameroon (Njile et al., 2019) and Nigeria (Adeniji, Adewale, Faleye, & Adewumi, 2017) to determine viruses that might be circulating in the environment and likely to cause disease. An environmental surveillance studied in Moscow, Russian Federation, applied trap sampling method and 3% beef extract in 0.05 M Tris-HCl at a pH of 9.1 was used to process the raw sewage (Ivanova et al., 2019) while another studied at United Kingdom applied filtration and centrifugation methods using Centiprep YM-centrifugal concentration devices (Merck) (Majumdar et al., 2018). Although WHO recommended two-phase separation method, the filtration and centrifugation methods are less laborious and quicker.

Almost all the sewage samples collected from the sample sites were not turbid which might be the outcome of few volume of the harvested pellets used for treatment and isolation of the PV and NPEV. The combination of the three compounds, PEG 6000, dextran T40 and NaCl might have facilitated the precipitation of the organic materials in the sewage samples to form the pellet

harvest during antibiotic treatment of sewage samples and isolation of viruses as compared with WHO (2003) manual that recommended two of the compounds, either PEG 6000 and Dextran T40 or NaCl.

Virus Isolation

The detection of human enterovirus HEV confirmed the efficacy of the methods used to isolate the PV. The isolation rate of HEV was 71.43% which was consisted with studies that was carried out in Dakar, Senegal, 79.7% (Ndiaye, Diop & Diop, 2014), as well as in Tehran province in Iran, 60.32% (Momou, et al., 2012). The HEV was relatively higher and was similar to the findings by Odoom et al., (2017) in Ghana and in Fars province in Iran (Momou, et al., 2012).

The isolation of HEV from site to site bases on the concentration of the sewage samples in the various sites were similar and are likely to be coxackiviruses, echoviruses, rhinoviruses and polioviruses. These HEV may be etiological agents associated in hemorrhagic conjunctivitis (AHC) also known as Apollo in Ghana. AFP and fulminant hepatitis may be caused by echoviruses whereas herpangina, Hand foot and Mouth disease may also be caused by coxsackieviruses. There are other echoviruses that may cause mild gastrointestinal and respiratory illness, myocarditis and encephalitis in children under 5 years of age. In the temperate climates HEV infection is common in the summer but the detection rate in this current studies shows that the seasonal pattern of detecting HEV is less evident in tropical areas where circulation trends are year round. The HEV might not necessarily be coming from the children under 5 years but adults

within the habitat who might be discharging these viruses in the environment that will mix up with the children.

Cell culture is the gold standard for the isolation of PV and the study used two cell lines in isolation as recommended by WHO. Cytopathic effect (CPE) on the L20B cell lines should have been replicated on the RD cell lines within 3 to 5 days' incubation. These shows the specificity in the isolation processes to ensure the specific type of viral isolation. Nevertheless, it was not all the CPE on the RD cell line was able to replicate on the L20B cell line. This also support the fact that no viruses are missed during isolation processes.

The isolation frequency of NPEV (3 isolates per month) was detected throughout the study period but there was a two-fold increment in January 2019. The detection of the NPEV in sewage was higher than detection of PV and it is consistent in work done on AFP surveillance on stools (Ivanova et al., 2019). There were other studies with even higher rate of NPEV detection in Rio de Janeiro, Brazil (Pereira et al., 2017) and Malaysia (Yusof et al., 2018) from urban sewage. The sensitivity of the procedure applied in collecting the sewage samples and processing them to be able to detect NPEV indicates that the ES is adequately been performed. According to WHO, ≥ 30% of NPEV is expected to be isolated in samples processed by Grab method (WHO, 2003).

Five (17.86%) out of the nine (26.47%) suspected poliovirus from the L20B cell lines to the RD cell line arm was confirmed as Sabin-like isolate using rRT-PCR. These findings were consistent with ES of PV carried out in Malaysia

(10.43%) after 5 years of withdrawal from OPV to IPV (Yusof et al., 2018). Also similar to the studies done in Cameroon (7.2%) where PV was detected and characterized from urban sewages (Njile et al., 2019) and Nigeria Sabin detection rate of 29% (Weldegebriel, Adeneji, Gasasiral, Okello & Elemuwa, 2015).

There were isolations of PV in the Eastern region samples but none in the Volta region samples after the cell culture. This could be as a result of the modernization of properly disposing off children under 2 years' stools which in turn could reduce the concentration of the virus if present in the large volume of waste that flows in the drainage system of the community. The habitat in the Volta region mostly use pit latrine to dispose off their human excreta while the few ones that use water closet would have their sewage water passing through the drainage system (Nakamura et al., 2015). This might reduce the concentration of the PV and NPEV if they are circulating within the community. There is also the possibility of low immunization coverage among the children under 5 years so the vaccine strain was also not detected in the environment. The increase in temperature of the environment during the time of sampling was higher than room temperature (25^0C) which might have been the effect of low survival of the virus in the sewage samples.

Characterization of PV

The main target of ES is to detect WPV and circulating VDPV from raw sewage in a community but neither of these PVs were isolated from all the sewage collected. This was in consistent with findings in Malaysia (Yusof et al., 2018), Senegal (Ndiaye, Diop & Diop, 2014), Ghana (Odoom et al., 2017) and Poland

(Figas et al., 2018). Probably, high immunization coverage in the selected districts and provinces may have contributed to the low isolation rates. Therefore, almost everyone in the population was protected from the WPV and VDPV infection. On the contrary, Nigeria (Gumede et al., 2018), Pakistan (Reilly et al., 2018) and Afghanistan (Kroiss et al., 2018) detected WPV and cVDPV in their environments. The findings indicated that wild polioviruses are still circulating among children in these nations.

The suspected PV after cell culture isolations were subjected to rRT-PCR for ITD to be able to determine the serotypes of the PV. The 5 isolates whose serotypes were determined came out to be Sabin like strain 1 and 3. It was determined that the most frequently detected serotype was PV3 which accounted for 66.67% of the total serotype strains. This finding is in consistent with similar findings from research works that have been carried out in Tehran (Matrajt et al., 2018) Japan (Majumdar et al., 2018) and Poland (Figas et al., 2018).

The presence of at least 1% of divergence in VP1 gene for serotypes 1 and 3 and 0.6% for serotype 2, in comparison with the prototype strains, classifieds them as VDPV. PVs with lower divergences than these are considered as “Sabin-like” (Pereira et al., 2017). This demarcation of ~1% difference in nucleotide sequence of VP1 indicates that replication of the vaccine virus has occurred for approximately one year (Kew et al., 2005).

The study did not detect any vaccine derived poliovirus from any of the sampling sites after all the sewage isolates were subjected to rRT-PCR to

determine intratypic differentiations using ITD and VDPV test kits from CDC. All the positives isolates detected were Sabin 1 and 3. The detection rate of the Sabin 3 was relatively more than Sabin 1 which was consistent with the findings in Poland (Figas et al., 2018) and Japan (Yoshida, Horie, Matsuura, & Miyamura, 2000). There were few occasions where singles and mixtures of the Sabin isolates were detected from the sample collection sites. New Juabeng and Asougyaman detected mixtures of Sabin 1 and 3 isolates while only New Juabeng again recorded single isolates of the Sabin. These detection of the Sabin 1 and 3 confirms the use of bivalent oral polio vaccine (bOPV) after the switch from the trivalent oral polio vaccine (tOPV) in immunization in 2016 by WHO.

Ho and Ketu South in the Volta region did not detect any Sabin, WPV or VDPV. Although the polio immunization exercise was routine for every child that is born in the region and the nation, the ES detected NPEV and non-enteroviruses (NEV). This study is consistent with a study that was carried out in Japan before the introduction of Inactivated Polio vaccine (IPV) (Nakamura et al., 2015). There could be low routine immunization coverage of the polio vaccine which could account for these situation. Some of the communities use pit latrine whereby the sewage does not go through any flowing sewage system. There is also the increase use of diapers for the children where they are properly discarded so the faeces that might contain the PV do not contaminate the sewage samples in the drainage system.

Genotyping of poliovirus

The VP1 region found on the surface of piconavirus viron is the main surface-accessible protein that could be detected. VP1 region contributes to the major neutralization sites that have been identified on the surface of PV. The polymerase chain reaction (PCR) amplifies the VP1 gene of PV and improves the sensitivity of the methods used for tracking specific sequences in PV isolates. The amplification of the complete VP1 regions were done using Y7/Q8 non-degenerate primers. These primers were efficient with Sabin isolates which serves as templates. Other primers that can be used as substitute were the degenerate sense Y7R or antisense PV8A primer to yield the desire amplicons.

The Y7/Q8 primers was observed to amplify all single isolates of the Sabin templates (100%) and displayed all the desired amplicon when they were ran on the gel electrophoresis. The mixed-serotype of the Sabin that were detected were amplified using 246S-248S Sabin strain-specific primer. Other studies also use 249A-251A anti-sense primers to obtain VP1 sequence for Sabin-like viruses (Kilpatrick, Iber, et al., 2011). Primers that could be used as antisense primers at the 3-end of the VP1 region may include 252A-254A primers combination (Kilpatrick, Iber, et al., 2011). There was also 100% amplification of the Sabin mixtures isolates from both Akosombo and Koforidua when the 246S-248S primers were used.

The VP1 region estimated for sequencing the Sabin strains that were used as a reference in this study were Sabin 1 (AY082688, 906 bp), Sabin 2

(AY082679, 903 bp), and Sabin 3 (AY082683, 900 bp) (Nakamura et al., 2015). The analysis of the VP1 for the Sabin isolates were not successful in this study to be able to determine if there were mutations in the VP1 sequence.

Limitations

There were several attempts to sequence the viral capsid VP1, but the desired fragments that needed to be sequenced were not attained. The time constrain to complete the study and cost involved in acquiring new set of primers to sequence the VP1 was a challenge that was why it has been left for future studies. However, the studies could not detect WPV and VDPV, emerging or circulating in the four district sampling sites over the course of the study.

Summary Chapter

The results from this study showed no circulation of WPV and cVDPV in the four districts where the sewage samples were collected. All the Sabins isolated were detected in the Eastern regions and their characterization showed that they were Sabin 1 and Sabin 3. This confirms the routine immunization of the bOPV carried out within the region. There was detection of NPEV in all the sampling collection sites which aid to determine the sensitivity of the procedure that was employed to collect and process the sewage samples.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATION

Studies on ES has been carried out in different countries where they have been certified by WHO as polio-free. Although they do not isolate PV using the AFP surveillance they were able to isolate circulating PV when the ES was implemented. Ghana is known to be one of polio-free countries in the African region and has only had three months' pilot study on ES but could not isolate any PV. This study seeks to apply various techniques as recommended by WHO in environmental surveillance for poliovirus to isolate and characterize PV in the Eastern and Volta regions of Ghana.

This study reported isolation of Sabin 1, Sabin 3 and NPEV among the sewage samples, VP1 region of the poliovirus genome was amplified.

Summary

Environmental surveillance for PV is important because it allows stakeholders to continually monitor the transmission and early detection of circulating PV in a defined population. This will help to put measures in place to stop the transmission of the PV. Ghana has been polio-free since it detected a case of WPV in 2008 was an importation from Nigeria through the AFP surveillance. The world has gotten to the last stage in eradicating PV through the effort and strategies adapted by the Global Polio Eradication Initiative (GPEI) and the government of every nation to immunized every growing child under 5 years with

either OPV or IPV. AFP surveillance is the gold standard in detect the isolation of PV but its detection rate is approximately 0.5% but the ES is able to detect 99.5%.

The aim of the study was to detect silent circulation of WPV and VDPV in four districts within the Eastern and Volta region of Ghana. The specific objectives of the study were to;

i. Determine the presence of PV and NPEV in the New Juaben, Asuogyaman, Ketu South and Ho districts.
ii. Characterize the PV isolates.
iii. Detect the genotypes of the PV strains isolated.

A systematic cross sectional design was use for the study. The convenient sampling method was used to collect the samples every four weeks. There were two open sewage systems located in the New Juabeng and Ho sampling sites while the Asougyaman and Ketu South sampling sites had close sewage systems. A total of 36 sewage samples were collected from September 2018 to May 2019.

The findings are presented below;

The estimated isolates that showed CPE on only RD cell line was 69.42%. There were 25.01% of the isolate that showed CPE on both L20B and RD cell lines. All the isolated that showed CPE on the RD cell lines were considered NPEV including some of the isolates that showed CPE on both RD and L20B cell lines but negative for ITD using rRT-PCR. The prevalence recorded for NPEV

was 82.14%. the highest prevalence was recorded in New Juabeng 28.57%, followed by Asuogyaman and Ho, 21.43% then Ketu South 10.71%.

Sabin isolated was 14.29% from the Eastern region among the two districts. The prevalence of isolating Sabin 1 and 3 among New Juabeng and Asuogyaman were 11.43% and 2.86% respectively. The isolation of these Sabin confirms the ongoing routine immunization within the Eastern region because the children who have received bOPV are shedding them in the environment.

The VP1 region of the viral capsid was determined for all the Sabin isolates by using conventional PCR after RNA extraction was carried out on the cell culture was aliquoted. The cDNA was viewed on gel electrophoresis. Several attempts were made to sequence the PCR product after purification of the cDNA but it was not successful. The desired fragments were not yielded. Due to time constrain and the cost involve in performing the sequencing, the process was not further repeated.

Conclusion

The hypothesis: there is no silent circulation of wild and vaccine-derived poliovirus in the selected districts of the Eastern and Volta regions of Ghana was supported. The ES detected circulation of Sabin 1 and Sabin 3 because of the bOPV that has been administered to children who are continually shedding the vaccine strain into the environment through their feaces. There was no Sabin isolation in the Volta region. There is also the possibility of low immunization

coverage among the children under 5 years so the vaccine strain was also not detected in the environment.

ES has proven to be very sensitive and it should be implemented in all the districts thought out the whole country. This will facilitate the early detection of any importation of the PV and reemergence strain of the PV as the world put forces together to eradicate polio.

Recommendation

Based on the findings from this study, the following are recommended:

i. Disease Control officers should train personnel to collect sewage samples from the various districts to be examined at the Regional Reference Polio Lab at Noguchi Memorial Institute for Medical research.

ii. Education on hand hygiene and the need to take polio vaccine should be given all the needed attention in order to increase the immunization coverage in the nation.

Suggestions for Future Research

Although the study provides insight into the processing of the environmental sewage samples, cell culture and characterization of the isolates, efforts to sequence the PV isolates could not yield the desired results. Future perspectives could include;

a. Expanding the environmental surveillance to other regions and districts in Ghana. This is crucial since the world is determine to eradicate poliovirus.

b. All poliovirus isolates and non-poliovirus isolates should be sequenced to determine the diversity of the genome.

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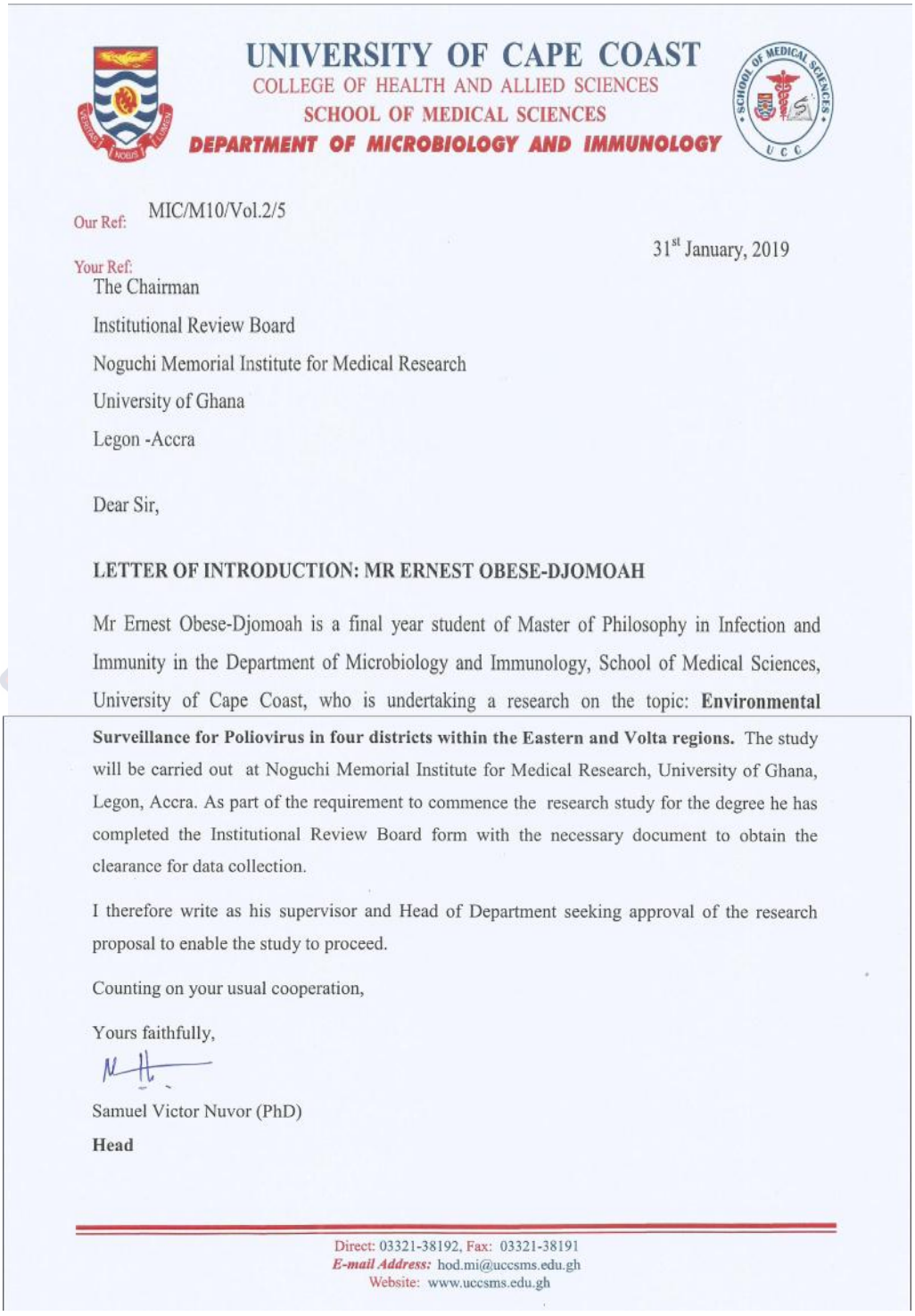
APPENDICES

APPENDIX A

INTRODUCTION LETTER



UNIVERSITY OF CAPE COAST
COLLEGE OF HEALTH AND ALLIED SCIENCES
SCHOOL OF MEDICAL SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

Our Ref: MIC/M10/Vol.2/5

Your Ref:

31st January, 2019

The Chairman
Institutional Review Board
Noguchi Memorial Institute for Medical Research
University of Ghana
Legon -Accra

Dear Sir,

LETTER OF INTRODUCTION: MR ERNEST OBESE-DJOMOAH

Mr Ernest Obese-Djomoah is a final year student of Master of Philosophy in Infection and Immunity in the Department of Microbiology and Immunology, School of Medical Sciences, University of Cape Coast, who is undertaking a research on the topic: **Environmental Surveillance for Poliovirus in four districts within the Eastern and Volta regions.** The study will be carried out at Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra. As part of the requirement to commence the research study for the degree he has completed the Institutional Review Board form with the necessary document to obtain the clearance for data collection.

I therefore write as his supervisor and Head of Department seeking approval of the research proposal to enable the study to proceed.

Counting on your usual cooperation,

Yours faithfully,

Samuel Victor Nuvor (PhD)
Head

Direct: 03321-38192, Fax: 03321-38191
E-mail Address: hod.mi@uccsms.edu.gh
Website: www.uccsms.edu.gh

APPENDIX B

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979
A Constituent of the College of Health Sciences
University of Ghana

Phone: +233-320-501180/513202 (Direct)
+233-320-501178/9 (S/board)
Fax: +233-320-502182/513202
E-mail: Director@noguchi..ug.edu.gh

Post Office Box LG 581
Legon, Accra
GHANA

My Reference:

Your Reference:

10th April, 2019

Mr. Ernest Djomoah-Obese
Department of Virology
NMIMR
Legon

Dear Sir,

APPROVAL OF PROTOCOL

The Scientific and Technical Committee of the Noguchi Memorial Institute at its meeting on 10th December, 2018, reviewed the protocol entitled: "Environmental Surveillance for Poliovirus in four Districts within the Eastern and Volta Regions", STC Paper 3(3) 2018-19, which was submitted by Mr. Ernest Djomoah-Obese.

The Committee after the review approved the protocol on Wednesday, 10th April 2019, and recommended that the study should be carried out.

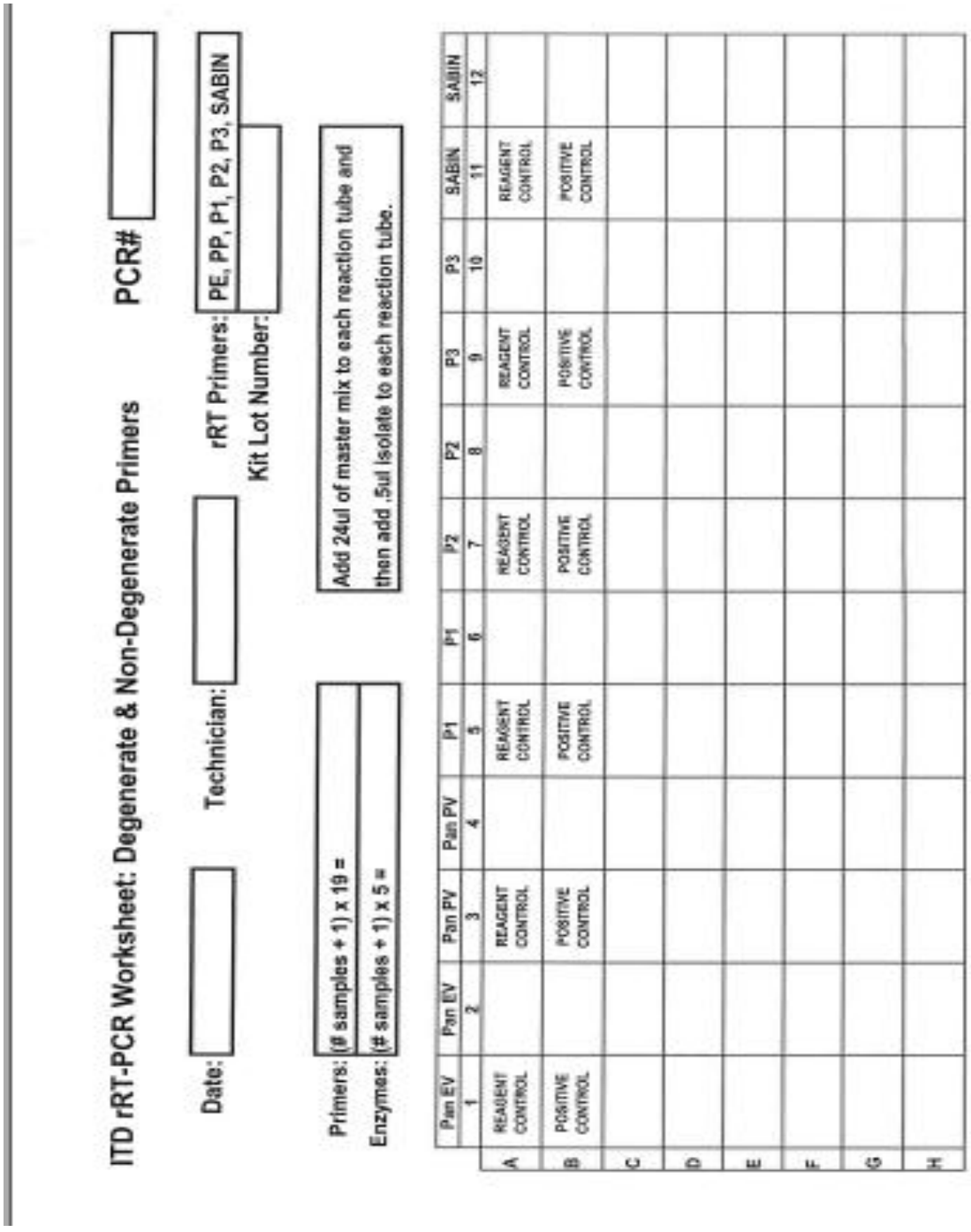
The Scientific and Technical Committee avails to you the assurances of its highest consideration.

Thank you.

Yours faithfully,

Professor Abraham Kwabena Anang, PhD
DIRECTOR

APPENDIX C



ITD rRT-PCR Worksheet: Degenerate & Non-Degenerate Primers PCR# [ ]

Date: [ ] Technician: [ ] rRT Primers: PE, PP, P1, P2, P3, SABIN

Kit Lot Number: [ ]

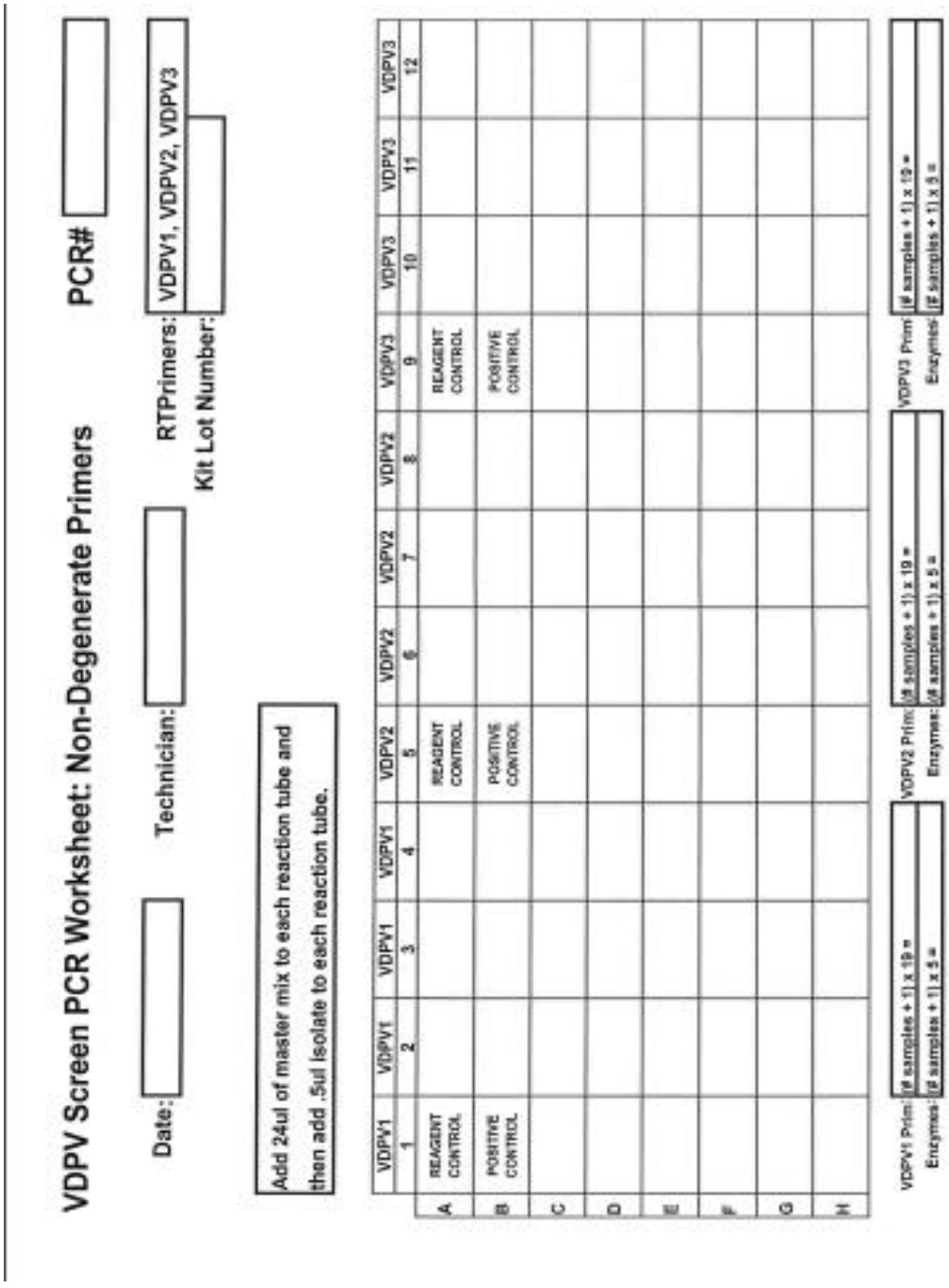
Primers: (# samples + 1) x 19 =

Enzymes: (# samples + 1) x 5 =

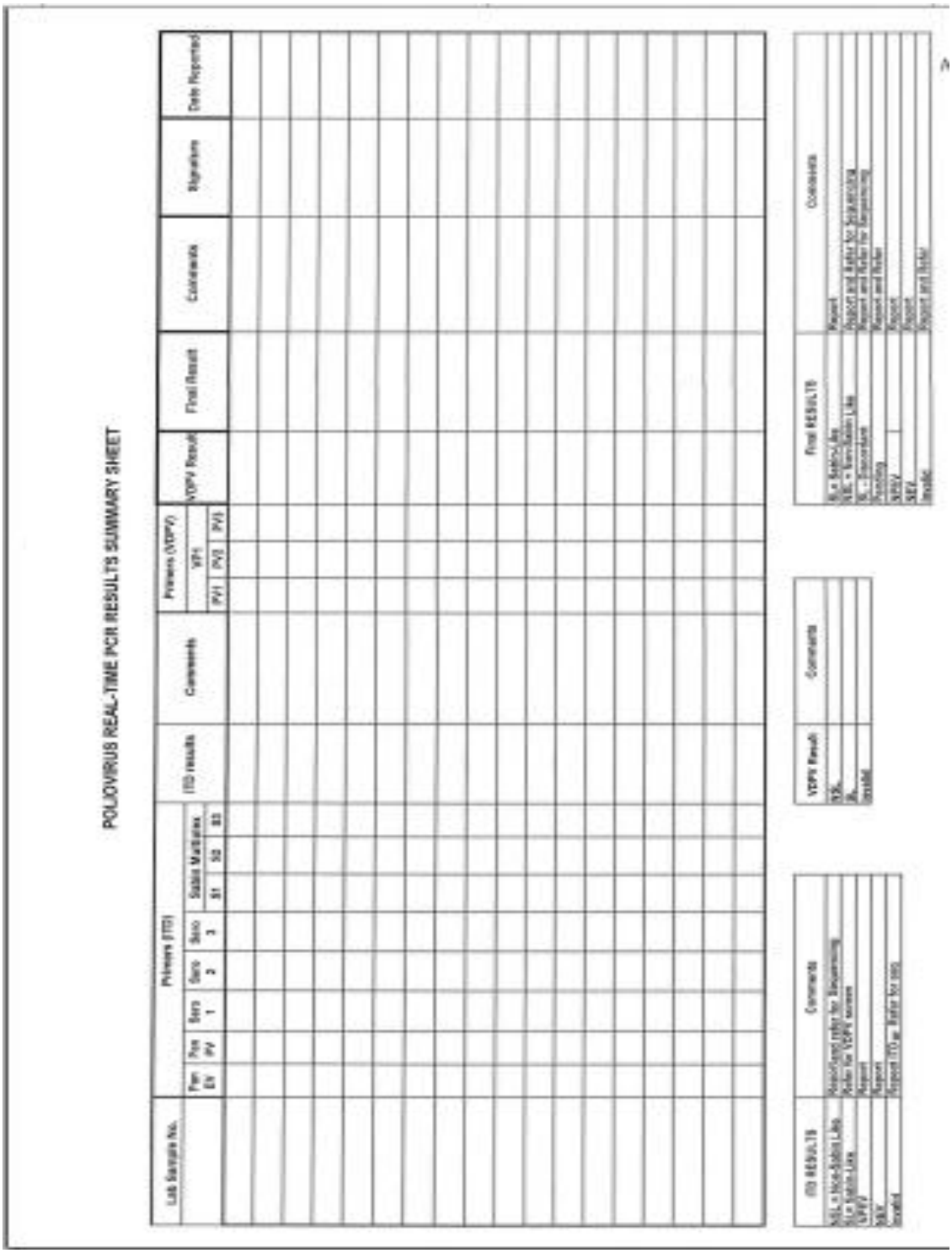
Add 24ul of master mix to each reaction tube and then add .5ul isolate to each reaction tube.

	Pan EV 1	Pan EV 2	Pan PV 3	Pan PV 4	P1 5	P1 6	P2 7	P2 8	P3 9	P3 10	SABIN 11	SABIN 12
A	REAGENT CONTROL		REAGENT CONTROL		REAGENT CONTROL		REAGENT CONTROL		REAGENT CONTROL		REAGENT CONTROL	
B	POSITIVE CONTROL		POSITIVE CONTROL		POSITIVE CONTROL		POSITIVE CONTROL		POSITIVE CONTROL		POSITIVE CONTROL	
C												
D												
E												
F												
G												
H												

APPENDIX D



APPENDIX E



APPENDIX F

POLIOVIRUS INVESTIGATION WORKSHEET

EPID NO	LAB ID	CELL LINE	DATE OF INO.	1	2	3	4	5	CELL LINE	DATE OF INO	1	2	3	4	5	ISO RESULT	DATE ARM COMPL	REVIEWED BY
		PASS NO.							PASS NO.							NEG/ NPENT/ SUSPECTED POLIO		
		L20B FLASK1							L20B FLASK1							NEG/ NPENT/ SUSPECTED POLIO		
		L20B FLASK2							L20B FLASK2							NEG/ NPENT/ SUSPECTED POLIO		
		L20B FLASK3							L20B FLASK3							NEG/ NPENT/ SUSPECTED POLIO		
		L20B FLASK4							L20B FLASK4							NEG/ NPENT/ SUSPECTED POLIO		
		L20B FLASK5							L20B FLASK5							NEG/ NPENT/ SUSPECTED POLIO		
		RD1 FLASK							RD2							NEG/ NPENT/ SUSPECTED POLIO		
									L20B									
CC		$L20B_1$ FLASK							$L20B_1$ FLASK									
		RD_1 FLASK							RD_1 FLASK									
		Negative: [-]		CPE: 1+ to 4+					Harvest/Freeze [H]		Cell degeneration/Dropped: [D]					Cell toxicity: [T] Contamination [C]		

APPENDIX G

Environmental Surveillance Form

Country: Country code:

ID Code:

(ENV/CCC/PPP/DDD/SSS/YY/###)

Region: Region code:

District: District Code:

Site name: Site code:

Village/Settlement:

Geo-coordinate of site (Latitude & Longitude): Lat: /Long:

Type of sewage plant or sewage system: Open Close WWTP

(tick correct response)

Time of sample collection: (hh:mm)

Atmospheric temperature at time of sample collection: ^{0}C

Date of sample collection: (dd/mm/yyyy)

Date sample sent to Laboratory: (dd/mm/yyyy

Name of Person who collected the sample…… ……

Phone no.:……...…….Signature……

Name of Supervisor during collection……...……..……

Phone no.:……..Signature:……

Laboratory Information

Date of sample receive at Laboratory: _____ (dd/mm/yyyy)

Name of person receiving sample at Laboratory: _____

Signature: _____

Sample Lab ID No: _____

Condition of sample at receipt: (1 = Good, 2 = Bad, 3 = Unknown)

If bad/ Unknown, specify:……..

Temperature of specimen carrier on arrival in the lab _____ ^{0}C

Volume of specimen: (1 > 1L, 2 < 1L)

Colour of specimen: (1= clear, 2= cloudy/ turbid., 3= dark)

Final Cell Culture results: _____

ITD results: _____

Sequencing results: _____

Date results sent out by lab: _____ (dd/mm/yyyy)

Date results received by surveillance (or WHO): _____ (dd/mm/yyyy)

APPENDIX H

MATERIALS

Reagent for laboratory work

QIAamp viral RNA kit (QIAGEN, USA)

Absolute ethanol (molecular biology grade) [SIGMA, USA]

Nuclease-free water (Ambion, USA)

Poliovirus rRT-PCR ITD 5.0 kit (CDC, Atlanta, GA USA)

Poliovirus VDPV 5.0 rRT-PCR kit (CDC, Atlanta, GA USA)

Agarose (SIGMA, USA)

Ethidium bromide (SIGMA, USA)

Tris-Borate-EDTA (TBE) [Ambion, USA)

DNA molecular weight 1000bp ladder (Biolabs, New England)

QIAquick PCR purification kit (QIAGEN, USA)

Laboratory equipment

Biological Safety Cabinet Class II (LABGARD, USA)

Biosafety Cabinet Class IIA (AirTech Services, India)

Vortex Genie-2 (Scientific Industries, USA)

Autoclave SS-325 (Tomy, Japan)

Microcentrifuge 5415D (Eppendorf, USA)

AirClean 600 PCR Workstation (AirClean Systems, USA)

GeneAmp PCR System 2700 and 2720 (ABI, USA)

Microwave oven (LG Electronics Inc., Ghana)

Gel logic 100 Imaging System (Eastman Kodak Company, USA)

APPENDIX I

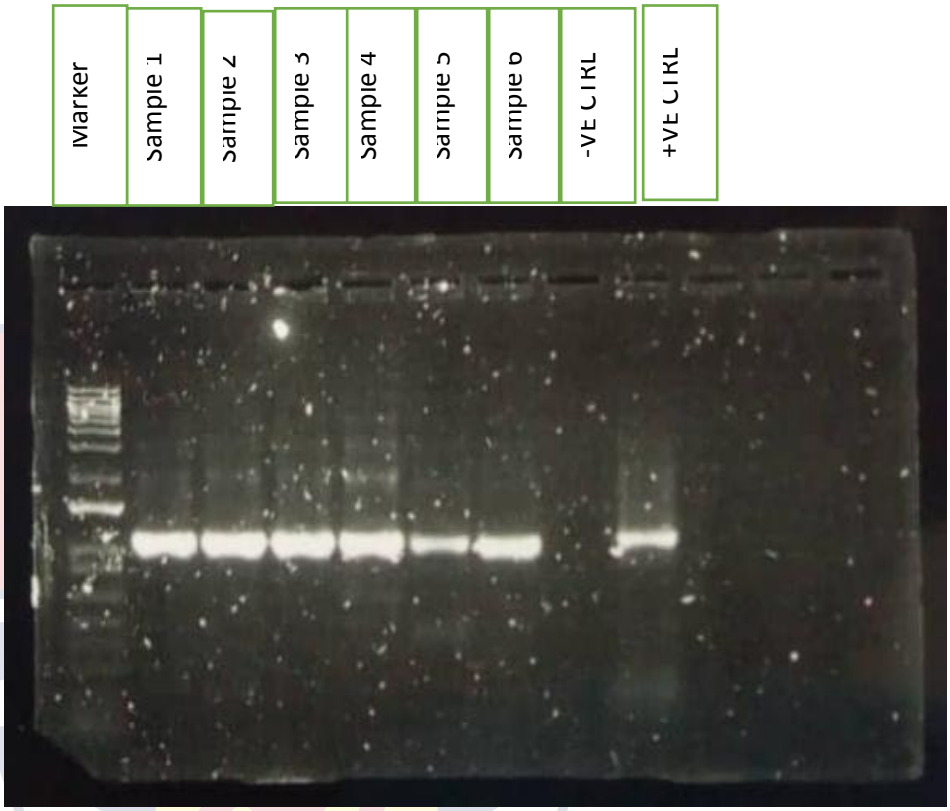
Media Preparation

Reagents	Growth Medium (ml)	Maintenance medium (ml)
Eagle's minimum essential medium	85.5	92.5
L-glutamine (200mM)	1.0	1.0
Fetal Bovine Serum	10.0	2.0
$NaHCO_3$ solution (7.5%)	1.5	2.5
HEPES (1M)	1.0	1.0
Penicillin/ Streptomycin solution	1.0	1.0

Preparation of Precipitation Solution

Reagents	Quantity (g)	Volume of sterile water (ml)
PEG 6000	90.75	222.0
Dextran T40	10.00	35.5
NaCl	10.25	30.0

APPENDIX J



An electrophoregram (1.0% agarose gel in 1X TBE buffer) of VP1 amplification. Lane 1 contained the 1000bp DNA ladder (Biolabs, New England). Lanes 8 is a negative control; Lanes 9 is a positive control and lanes 2 to 6 contained positive samples from cDNA products of sewage samples. Expected size of positive amplification product is ~900bp and ~906bp.