MOLECULAR DETECTION OF NOROVIRUS IN CHILDREN PRESENTING WITH DIARRHEA IN OGUN STATE, NIGERIA

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA.

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE DEGREE (B.Sc.) IN MICROBIOLOGY

SEPTEMBER, 2022

DECLARATION

I hereby declare that this project report written under the supervision of Dr (Mrs) C. I. Ayolabi is a product of my own research work. Information derived from various sources has been duly acknowledged in the text and a list of references is provided. This project report has not been previously presented anywhere for the award of any degree or certificate.

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OLAWALE, EUNICE AYOMIDE

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Date

CERTIFICATION

This is to certify that this project report titled **"MOLECULAR DETECTION OF NOROVIRUS IN CHILDREN PRESENTING WITH DIARRHEA IN OGUN STATE, NIGERIA"** was carried out by OLAWALE, Eunice Ayomide, with the matriculation number 18010101040**.** This project meets the requirements governing the award of BACHELOR OF SCIENCE (B.SC.) Degree in MICROBIOLOGY department of biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

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Dr (MRS) C.I. Ayolabi

Supervisor

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DEDICATION

This work is dedicated to the Lord God Almighty, EL SALI (THE GOD OF MY STRENGTH) for his strength, mercy and favour in overseeing this report's positive outcome. To my most wonderful parents, MR AND MRS OLAWALE and my ever-supportive siblings, I dedicate this work to you. I also dedicate this work to my supervisor DR (MRS) C. I. Ayolabi for her motherly care and support in helping us with the resources needed to make this work achievable and to MR and MRS TANIMOWO for their care and love throughout my university days. Lastly, I dedicate this work to MR OLUMIDE ADESINA and DR A.O YOUNG, the best fire duo, both spiritually and physically for their discipline and love.

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ABBREVIATIONS

NoV -Norovirus.

RT-PCR – Reverse transcriptase polymerase chain reaction.

GI, GII, GIV – Genogroup I, II, IV.

ORFs – Open Reading Frames.

RdRp - RNA-dependent RNA polymerase.

RNA- Ribonucleic Acid.

ssRNA- Single Stranded RNA.

VP –Viral Protein.

NTPase - Nucleoside-Triphosphatase.

HBGAs - Histo-Blood Group Antigens.

HuNoV -Human Norovirus Molecular Model.

AGE- Acute Gastroenteritis.

VPg- Viral Protein genome-linked.

COP- Coat proteins

Pol-protein- polyprotein.

DALYs- Disability-adjusted life-years.

CI- Confidence Interval.

UI- Unit Interval.

FUT- Fucosyltransferase.

Ig – Immunoglobulin.

TBE -Tris Borate EDTA

WASH- Wash, Sanitation and Hygiene.

Bp- Base pair.

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ABSTRACT

Norovirus (NoV) is amongst the eminent etiological agent of viral gastroenteritis outbreaks worldwide. Apart from rotavirus, NoV plays a rising and crucial role in enteric infection in children, particularly during the post-rotavirus vaccination period. Among the three human norovirus strains discovered, GII genogroup is more prevalent in Nigeria and they also occur during the dry season. Children infected with NoV typically exhibit classic clinical signs of acute viral gastroenteritis, such as vomiting and liquid stools, and paediatric patients are more likely to develop dehydration that necessitates hospitalisation. Although most NoVs infection symptoms are self-limiting, recurring infection is not uncommon in children. However, its incidence is rarely reported in developing countries such as Nigeria. The aim of this study was to investigate the prevalence of Norovirus in children below the age of five presenting with diarrhea in Ogun State, Nigeria. With the consent of the caretakers, a questionnaire was provided, and 100 stool samples were obtained from children who had diarrhoea. Viral RNA was isolated from the samples using molecular methods and tested for the presence of Norovirus using RT-Nested PCR. Samples were amplified by PCR using specific primers to make the result more legible. The collected data were statistically analysed using SPSS version 20. Analysis of the samples showed the prevalence of Norovirus to be 6%, of which 4% were male and 2% were female. Subsequent investigation found that children under the age of two were more impacted than children beyond the age of two. This, however, was not statistically relevant. The P-value of > 0.05 is significant. In conclusion, this study established the importance of NoV as a cause of paediatric diarrhoea and identified norovirus as one of the common causative agents of paediatric illness in circulation in Ogun State, Nigeria. Norovirus gastroenteritis is a public health hazard that requires quick action to prevent future spread.

KEYWORDS: Children, Diarrhoea, Norovirus, Nigeria, RT-PCR

CHAPTER ONE

INTRODUCTION TO NOROVIRUS

Norovirus is a virus belonging to the family Caliciviridae. They are known to cause sporadic and epidemic acute viral gastroenteritis (Glass *et al.*, 2000; Patel *et al.*, 2008). This group of viruses were formerly named Norwalk virus (Britannica, 2022). It is the second most prevalent cause of acute viral gastroenteritis after rotavirus (Al-Mashhadani *et al.*, 2008). The "Norwalk virus" was named after an outbreak that occurred in 1968 at Norwalk, Ohio, elementary school (Knowable magazine, 2017). Noroviruses (NoV) are well-known aetiology of severe gastroenteritis and have been implicated in outbreaks in places like schools, cruise ships, nursing homes, and communities (Glass *et al.*, 2000). Human norovirus strains can be classified into 3 groups: GI, GII, or GIV. Norovirus-associated gastroenteritis can be distinguished from other viral aetiology by the abrupt onset of diarrhoea and/or vomiting, although it may also be accompanied by nausea, stomach discomfort, fever, headache, and body pains (Atmar and Estes, 2006). These symptoms often appear 12 to 48 hours after norovirus exposure and are self-limiting, lasting 1 to 3 days (Atmar and Estes, 2006). More serious effects are conceivable and are most commonly linked with severe dehydration in adolescents. Moreover, molecular methods have identified this virus as sporadic self-limiting gastroenteritis in children and often times requiring hospitalization (de Wit *et al.,* 2001; Marshall *et al.,* 2003; Oh *et al.,* 2003; Nakagomi *et al.*, 2008). Despite the misconception, that norovirus only causes self-limiting, mild gastroenteritis, there have however been reports of cases necessitatng medical attention, resulting in severe illness or death. Annually, there are an estimated 70,000 child fatalities globally associated with norovirus infection (Lanata *et al.*, 2013). Although norovirus (NoV) has drawn more attention as a cause of childhood diarrhoea, its prevalence in underdeveloped nations like Nigeria has not been adequately reported (Ayolabi *et al.,* 2010).

1.1 Statement of Problem

In spite of the advances in sanitary and hygiene conditions, Noroviruses have been identified as important agents of intermittent gastroenteritis in developed countries. However, there is little or no epidemiological data on the disease burden affiliated with infrequent norovirus infection in clinical or hospitalised patients in developing countries, particularly in Africa. The purpose of this paper is to look at NoV in young patients with diarrhoea in Ogun State, Nigeria.

1.2 Justification of Study

The justification of this study will prove beneficial to one's advantage in society by implementing a faster method in the detection of Norovirus in samples. It also provides epidemiological data on this virus as a cause of gastroenteritis in children and also monitors the prevalence of this virus in Ogun state, Nigeria.

1.3 Aim and Objectives

This study aims to ascertain the prevalence of Norovirus infection among children less than the age of five in Ogun State, Nigeria.

The specific objectives are:

- To investigate the prevalence of Norovirus infection among children less than the age of five in Ogun State using RT-nested PCR.
- To determine the associated risk factor of Norovirus infection among children less than the age of five in Ogun State using questionnaires.
- To determine the seasonal variation of Norovirus infection among children less than the age of five in Ogun State using questionnaires.

CHAPTER TWO

LITERATURE REVIEW

Norwalk agent was the first virus identified as causing gastroenteritis in humans. However, identification of its significance as a pathogen has been limited due to a lack of affordable, sensitive, and regular diagnostic tools (Glass *et al.,* 2009). Interesting developments in understanding the molecular biology of noroviruses, along with the use of innovative diagnostic tools, have drastically transformed our knowledge of their impact. Noroviruses are currently recognised as the major cause of gastroenteritis outbreaks and a significant source of sporadic gastroenteritis in both children and adults (Glass *et al.,* 2009). Even as norovirus gastroenteritis is normally moderate and short-lived, emerging research reveals that the sickness can be severe and occasionally deadly, particularly in susceptible groups such as small children and the elderly, and is a common cause of gastroenteritis hospitalisation (Glass *et al.,* 2009). Epidemiologic studies have revealed both fast local transmission and the creation of new norovirus strains that spread globally, comparable to the epochal patterns of influenza (Dolin, 2007). Therefore, controlling norovirus epidemics presents significant hurdles.

2.1 Classification and Strains of Norovirus

Norovirus is a minuscule, single-stranded, non-enveloped virus that needs particular cell systems to replicate in vitro (Straub *et al.,* 2007). The capsid is icosahedral in shape and is 20 to 40 nm (1 nm = 109 metres) in diameter (Britannica, 2022). There are 5 different genogroups of noroviruses (GI-GV), and only GI, GII, and GIV infect humans (Zheng *et al.,* 2006). Premised on sequence diversity, the norovirus genogroups are further classified into genotypes and variations (sub-genotypes) (Kroneman *et al.,* 2011). Genogroups have been categorised in various ways based on the three open reading frames (ORFs)' diverse amino acid composition, the RNA-dependent RNA polymerase (RdRp) and VP1 regions, or VP1 (Green *et al.,* 1994; Oliver *et al.,* 2003; Vinje *et al.,* 2003). The current classification into 5 genogroups was developed, on the grounds of the divergence of the VP1 protein. Either the RdRp sequence or the capsid sequence serves as the basis for genotype definition (Vinje *et al.,* 2000).

Since the mid-1990s, GII.4 strains have been the most common genotype circulating globally, accounting for roughly 50-70% of outbreaks (Cannon *et al.,* 2017). GII.4 variants have developed every 2 to 4 years since 2002, with noteworthy pandemic GII.4 variants appearing in Farmington Hills in 2002, Hunter in 2004, Den Haag in 2006b, New Orleans in 2009, and Sydney in 2012 (van Beek *et al.,* 2018). For several years, this pattern of GII.4 variant

emergence and epochal development has been seen, with the appearance of phenotypically unique variations, several of which have been linked to an increasing number of outbreaks. While GII.4 subtypes continue to predominate extensively, non-GII.4 viruses also play a major role in norovirus disease burden and propagation (Kwok *et al.,* 2017).

2.2 Genomic Structure of Norovirus

A single-stranded, positive-sense, polyadenylated RNA with three open reading frames (ORFs 1–3) and a length of about 7.5 kb make up the norovirus genome (Dinkle *et al.,* 1995). In the norovirus genome, six non-structural proteins including the RNA-dependent RNA polymerase (RdRp), is encoded by the ORF1 (approximately 5.5 kb) (Bull *et al.,* 2010). The primary capsid protein, VP1, which has two protruding (P) domains, P1 and P2, and a shell (S), is encoded by ORF2 (Prasad *et al.,* 1999). The P1 domain improves virus particle stability, while the S domain is in charge of VP1 assembly (Tan *et al.,* 2005). The P2 domain, the most visible outer layer of the virion (Prasad *et al.,* 1999), interacts with potential neutralising antibodies as well as histo-blood group antigens (HBGAs), which are the presumed initial binding site in human infection (Cao *et al.,* 2007; Donaldson *et al.,* 2008). The minor capsid protein, VP2, which is encoded by ORF3, is thought to contribute to VP1's increased synthesis and stability (Bertolotti-Ciarlet *et al.,* 2003).

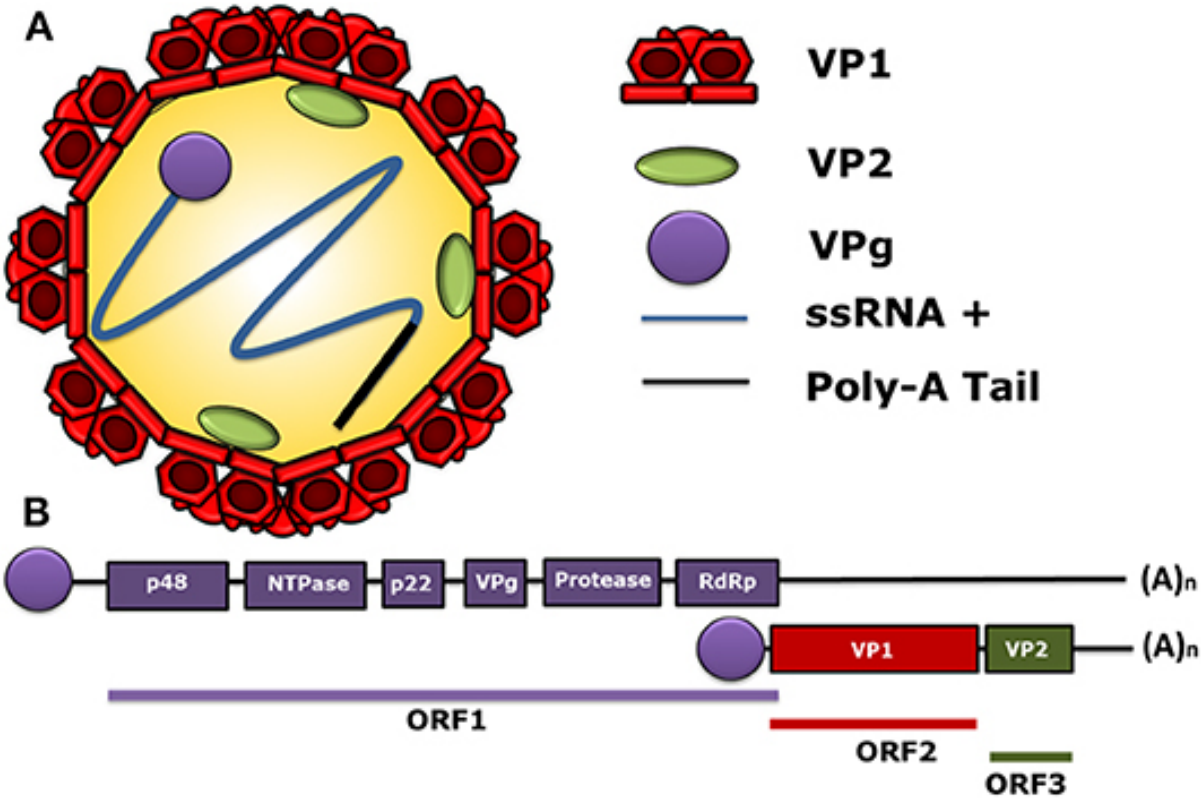

Figure 2.1: Human Norovirus Molecular Model (HuNov). **Source:** Campillay-Véliz *et al.,* (2020).

(A) A schematic illustration of a human norovirus viral particle displaying the 90-dimer protein surface of the capsid's VP1 structural protein. Within the viral capsid is the structural protein VP2 (1-8 proteins per virion). The non-structural VPg protein is shown covalently bound to the 5′ end of the RNA genome (ssRNA+) in a positive sense, as well as a poly-adenine tail at the 3′ ends (Jiang *et al.,* 1993; Glass *et al.,* 2000; Vongpunsawad *et al.,* 2013).

(B) A general depiction of the HuNov genetic structure. ORF1 is responsible for the production of non-structural proteins such as p48, NTPase, p22, VPg, Protease, and RdRp. The *ORF2* gene encodes the major structural protein VP1, while the *ORF3* gene encodes the minor structural protein VP2. Below the ORFs is the sub genomic RNA bound to VPg encoding VP1 and VP2. VPg is shown as a circle connected to genomic and sub genomic RNAs (Jiang *et al.,* 1993; Glass *et al.,* 2000; Vongpunsawad *et al.,* 2013).

Figure 2.2: Depiction of the Human Norovirus genomic structure. **Source:** Campillay-Véliz *et al.,* (2020).

2.3 Epidemiology of Norovirus

Norovirus (NoV) acute gastroenteritis (AGE) is most common in children under the age of five. The disease severity in this age group is predicted to be 21 400 (15 900-27 700) per 100 000 population or nearly 6.5 times the incidence in the ≤ 5-year-old population (Phillips *et al.,* 2010). This age cohort also has the highest rates of NoV-related outpatient visits, emergency department visits, and hospitalizations (Belliot *et al.,* 2014).

The universal frequency of norovirus among acute gastroenteritis cases is estimated to be 17% in hospitalised patients and 24% in the regular populace (Ahmed *et al.,* 2014). A World Health Organization-commissioned study estimated 685 million annual norovirus infections (95% confidence band 491 million-1.1 billion) and 212,000 annual norovirus deaths (95% confidence interval 161,000-278,000) (Pires *et al.,* 2015). The broad ambiguity intervals reflect current data gaps at the country level, particularly in low-income, high-mortality countries. Winter months account for more than half of all cases worldwide (Ahmed *et al.,* 2013). The start of the school year may be a factor in the seasonality of norovirus in high-income nations, with research suggesting that outbreaks in children begin with the school year and later expand to outbreaks in adults (Kraut *et al.,* 2017). According to a comprehensive review, GII.4 is the most prevalent genotype in endemic norovirus gastroenteritis in children, accounting for nearly two-thirds of cases (Hoa Tran *et al.,* 2013). Genotype II subtype 4 (GII.4) is especially noteworthy because it has been the principal cause of NoV-associated acute gastroenteritis in people since the mid-1990s (Shinohara and Kageyama, 2002). Following the evolution of molecular identification methodologies over the last century, it has become viable to further genotype circulating NoV and detect its genetic diversity in different nations and continents (Chen and Chiu, 2012).

NoVs have been extensively investigated in the developed world, but little research has been undertaken on the subject in developing countries such as Nigeria (Ayolabi *et al.,* 2010). NoV is also frequently discovered in diarrhoea-free children's faeces, making it difficult to definitively ascribe a portion of the diarrhoeal disease burden to NoV in low-income countries (Kotloff *et al.,* 2013; Lopman *et al.,* 2014). Eight unique NoV genotypes have been identified in Africa (GI.2, GI.4, GII.1, GII.4, GII.8, GII.14, GIIb/GII.2 and GIIb/GII.3). GII.4 emerged as the most common, accounting for 83 (64.8%) of the cases (Sdiri-Loulizi *et al.,* 2009). In Nigeria, GII is more prevalent (Ayolabi *et al.,* 2010). According to Ayolabi *et al*., (2010) the prevalence of Norovirus infection in Lagos, Nigeria is 37.3%.

2.4 Norovirus disease burden

The infection load is comparatively high in developing countries for a variety of reasons some of which includes potentially lower water, sanitation, and hygiene conditions, as well as a weaker immunological response to infection. Diarrheal illness is predicted to cause 1.45 million deaths and 89.5 million disability-adjusted life-years (DALYs) lost globally each year (Lozano *et al.,* 2012; Murray *et al.,* 2012).

In a major systematic analysis of 137 research, NoV was predicted to be responsible for 18% (95% CI 17-20%) of gastroenteritis cases worldwide (Lopman *et al.,* 2013). Norovirus was projected to cause 699 million illnesses [95% UI: 489-1,086 million] and 219,000 fatalities (95% UI: 171,000-277,000) worldwide each year (Bartsch *et al.,* 2016). The overall societal expenses of disease among children less than five years were roughly $39.8 billion (95% UI: $27.2-$58.1 billion), equating to $20.4 billion (95% UI: $16.9-$25.4 billion) for all other age demographics combined. Globally, viruses of the GII.4 genotype are the leading cause of norovirus disease (Siebenga *et al.,* 2009), include new variants that emerge every 2 years to 4 years (Zheng *et al.,* 2010; Vega *et al.,* 2014), and are associated with greater symptom severity and health care burden (Desai *et al.,* 2012).

2.5 Mode of Transmission

Norovirus is extremely contagious, with an infectious dosage of as low as 20 viral particles (Teunis *et al.,* 2008). Person-to-person transmission is the most common route, either directly through the faecal-oral route, by consumption of aerosolized vomitus, or indirectly through fomites or contaminated ambient surfaces (Hall *et al.,* 2009). Norovirus is also the primary known cause of both sporadic cases (Scallan *et al.,* 2011; Kirk *et al.,* 2015) and foodborne disease outbreaks, with contamination happening either via infected food handlers or directly from foods (Hall *et al.,* 2014). Leafy greens, fresh fruits, and seafood are frequently implicated in norovirus outbreaks, but any meal lightly cooked or mishandled after being cooked might be contaminated. Waterborne transmission is infrequent, nevertheless, it is probable if drinking or recreational water is not sanitized (Hall *et al.,* 2013).

2.6 Pathophysiology

Much of what is known about the pathophysiology of norovirus infections, as well as susceptibility and immunity to them, comes from data collected from over 1000 volunteers who took part in challenge trials (Glass *et al.,* 2009). Proximal jejunal biopsy specimens from

ailing volunteers revealed enlargement and blunting of the intestinal villi, crypt-cell hyperplasia, cytoplasmic vacuolization, and infiltration of polymorphonuclear and mononuclear cells into the lamina propria while the mucosa remained intact (Glass *et al.,* 2009). There are no histologic alterations in the stomach fundus, antrum, or colonic mucosa (Levy *et al.,* 1976), nor are they found in biopsy specimens acquired during the convalescent phase of the disease. Perhaps because distal involvement of the small intestine could not be explored, and the location of viral replication has not been established, the amount of participation of the small intestine remains uncertain (Glass *et al.,* 2009). Enzymatic activity (alkaline phosphatase, sucrase, and trehalase) near the small intestine brush boundary is reduced, causing moderate steatorrhea and transitory carbohydrate malabsorption (Agus *et al.,* 1973). Jejunal adenylate cyclase activity is not increased (Levy *et al.,* 1976), and these histologic alterations have been linked to increased gastric production of hydrochloric acid, pepsin, and intrinsic factor. Stomach emptying, on the other hand, is delayed (Meeroff *et al.,* 1980), and the decreased gastric motility may be responsible for the nausea and vomiting associated with this gastroenteritis. The reason of the sudden and frequently explosive sickness has yet to be completely addressed at the molecular level.

2.7 Replication Cycle

A proposed model of replication cycle of Human Norovirus in human enterocytes is sequentially explained below, accompanied with its diagrammatical representation in Figure 2.3:

(1) The replication process begins with the binding of the P2 region present in the P domain of VP1 to an unknown receptor and some host co-receptors such as HBGA (Lin *et al.,* 2014); (2, 3) following this union, there is an internalization of the virus in the cell and dissemble of the virus releasing the RNA in the cell cytoplasm (Daughenbaugh *et al.,* 2003); (4, 5) once in the cytoplasm, the covalently linked VPg protein at the 5′ end induces the binding (Hosmillo *et al.,* 2019); (6) alongside this, the Proprotein cleaves the polyprotein, yielding three precursors, but only the Pro-Pol precursor has enzymatic activity; these precursors are then cleaved, yielding the six separate proteins (Huang *et al.,* 2005); (7) (A) The P48 protein is transported to the reticulum and then to the Golgi (Ettayebi *et al.,* 2003). (B) The replication process will be aided by the NTPase protein (Pfister and Wimmer, 2001). (C) The P22 protein attaches to vesicles coated with COPII and aids in the breakdown of the Golgi, and this protein, together with the NTPase, promotes the cell's pro-apoptotic activity (Sharp *et al.,* 2010). (D)

The VPg protein is involved in both the recruitment of translation initiation factors and the replication process, as well as the creation of replication complexes (Daughenbaugh *et al.,* 2003). (E) Proprotein is capable of non-structural polyprotein cleavage (Cao *et al.,* 2007). (F) The Pol protein functions in viral replication, and its activity is considerably boosted by P48. The VP1 and VP2 proteins, on the other hand, are produced by a sub genomic RNA, after which the virions are assembled and the output proteins are produced (Subba-Reddy *et al.,* 2011).

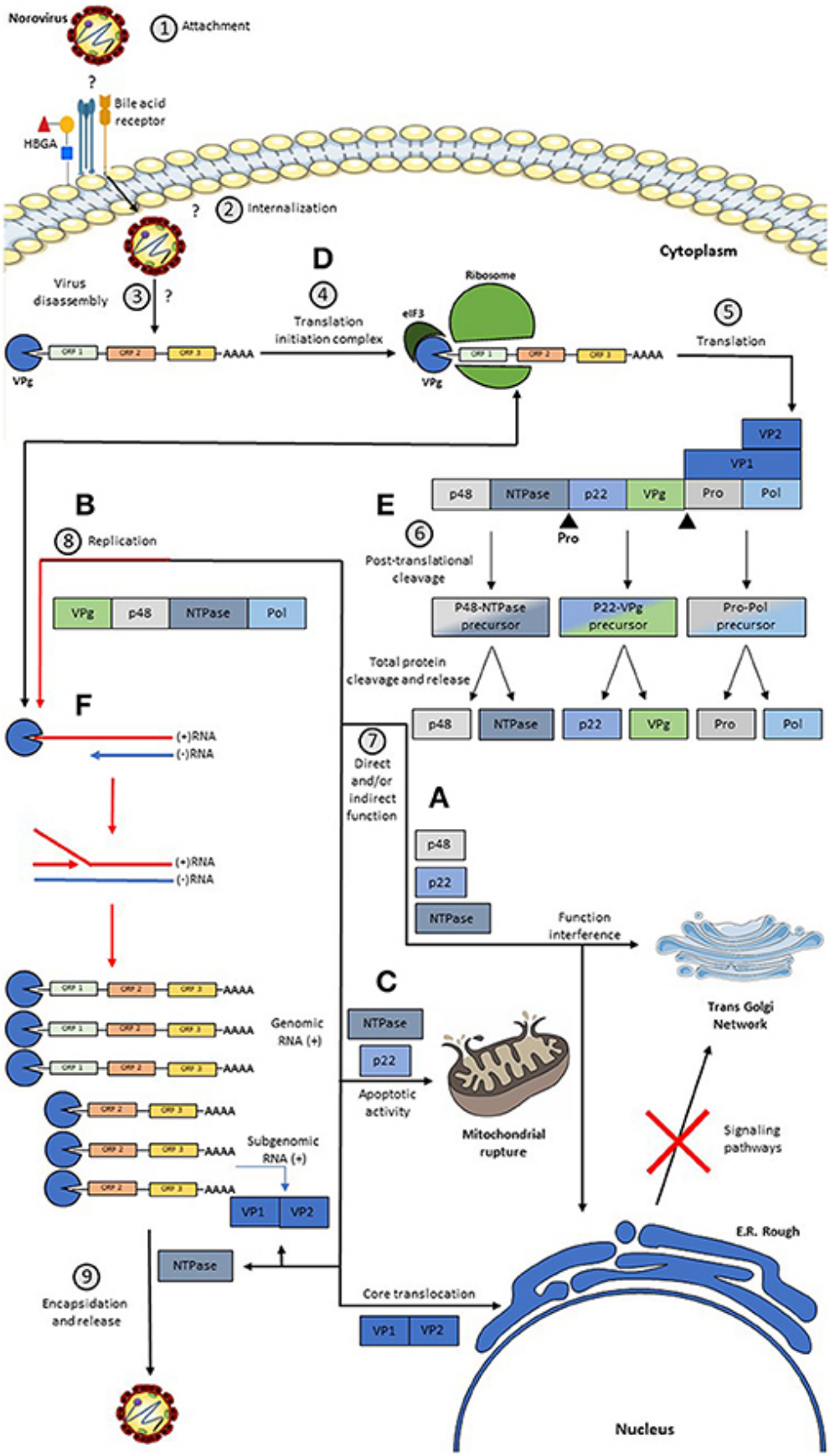

Figure 2.3: Model of a Replicative HuNov Cycle and Function of its Proteins. **Source:** Campillay-Véliz *et al.,* (2020).

2.8 Pathogenesis

The gestation period for NoV is estimated to be 1.2 days on average preceding exposure and the initiation of gastrointestinal symptoms (Lee *et al.,* 2013). Diarrhoea constitutes the most common symptom, occurring in around 90% of patients, with vomiting occurring in approximately 75% of cases (Rockx *et al.,* 2002). The onset can occur without a prodrome, culminating in public vomiting events, which may be an especially effective mode of transmission (Evans *et al.,* 2002; Thornley *et al.,* 2011). In the absence of diarrhoea, vomiting may ensue. Abdominal pains, fever, headache, chills, and myalgia are all possible symptoms. Clinical manifestations last 2-3 days, but may persist longer in little adolescents and the elderly infected in healthcare facility epidemics (Rockx *et al.,* 2002; Lopman *et al.,* 2004). The shedding of virus in faeces occurs before the onset of symptoms, invariably surges (at around; 10^{10}viral particles per gramme of stool) on day 4 after exposure, and can persist for several weeks in the general population or months in immunocompromised persons (Atmar *et al.,* 2008). There are insufficient quantitative data on severity (number of bouts of diarrhoea, vomiting, and dehydration) in adults, whereas NoV gastroenteritis is less severe in children than rotavirus gastroenteritis (Wikswo *et al.,* 2013).

2.9 Immune Response

Resistance to norovirus is an extensive research topic relevant to vaccination prospects. Accumulated immunity after infection is probable to be short-lived, with protection lasting weeks to two years in volunteer challenge studies (Parrino *et al.,* 1997), whereas modelling studies suggest protection lasting up to nine years (Simmons *et al.,* 2013). The identification of multiple norovirus infections in children monitored in prospective birth studies, with 25% to 40% of toddlers from conception to 3 years in various environments having at least two episodes of norovirus gastroenteritis, lends support to a limited duration of immunity in children (Mennon *et al.,* 2016). Although repetitive infections by other genotypes occur, immunity may be confined to the preliminary genotype when acquired (Malm *et al.,* 2014).

In addition to acquired immunity, wild-type mutations in the alpha (1,2) fucosyltransferase *(FUT2)* gene can confer innate immunity by controlling the expression of histo-blood group antigens on the gastrointestinal surface epithelium that bind to norovirus (Kambhampati *et al.,* 2016). These mutations vary by ethnicity and are found in 5% to 50% of the world's populations (Nordgren *et al.,* 2016).

2.10 Laboratory Diagnosis

Ever since the norovirus sequence was determined, a molecular-based reverse-transcriptase-polymerase chain reaction (RT-PCR) test became the gold standard for detecting norovirus in faeces, water, and food (Atmar and Estes, 2001). In immunoassays to detect antibody responses to infection, virus like particles were employed as antigen, and antibodies to virus like particles were created to detect norovirus antigen in faecal specimens (Trujillo *et al.,* 2006). To account for the wide range of strains, RT-PCR requires a cocktail of primers, while immunoassays require a collection of cross-reactive antibodies (Trujillo *et al.,* 2006). As diagnostic technologies advance, RT-PCR is being phased out in favour of real-time RT-PCR, which is more sensitive and efficient and, when combined with a Taqman probe, enables both confirmation and quantification in a single experiment (Koopmans, 2008).

2.11 Treatment

If the patient is aware and able to drink, the therapy for norovirus gastroenteritis is oral rehydration with fluids and electrolytes, or IV fluids if the vomiting and dehydration are severe. Adults can benefit from antimotility and antisecretory drugs to reduce diarrhoea in instances when a person's performance is crucial (Glass *et al.,* 2009). Although no antiviral medications have yet been discovered, the x-ray crystallographic structures of viral polymerase and proteases, as well as the binding location of histo-blood group antigens in particles, are known and might be used to build therapeutics (Glass *et al.,* 2009). Interferons and ribavirin efficiently reduce Norwalk virus replication in replicon-bearing cells (Chang and George, 2007), and their potential therapeutic utility should be investigated further. Parenteral or oral administration of hyperimmune human immune globulin has been proposed, however, this medication has never been evaluated in a clinical study.

2.12 Prevention and Control

The control of norovirus outbreaks has been particularly difficult because outbreaks that begin with a single common exposure to contaminated food or water can quickly spread through person-to-person contact (Glass *et al.,* 2009). Tracing and studying the epidemic necessitates distinguishing the very first instances from later cases, the mechanism of transmission of which may differ from the first (Yee *et al.,* 2007). Stopping an outbreak typically involves Herculean efforts to clean the environment aboard cruise ships, hospital wards, or catastrophe sites, and even then, epidemics usually end only after the vulnerable pool is depleted (Widdowson *et al.,*

2004). Knowledge of the epidemic's precise sequence can link patients to a common exposure — such as raw oysters or contaminated meals — and occasionally identify the relevant virus in the food (Widdowson *et al.,* 2004).

Sequencing a specific variable projecting the area of an epidemic strain has been effective in correlating strains to a single outbreak, tracking its development as the outbreak expands, and identifying individual strains linked with the extended transmission (Xerry *et al.,* 2008). Assays that have been utilized in particular outbreaks to identify noroviruses directly in contaminated food and water are currently being developed for routine food and water screening (Daniels *et al.,* 2000). Current control efforts, which are only marginally effective at best, are geared at reducing exposure to foods that have been contaminated at the source, either by environmental contamination (as has been the case with raspberries and oysters) or through contamination by food handlers (Baert *et al.,* 2009). Preventing the virus's secondary transmission through person-to-person contact and contaminated environmental surfaces is critical to halting the spread of epidemics like those seen in hospital wards and on cruise ships. Personal cleanliness, gastrointestinal precautions, and decontaminating ambient surfaces may all be beneficial (Glass *et al.,* 2009). A school-based research found that using alcohol-based hand sanitizers and disinfecting classroom surfaces daily with quaternary ammonium wipes decreased absenteeism for norovirus infection compared to control classes that employed standard hand-washing and cleaning methods (Sandora *et al.,*2008).

CHAPTER THREE

METHODOLOGY

3.1 Study Site and Target Demographic

Hospital-based case control research was undertaken for 1 year and 6 months from selected primary health care centres in Abeokuta Local Government Area and Obafemi Owode Local Government Area of Ogun State, Nigeria (6.9980°N 3.4737°E). The locations of the selected primary health care centres are shown on an Ogun state map (Fig 3.1). In the research region, the chosen primary health care centres serve roughly 90% of new-borns and children under the age of five. The children who visit these primary health care clinics are frequently treated for severe diarrhoea, which is why the research population was chosen during the course of the study. This study focused on places with a high population density and low middle-income earners (Fig 3.1).

3.2 Sample Collection

The samples were obtained from kids below the age of five who had diarrhoea. With the assistance of nursing staff, permission to conduct the study was obtained from the child's caregiver prior to sample collection. Stools were collected from children who had gastroenteritis and placed in a clean, aseptic universal bottle that had been prefilled with Cary Blair transport medium (Cary and Blair, 1964). The samples were labelled, packed, sealed, and sent to the laboratory for analysis within 24 hours.

3.3 Social Demographic

In a face-to-face interview with the patient's parents and guardians, a systematic (closed-ended questions) questionnaire was used to acquire a preliminary demographic profile on the patients, encompassing gender, age, medical history, occupation, housing, and sanitary supplies. The data collected was used to determine the risk factors that increase a child's probability of having diarrhoea.

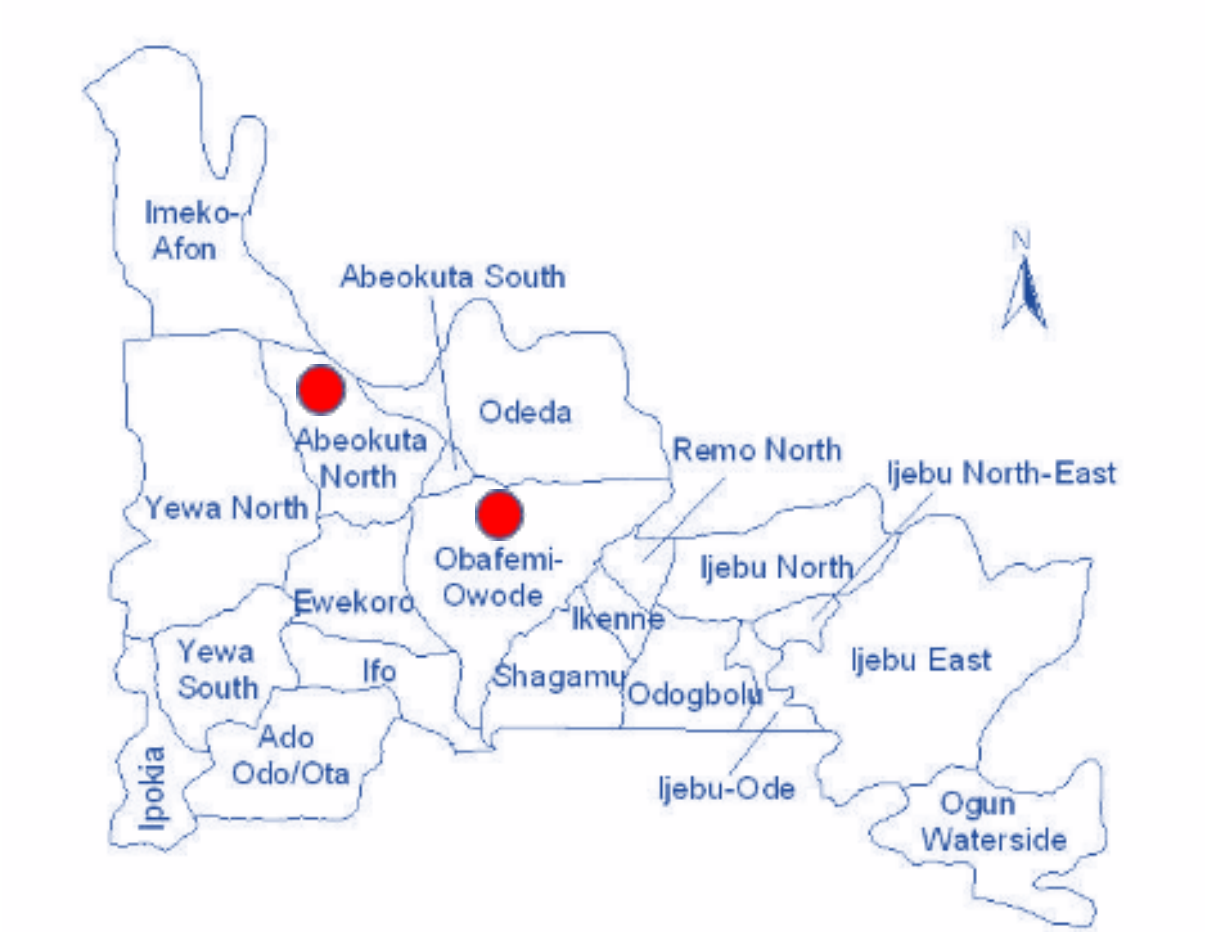

Figure 3.1: Map of the research region with red dots designating the sampling sites. **Source:** Google map (2022).

3.4 Stool Processing

Prior to the processing of the stool, safe lab practices were undertaken such as; wearing laboratory coats and gloves.

Normal saline solution for 100 samples was prepared according to manufacturer instruction using the saline tablets (Oxoid Limited, England)

Procedure

- Dissolve 1 tablet of normal saline in 500ml of distilled water.
- Stir using a hot plate stirrer, till the tablet dissolves.
- Label 100 pieces of 2.0ml Eppendorf tubes (1V-100V).
- Pipette 700 µl of normal saline into each Eppendorf tubes.
- Pipette 700 µl of stool sample into each Eppendorf tube containing the saline.

Purpose of the saline: Normal saline is an isotonic medium used to preserve the virus's composition as well as the cells found in stool.

3.5 Extraction of the RNA and Dilution of the Primer

(Using the QIAamp Viral RNA Mini Kit)

The genomic RNA of Norovirus was extracted using the QIAamp Viral RNA extraction method. The reagents were prepared according to the manufacturer's instructions in QIAamp Viral RNA Mini Kits (QIAGEN, GERMANY) (Appendix 1). Below are the procedures carried out during the extraction process:

Extraction Process

I. Using the 1000 µl micropipette, 1000 µl of AVL buffer was pipetted 56 times into a falcon tube to make a total of 56000 microliters of AVL buffer (QIAGEN, Germany) in falcon tube.

II. Using the 20-200 µl micropipette, 560 µl of the carrier RNA (cRNA) was dispensed into the AVL buffer and it was gently swirled 10 times rather than vortex to prevent foaming.

III. 100 Eppendorf tubes (Desco, India) were labelled and 560 µl of prepared AVL buffer solution was dispensed in each Eppendorf tube and 140 µl of the stool sample were dispensed into the Eppendorf tube,

IV. The solution was incubated at room temperature for 10 mins, pulse vortexed for 15 seconds using the vortex mixer (Scientific Industries, United States of America) and centrifuged for nine seconds.

V. 560 µl of absolute ethanol (96-100%) was added to the sample solution, pulse vortexed, and pulse centrifuged for 15 secs. 630 µl of the already prepared solution was dispensed into QIAamp mini columns (in a 2ml collection tube) (QIAGEN, Germany) carefully without wetting the rims and it was centrifuged at 6000 x g (8000rpm) for 1 min. Each QIAamp mini-column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded.

Note: To avoid cross-contamination during centrifugation, ensure that each spin column is closed.

VI. 500 µl of Buffer AW1 was added to each QIAamp mini-column and it was centrifuged at 6000 x g (8000rpm) for 1 min. Each QIAamp mini-column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded 500 µl of Buffer AW2 (QIAGEN, Germany) was added to each QIAamp Mini column and it was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3mins.

VII. Each QIAamp mini-column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded and it was centrifuged at full speed for 1 min. Each QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube and the previous collection tubes were discarded.

VIII. 60 µl of Buffer AVE was added to each QIAamp Mini column and it was incubated at room temperature for 1 min. After the incubation, it was centrifuged at 6000 x g (8000 rpm) for 1 min and it was stored at -30 °C.

3.7 Molecular Confirmation of Norovirus

The cDNA was first generated from the 100 extracted RNA templates according to the manufacturer's instruction using the Script cDNA synthesis KIT (Jena Bioscience, Germany). The reaction table for the cDNA synthesis can be found in Table 3.1 below and its calculations can be found in (Appendix 2)

Table 3.1: Reaction table of the 101 samples (1V-100V cDNA generation

Reagents	Initial Concentration	Final Concentration	Volume per Reaction V/R (µL)	No of samples (n=101)
Random hexamers	100µM	2.5 µM	0.5 µL	50.5 µL
Script RT	200 units/ µL	100 units/ µL	0.5 µL	50.5 µL
Script RT buffer	5X	1X	4 µL	404 µL
dNTP mix	10 µM	500 µM	1 µL	101 µL
RNase inhibitor	40 units/ µL	20 units/ µL	0.5 µL	50.5 µL
RNase free water			8.5 µL	858.5 µL
RNA template			5 µL	
Total			20 µL	

3.8 Protocol for Generating the cDNA for Samples (1v-100v).

I. Firstly, 100 PCR strip tubes were labelled, with one extra tube serving as a positive control.

II. Pipetting 858.5µL of RNase-free water into a sterile Eppendorf tube (labelled RT PCR master mix for easy identification), 404 µL of script Reverse Transcriptase buffer (Script RT PCR buffer), 101 µL of dNTP mix (deoxynucleotide triphosphate), 50.5 µL of RNase inhibitor, and 50.5 µL of random hexamers into the Eppendorf tube completed the one-step RT PCR mixture.

III. For 8 seconds, the solution was pulse centrifuged.

IV. Pipette 15 µL of the prepared Master Mix Cocktail (MMC) into the PCR strip tubes that have already been labelled.

V. 5 µL of the RNA template was pipetted into the prepared MMC-containing PCR strip tubes (1-30).

After that, the samples were placed in the thermal cycler under the following cycling conditions:

- 42ºC for 10minutes
- 50ºC for 30-60 minutes (50mins)
- 70ºC for 10 minutes (to inactivate the RT enzyme)
- 10ºC for ∞ (final extension)

Primer Dilution

The Norovirus genes were genetically typed using Nested reverse transcriptase polymerase chain reaction (RT-Nested-PCR) as described by Oh *et al.* (2003). The primers and their sequence can be found below in Table 3.2. Their working solution was prepared according to the manufacturer's instruction, Ligo (Macrogen, South Korea) (Appendix 3).

Table 3.2: Primer sequences used in the Nested PCR assay and their required base pairs products (Oh *et al.,* 2003).

Primer code	Sequence (5'-3')	Base pair size
NV 32	ATGAATATGAATGAAGATGG	4226-4246
NV 32A	ATGAACACAATAGARGATGG	4226-4246
NV 36	ATTGGTCCTTCTGTTTTGTC	4688-4707
NV 33	TACCACTATGATGCAGATTA	4280-4299
NV 33A	TATCACTATGATGCTGACTA	4280-4299
NV 35	TATCACTATGATGCTGACTA	4598-4617
NV 35A	ACAATYTCATCATCICCAT	4593-4611

The working solution was prepared as follows:

I. The primers were diluted by pipetting 80 µL of PCR grade water into 7 sterile Eppendorf tubes that were labelled with each primer's code.
II. 20 µL of each primer were pipetted into each Eppendorf tube. Vortex and centrifuge for a few seconds.

1ST and 2ND round RT-PCR reaction procedures

First round RT Nested PCR procedure for the 100 samples

- Firstly, 1030.2 µL of PCR grade water (QIAGEN, Germany) was pipetted into a new Eppendorf tube labelled master mix (MM), followed by 404µL of master mix, and 60.6 µL of each primer was added into the Eppendorf tube.
- After that, the Master Mix Cocktail (MMC) was pulse vortexed and centrifuged. After labelling 30 PCR strip tubes (1v-30v), 16µL of the MMC was pipetted into each of the 100 PCR strip tubes.
- Afterwards, 4µL of the cDNA was pipetted into each PCR strip tube and placed into a MiniAmp thermocycler (Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany).
- The thermocycler was set at the following cycling conditions:

  - 95ºC for 2 minutes (Initial Denaturation)
  - 94ºC for 30 seconds
  - 42ºC for 30 seconds — **35 cycles**
  - 72ºC for 45 seconds
  - 72ºC for 3 minutes (Final elongation)
  - 10ºC for ∞
  - Storage at -4ºC

The reaction table for the first round can be found in Table 3.3 below.

Second round RT Nested PCR procedure for the positive samples

- 69.6 µL of PCR grade water (QIAGEN, Germany) was pipetted into a new Eppendorf tube labelled master mix (MM), followed by 16µL of master mix, and 3.6 µL of each primer was added into the Eppendorf tube.

- After that, the Master Mix Cocktail (MMC) was pulse vortexed and centrifuged. After labelling 4 PCR strip tubes (18V, 19V, 21V, 29V, 68V & 79V), 18µL of the MMC was pipetted into each PCR strip tube.
- Afterwards, 2µL of each amplicon was pipetted into each PCR strip tube and placed into a MiniAmp thermocycler (Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany). The thermocycler was set at the following cycling conditions:

  94ºC for 2 minutes (Initial Denaturation)

  94ºC for 30 seconds
  42ºC for 30 seconds
  72ºC for 45 seconds
  35 cycles

  72ºC for 3 minutes (Final elongation)

  10ºC for ∞

  Storage at -4ºC

- The reaction table for this setup can be found in Table 3.4 below.

3.9 First Round Reverse Transcriptase (Nested)-Polymerase Chain Reaction (RT Nested PCR) for the 100 Samples' Generated cDNA.

Table 3.3: NoV 1st round reaction table

The primers used in the first round of PCR were NV 32, NV 32A and NV 36' (Oh *et al.,* 2003).

Reagents	**Initial concentration**	**Final concentration**	**Volume per reaction (V/R)**	**Number of samples n = 101**
Master mix	5X	1X	4 µL	404 µL
NV32	20 µM	0.6 µM	0.6 µL	60.6 µL
NV32A	20 µM	0.6 µM	0.6 µL	60.6 µL
NV 36	20 µM	0.6 µM	0.6 µL	60.6 µL
dH20			10.2 µL	1030.2 µL
cDNA			4 µL	
Total reaction volume			20 µL	

*The calculation for this reaction table can be found in Appendix 4.

Table 3.4: NoV 2nd round reaction table for the positive samples from the 1st round (Using RT-nested-PCR).

The primers used in the second round of PCR were NV 33, NV 33A, NV 35 and NV 35A (Oh *et al.,* 2003)

Reagents	**Initial concentration**	**Final concentration**	**Volume per reaction (V/R)**	**Number of samples n = 6**
Master mix	5X	1X	4 µL	24 µL
NV33	20 µM	0.6 µM	0.6 µL	3.6 µL
NV33a	20 µM	0.6 µM	0.6 µL	3.6 µL
NV35	20 µM	0.6 µM	0.6 µL	3.6 µL
NV35a	20 µM	0.6 µM	0.6 µL	3.6 µL
dH20			11.6 µL	69.6 µL
Amplicon from 1st round			2.0 µL	
Total reaction vol.			20 µL	

*The calculation for this reaction table can be found in Appendix 5.

3.10 Agarose Gel Electrophoresis

Following each PCR round, agarose gel electrophoresis was carried out using 1.8% agarose gel stained with ethidium bromide and viewed using the UV-transilluminator. The agarose gel was prepared according to the manufacturer's instruction, Multi-Purpose Agarose (Cleaver Scientific, United Kingdom). The calculations can be found in Appendix 6.

Procedure

I. In a conical flask, 45mls of TBE were measured using a 100ml measuring cylinder and dispensed then 0.81g of agarose powder was added.
II. It was microwaved several times to prevent solidification before being dispensed into a falcon tube containing 4 µL of ethidium bromide and swirled to mix before being dispensed into the tank and allowed to solidify. The combs were placed into the tank before pouring the gel in order to create the wells.
III. The box has one end connected to a positive electrode and the other end connected to a negative electrode. Tris Borate EDTA (TBE) buffer solution was poured into the tank with the already solidified gel, with one end of the gel in the well facing the negative electrode and the other facing the positive electrode.
IV. Each PCR reaction was transferred into a separate well, with one well reserved for a DNA ladder (a standard reference that contains DNA fragments of known lengths).
V. The gel box's power was turned on, and the current began to flow through the gel. Norovirus is an RNA virus, and because RNA molecules have a negative charge due to the phosphate groups in their sugar-phosphate backbone, they will be drawn to the gel's positively charged end. The gel ran for 30 minutes at 90 volts before being viewed with the UV transilluminator from the Gel Documentation system.

CHAPTER FOUR

RESULTS AND DISCUSSION

Out of the 100 samples, 59% were female out of which only 2 were positive for Norovirus whereas 4% were positive out of 41% of the male gender present. **Appendix** 9 shows the positive samples and their respective codes.

4.1 Results

Amplification of 1st Round PCR

For the first-round polymerase chain reaction using primers NV32, NV32a and NV36, a total of 100 amplicons were electrophoresed on 1.8% agarose gel stained with 4 µl ethidium bromide at 90 Volts. Among the 100 samples only 6% were positive for the norovirus-specific gene with the required band size of 481 base pairs. A representative of this result can be seen in **Plate** 4.1. This result confirmed the existence of Norovirus among the 100 stool samples of children with gastroenteritis. This gave the basis for the second-round reaction. In **Plate** 4.1 Samples A, B and K can be seen to be positive showing the expected 481 base pair.

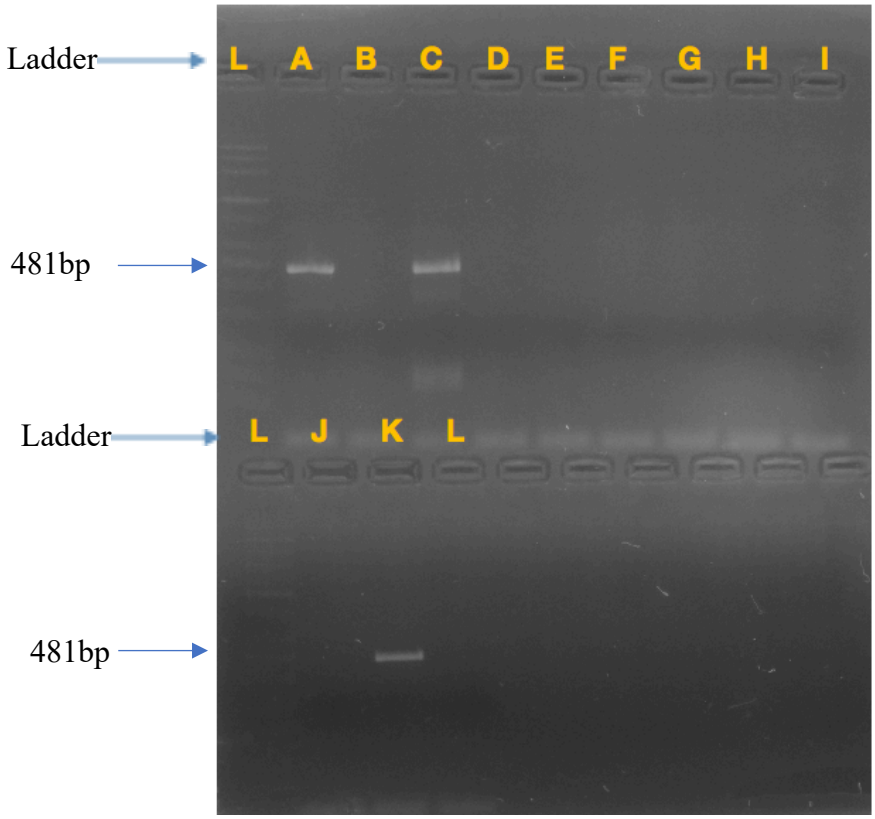

Plate 4.1: A representative gel image showing the 1st round of the Reverse transcriptase-polymerase chain reaction where the first two wells represent the DNA ladder (L) and letter A-L represents the samples.

Amplification of the Nested (2nd Round) PCR

For the second round, Nested PCR was used to amplify the positive samples from the first round. The primers used to amplify the samples were NV 33, NV 33A, NV 35 and NV35a accordingly. The required band size for the second reaction was 331 base pair. **Plate** 4.2 shows a pictural representative of the amplified samples with the expressed 331 base pair.

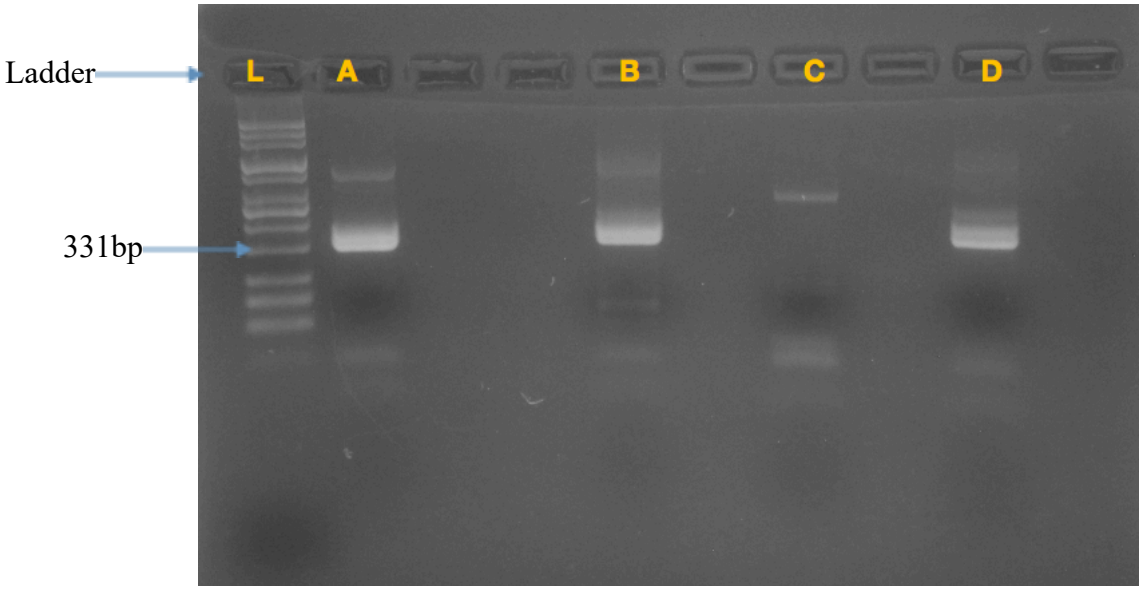

Plate 4.2: Image representation of Positive NoV samples identified by RT-Nested-PCR. Amplicons with band size of 331bp were positive for Norovirus.

Statistical Analysis of Associated Risk Factors

Table 4.1 shows that amongst 41 male gender only 4 presented with Norovirus, meanwhile among 59 females only 2 presented with Norovirus. This correlates to Norovirus being more prominent in male than in female. Furthermore, this analysis shows that children less than the age of 2 had the virus which is relevant because Norovirus is known to be predominant in children less than the age of five. In **Table 4.2**, the relevance of fever in Norovirus-infected infants was well described as fever was seen to be more predominant than in those without Norovirus. There is an equivalence in patients showing symptoms of vomiting which may not add to the effect of the virus in the patients whereas a total of 18 patients exhibited nausea as one of the effects of Norovirus. Less patients had abdominal pain but the odd part is those without Norovirus had abdominal pain more than those with the virus, this could be due to other causative agents of gastroenteritis present in their system. Out of 50 presenting with NoV five had a loss of appetite which could be linked to the effect of the viral agent in their body making them vomit every food they eat and also making them nauseated which may occur during the Norovirus infection cycle. All of the patients infected with NoV had water closet has their toilet type which might seem safe but also has its risks such as the toilet handle whereby a child may use the toilet without washing their hands and another child comes in contact with that handle and touches it as well causing cross faecal-oral contamination. Whereas there was an equal number of those who drink from sachet water and wells or borehole which could be a possible pathway for NoV to enter the patient due to the unhygienic position of some wells and boreholes as well as sachet water factories. Some boreholes or wells are dug close to the house's excreta waste holes which could then leak into their drinking water thereby making it easy for the child to get the viral infection. Most of the caregivers are office workers which may have significance in this analysis as most office workers give less attention to their children thereby leaving them to wander on their own to places, they could get the infection unanimously. This can be seen in **Table 4.3.**

Table 4.1: The relationship between Norovirus infection and adverse outcomes in children under the age of five in Ogun State and their respective gender (P value of < 0.05 was statistically significant).

Variable	Total no.	No. with NoV	OR (95% CI)	P value
Gender				
Female	59	2	1	
Male	41	4	3.1 (0.5-17.7)	0.37
Age (year)				
< 2	67	4	1	
≥2	33	2	1.0 (0.2-5.9)	1.00

The statistical analysis shows that out of 100 samples tested, male were recorded the most positive compared to the female. This also shows that children less than 2 years of age are most likely to be infected with Norovirus induced diarrhea.

Table 4.2: The relationship between Norovirus infection and adverse outcomes in children under the age of five in Ogun State.

Variable	Total no.	No. with NoV	OR (95% CI)	P value
Fever				
Absent	33	2	1	
Present	67	4	0.98(0.2-5.7)	0.99
Vomiting				
No	62	3	1	
Yes	38	3	1.7 (0.3-8.8)	0.53
Loss of Appetite				
Yes	50	4	1	
No	50	2	0.5 (0.8-2.7)	0.40
Nausea				
No	82	1	1	
Yes	18	5	31.2 (3.4-288.4)	0.00
Abdominal pain				
Absent	78	5	1	
Present	22	1	0.70 (0.7-6.3)	0.75
ORS before Hospital presentation				
No	47	3	1	
Yes	53	3	0.9 (0.2-4.6)	0.87
Site of enrolment				
School/day care	77	3	1	
Others	23	3	3.7 (0.7-19.8)	0.11
Street food consumption				
Absent	83	6		
Present	17	0	0.9 (0.9-1.0)	0.25

Table 4.3: The relationship between Norovirus infection and adverse outcomes in children under the age of five in Ogun State.

Variable	Total no.	No. with NoV	OR (95% CI)	P value
Type of toilet used				
Water closet	84	6	1	
Pit Latrine	6	0	1.0 (0.0-0.0)	
Potty	10	0	1.0 (0.0-0.0)	0.4
Source of drinking water				
Bottled water	20	0	1	
Sachet water	34	3	0.7 (0.3-7.3)	
Wells/borehole	46	3	0.7 (0.3-7.3)	
Streams	0	0		0.4
Occupation of caregiver				
Office worker	51	5	1	
Merchant/trader	19	1	1.0 (0.0-0.0)	
Artisan/craftsman	22	0	1.0 (0.0-0.0)	0.5

Seasonal Variation of Norovirus in Ogun State

Figure 4.1 shows the viral infection peaked during October also in-between November and January 2020, but then also surged again in-between April and June 2021, then peaked up during March again. In this study, Norovirus surged during the dry and wet seasons but emerged more during the dry seasons.

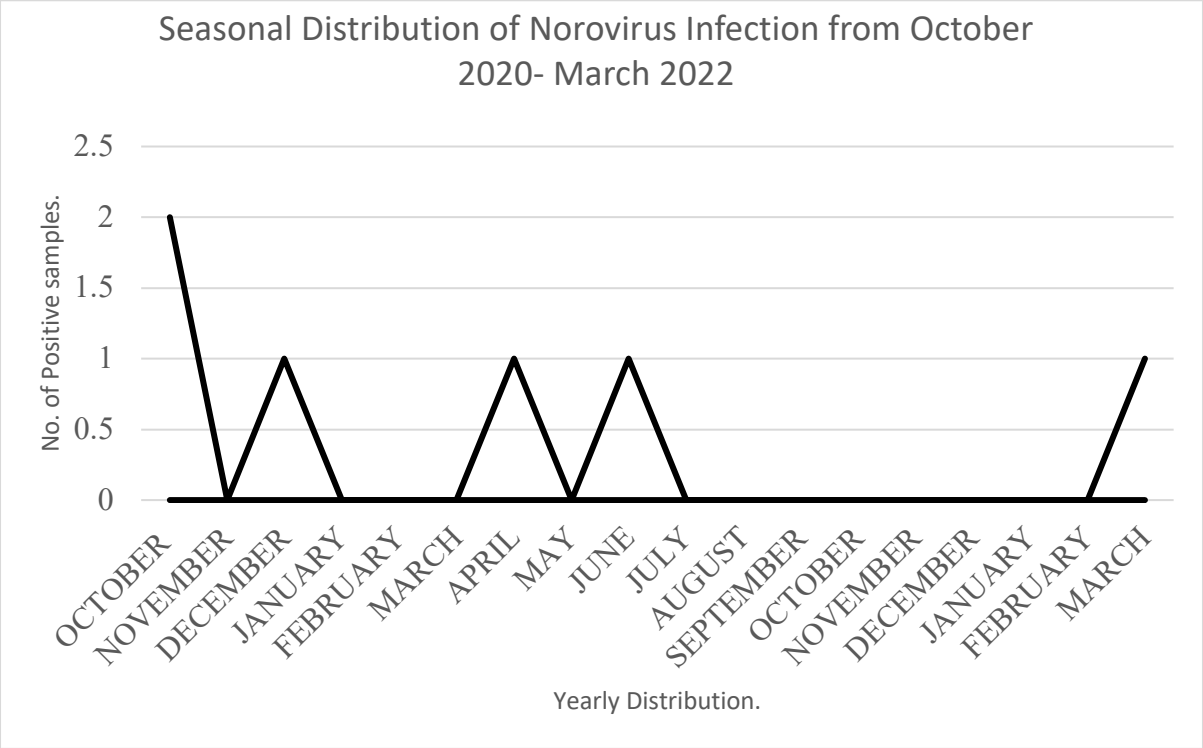

Figure 4.1: A graphical representation of the seasonal variation of Norovirus infection from October 2020 to March 2022.

4.2 Discussion

Gastroenteritis is amongst the most common childhood illnesses, and it can be caused by a variety of infectious agents, including bacteria, parasites, and viruses, the aetiology of which differs between developing and developed nations. Noroviruses have been shown to be an important cause of sporadic gastroenteritis in the developed world (Kirkwood *et al.,* 2005), but there are little or no epidemiological data on the disease burden associated with sporadic norovirus infection in clinical or hospitalised patients living in developing countries (Borges *et al.,* 2006; Gallimore *et al.,* 2004; Victoria *et al.,* 2007), particularly in Africa (Armah *et al.,* 2006). In the current study, an overall prevalence of 6% Norovirus infection was observed among infants in Ogun State, Nigeria. The prevalence observed in this study is somewhat similar (6.7%) to that reported in North-East, Nigeria (Oyinloye *et al.,* 2016) but less than the 15.3 % reported in the South-South geopolitical zone of Nigeria (Osazuwa *et al.,* 2019) and the 37.3% reported among children in Lagos State, Nigeria (Ayolabi *et al.,* 2010).

Compared to the findings from other African countries, the prevalence was less than the reported prevalence of 12.6% in other countries within Sub- Saharan Africa (Munjita, 2015), and the reported 11.3 % and 11.8% in community-based studies in Malawi (Trainor *et al.,* 2013) and Tanzania (Elfving *et al.,* 2014) respectively. The discrepancy observed in the prevalence could be accounted for by the lower sample size for this study. However, this shows that Norovirus is still part of the aetiology of gastroenteritis in the state. This observation suggests that Norovirus infection can be considered a significant cause of diarrhoea in Ogun State. The variation in NoV infection between this research and others might be attributed to changes in food and water sources, sterilisation techniques, climatic conditions, and environmental/household features (Munjita, 2015).

The seasonal pattern for Norovirus in this study could not be established. However other report from Nigeria show that norovirus peaks during the dry season (Ayolabi *et al.,* 2010) in contrast to Ghana where it peaks in both the dry and rainy seasons (Armah *et al.,* 2003).

In this study, the statistical analysis of the risk factors reveals that amongst the 100 samples, 2 out of 59 females were positive for Norovirus, while 4 out of 41 males were positive. Furthermore, the age demographics of children exhibiting norovirus were considerable, with shedding occurring primarily in infants and toddlers and the majority of infections occurring in children aged 6 to 12 months ($P < 0.05$). This is not unexpected given that this is the age at which children are often weaned from maternal antibodies and introduced to semisolid meals that may be contaminated owing to inappropriate handling. Similarly, it is the time when they

crawl around, putting whatever they see into their mouth. This conclusion is congruent with findings in Ghana (Armah *et al.,* 2006) and Bangladesh (Black *et al.,* 1982), where the infection was shown to be prevalent in children under the age of two. However, the specimen collection period was a comprehensive year, to make a conclusive remark on the seasonal fluctuation, norovirus was observed to emerge all through the research period and to surge between February and April. Although the incidence of norovirus infections in Ogun State is around 6%, the role of noroviruses in acute and chronic diarrhoea in this part of Nigeria remains unknown.

The presence of norovirus in a stool sample from a kid suffering from gastroenteritis does not always indicate that this is the source of the illness; it could be combined infections with several other bacterial and viral agents (Munjita, 2015). As accurate as this may sound, several of the research included in this review did not look into the presence of noroviruses as single or mixed viral infections in norovirus-confirmed diarrhoea specimens (Munjita, 2015). Due to urbanisation and work laws, parents and guardians who work in offices often seek out daycare centres for their children/wards. Thus, poor hand hygiene (Lyman *et al.,* 2009) and close contact with sick people at these Facilities must have contributed to the considerable NoV infection seen in this study (Heijne *et al.,* 2009; Moore *et al.,* 2015). This observation is consistent with the findings of Osazuwa *et al*. (2019) investigation (Osazuwa *et al.,*2019).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The occurrence of norovirus infection in hospitalised children under the age of five with gastroenteritis in Ogun State is 6%. The study validated noroviruses' importance as a primary cause of acute watery diarrhoea and fatal illness in children leading to their hospitalization. Also, this study shows that the cause of the viral infection could be contaminated water consumption as none of them that presented with Norovirus infection consumed food gotten from the streets according to the survey and analysis.

5.2 Recommendation

Considering norovirus epidemiology changes fast, establishing continuous surveillance within sentinel sites across the state will improve monitoring of circulating norovirus strains and offer a continual awareness of the state of norovirus infection in our settings. This will enable the public to better assist public health intervention measures against norovirus-associated gastroenteritis in children. Investigations that improve our awareness of norovirus evolution and adaptation to immunological stimuli are also important for future vaccine efficacy studies. Caregivers and parents should consequently implement adequate environmental cleaning and hand washing measures, particularly for young children after faeces. Government and non-profit groups should also invest in the establishment of WASH facilities in the Ogun State regions. The rapid development of the norovirus vaccine is also needed as it could help eradicate the virus in society quicker thereby reducing its prevalence, burden and morbidity rate. Early screening and management of breakouts in hospitals, professional education through the implementation of virus-specific hazard analysis and key control point procedures in the food and catering sectors and also water sectors, and the protection of at-risk communities with low-cost vaccinations are all options for reducing the NoV burden.

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APPENDICES

Appendix 1

Materials and Equipment Used

- Measuring cylinder (100ml),
- Jena bioscience kit,
- Micropipette (10µl,100µl,1000µl),
- Pipette tips (10µl,100µl,1000µl),
- Vortex mixer,
- Centrifuge,
- Eppendorf tubes (2.0ml),
- Spin column (2.0ml),
- Agarose tank,
- Conical flask,
- Measuring scale,
- Falcon tube,
- Powdered gloves,
- Nitrite gloves,
- Thermocycler,
- Hot plate stirrer,
- Microwave,
- PCR strip tubes.

Appendix 2

Reagents Used

- Buffer AVL (Viral lysis buffer),
- Buffer AVE,
- Buffer AW1 (Wash buffer 1),
- Buffer AW2 (Wash buffer 2),
- AVL cRNA (Carrier RNA),
- Normal saline (0.85% isotonic saline in tablet form)
- Random Hexamers,
- Script RT Buffer,
- RNase Inhibitor,
- Script Reverse Transcriptase,

- RNase-free water,
- RNA template
- Master mix,
- PCR grade water,
- Primers (NV 32, NV 32a, NV33, NV33a, NV35, NV 35a)
- Ethidium Bromide,
- Agarose gel powder,
- TBE Buffer (1X, 1.5X),
- Mid-Range DNA Ladder.

Appendix 3

Reagent Calculation for Sample Preparation

n x 0.56ml = **y** ml

y ml x 10 µl/ml =**z** µl

} Determination of Lysis Buffer AV1. Each sample requires 0.56ml of Buffer AVL.

where: **n**= the number of samples to be processed simultaneously

y= calculated volume of Buffer AVL

z= volume of carrier RNA-Buffer AVE to add to Buffer AVL

100 samples x 0.56ml of buffer AVL = 56ml (buffer AVL)

56 ml was converted to microliter i.e., 56 x 1000= 56000 microliters of buffer AVL

56 ml x 10 microliter per ml= 560 microliter(µl) (carrier RNA)

Therefore 560 µl of carrier RNA was added to 56000 µl of buffer AVL

Appendix 4

Preparation of The Working Solution of Each Reagent

Each reagent appears as a stock solution; therefore, they are to be diluted to their required working solution by the use of the general equation:

$$C_1V_1=C_2V_2$$

Denotation;

C_1 – Initial concentration

V_1 – Initial Volume

C_2 – Final concentration

V_2 – Final Volume

To get the total volume per reaction (V/R) for the random hexamers,

$$C_1V_1=C_2V_2$$

Where, C_1 = 100 µM C_2 = 2.5 µM V_1 =? V_2 = 20 µL

$$100 \text{ x } V_1 = 2.5 \text{ X } 20$$

∴ Total volume per Reaction (V/R); V_1 = 50/100 = 0.5 µL

To get the total volume per reaction for RNase-free water;

Total volume of the whole reaction – (Addition of the total volume per reaction for all the reagents) i.e., 20 – (0.5 + 0.5 +4 + 1 + 0.5 +5) = 20 – 11.5 = 8.5 µL

N.B.: The total volume of the mix to be pipetted into each of the 30 Eppendorf tubes is gotten by subtracting the volume of the RNA template from the total volume of the reaction.

i.e., 20 µL - 5 µL = 15 µL

Appendix 5

Preparation of Working Solution for NoV Primers

Primers come lyophilized in their respective kits and need to be centrifuged to bring them down as they may be stuck to the lid which could lead to accidental loss of the primer if not centrifuged. They are also to be diluted from the stock solution to the working solution.

Micro molar (µM) concentration of primers = 100 µM

Working Solution = 20 µM

Using $C_1V_1=C_2V_2$,

Where; C_1 = 100 (Micro molar (µM) concentration of primers)

V_1 =?

C_2 = 20 (Working Solution)

V_2 = 100

$$100 \text{ x } V_1 = 20 \text{ x } 100$$

V_1= 2000/100 = 20 µL of primer + 80 µL of dH_2O (PCR grade water)

Appendix 6

Calculations for the 1st Round RT-PCR Reaction

To get the total volume per reaction of the Master mix,

$$C_1V_1=C_2V_2$$

Where; $C_1 = 5$, $V_1 =?$ $C_2 = 1$, $V_2 = 20$,

$$5 \times V_1 = 1 \times 20$$

$$V_1 = 20/5 = 4\ \mu L$$

To get the total volume per reaction of the primers **NV32, NV32a** and **NV36**.

$$C_1V_1=C_2V_2$$

Where; $C_1 = 20$, $V_1 =?$ $C_2 = 0.6$ $V_2 = 20$

$$20 \times V_1 = 0.6 \times 20$$

$$V_1 = 12/20 = 0.6\ \mu L$$

This volume is the same for all the primers because they all have the same final and initial concentration.

To get the total volume of dH_2O,

The total volume of the whole reaction – (Addition of the total volume per reaction for all the reagents)

i.e., 20 – (4 + 0.6 +0.6 +0.6 +4) = 20 – 9.8 = 10.2 µL

Appendix 7

Calculations for the 2nd Round RT-PCR Reaction

To get the total volume per reaction of the Master mix,

$$C_1V_1=C_2V_2$$

Where; $C_1 = 5$, $V_1 =?$ $C_2 = 1$, $V_2 = 20$,

$$5 \times V_1 = 1 \times 20$$

$$V_1 = 20/5 = 4\ \mu L$$

To get the total volume per reaction of the primers **NV33, NV33a, NV35** and **NV35a.**

$$C_1V_1=C_2V_2$$

Where; $C_1 = 20$, $V_1 = ?$ $C_2 = 0.6$, $V_2 = 20$,

$$20 \times V_1 = 0.6 \times 20$$

$$V_1 = 12/20 = 0.6\ \mu L$$

$dH_2O = 20 - (4 + 0.6 + 0.6 + 0.6 + 0.6 + 2) = 11.6\ \mu L$

Appendix 8

Agarose Gel Calculation

1.8% of agarose gel was prepared

Total volume of gel slabs = 45mls

Therefore $\frac{1.8}{100} \times \frac{45}{1} = 0.81g$

Appendix 9

Positive Samples Among Children in Ogun State

SAMPLES NUMBER	SAMPLES CODE
18V	FMC 004
19V	FMC 005
21V	OF 3
29V	SH 38
68V	SH 262
79V	SH 243

*Table representation of the six positive samples and their codes.