MOLECULAR DETECTION OF HEPATITIS A VIRUS (HAV) IN CHILDREN PRESENTING WITH DIARRHOEA IN OGUN STATE

BY

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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 FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY

SEPTEMBER, 2022

DECLARATION

I hereby declare that this project report has been written by me and is a record of my own research work. It has not been presented in any previous application for a higher degree of this or any other University. All citations and sources of information are clearly acknowledged by means of reference.

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UNACHUKWU, ADAOBI EMMANUELLA

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Date

CERTIFICATION

This is to certify that this research project entitled **'Molecular Detection of Hepatitis A Virus (HAV) in Children Presenting with Diarrhea in Ogun State'** was prepared and submitted by UNACHUKWU ADAOBI EMMANUELLA in partial fulfillment of the requirements for the degree of **BACHELOR OF SCIENCE IN MICROBIOLOGY.** The original research work was carried out by her under my supervision and is hereby accepted.

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Head of Department

DEDICATION

This report is dedicate to God, my lovely and ever supporting family.

ACKNOWLEDGEMENT

Firstly, all thanks to the good God almighty for the wisdom, knowledge and understanding He blessed me with to carry out this project. I would also like to extend my earnest gratitude to my family for all the support they have given to me.

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ABBREVIATIONS

HAV	Hepatitis A Virus
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
-ssRNA	Negative Single Stranded RNA
+ssRNA	Positive Single Stranded RNA
ELISA	Enzyme Linked Immunosorbent Assay
ICA	Immunochromatographic Assay
IgM	Immunoglobulin M
IgG	Immunoglobulin G
Anti HAV IgM	IgM (immunoglobulin M) antibodies.
Anti HAV IgG	IgG (immunoglobulin G) antibodies
PCR	Polymerase Chain Reaction
cDNA	Complementary DNA
ALT	Alanine transaminase
AST	Amino transferase
HBV	Hepatitis B Virus
Script RT	Script Reverse Transcriptase
dNTP	Deoxy nucleoside Triphosphate
RNase	Ribonuclease
UV	Ultra Violet
EDTA	Ethylene diamine tetra acetic acid
Bps	Bits per seconds
µM	Micro molar
µL	Micro Liter

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ABSTRACT

Hepatitis is a viral infection that causes the inflammation of the liver. Hepatitis A is one of the viral types of hepatitis common world wide with approximately 1.5 million cases annually. It is caused by the hepatitis A Virus (HAV) and can be transmitted via faecal oral route. Symptoms of hepatitis A virus infection include: Jaundice, dark colored urine, fever, malaise, diarrhea and loss of appetite. HAV is presumed to be prevalent in developing countries where there is an unavailability of safe drinking water, bad hygiene and sanitary practices. Information on the infection of HAV in Nigerian children is limited. The aim of this hospital based cross sectional study is to know the prevalence of HAV infection in children in Ogun state, Nigeria by detecting the presence of hepatitis A virus in the stool samples of children presenting with diarrhoea using molecular techniques. A hospital based cross sectional study was carried out in primary health care centres in Abeokuta Local Government Area and Owode Local Government Area of Ogun State**.** Consents were obtained from the child's caregiver, with the help of the nursing staff and a total of one hundred (100) stool samples were collected from children presenting with diarrhea aged 5 years and below. Stool samples were analysed for the presence of hepatitis A virus using molecular techniques. The viral RNA was extracted from the samples, the cDNA was generated and was screened for the presence of Hepatitis A Virus (HAV) using 2-step RT-PCR and results were obtained after agarose gel electrophoresis. Absence of bands showed that no subject tested positive for hepatitis A virus (HAV) infection. In conclusion this study showed that HAV was not prevalent in the study population and this led to the conclusion that HAV was not endemic in the area.

Keywords: Hepatitis A virus (HAV), PCR, Prevalence, Gel electrophoresis, Result

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Hepatitis A Virus (HAV) infection is an acute type of hepatitis which is also vaccine preventable and is caused by hepatitis A virus (HAV). HAV was first isolated in 1973 by Stephen Mark Feinstone, Albert Kapikian, and Robert Purcell (Feinstone *et al.,* 2018). HAV is a member of the genus *Hepatovirus* that is found within the family *Picornaviridae* and is the causative agent of HAV infection (Dotzauer, 2008). The endemicity of HAV infection is highly dependent on hygiene and sanitation practices (Anita *et al.,* 2019). Yearly, about 1.5million clinical cases of HAV infections are reported worldwide (Elisabetta *et al.,* 2012). Many young children that have been infected with HAV are asymptomatic unlike adults and older children who show symptoms (symptomatic) such as jaundice (Jacobsen, 2014). HAV infection is found to be common among developing countries with poor sanitary practices and it is endemic in areas like Africa, Asia, South and Central America, the Middle East and Western pacific (Monique *et al.,* 2021). Although Africa is included as one of the regions with high endemicity of HAV, there has been limited information on the infection of HAV in Africa (Franco *et al.,* 2012).

The main mode of transmission of Hepatitis A virus is through faecal-oral route but other modes have been identified which include parenteral mode and sexual intercourses (anal sexual intercourse) that is common among men who have sex with men (MSM) (Anita *et al.,* 2019). The most reported risk factors associated with HAV include: intravenous drug use, house hold contact with HAV infected individuals, sexual contacts with HAV infected persons and individuals going to and fro countries with high endemicity of HAV infection (Foster *et al.,* 2019). All age groups are susceptible to HAV infection with young children being asymptomatic because of absence of jaundice in their symptoms so they are not diagnosed with HAV infection (Jacobsen, 2009). Approximately 80-95% of children less than 5 years have asymptomatic infection while for adults just 10-25% sometimes appear to be asymptomatic (Hollinger *et al.,* 1996). For those who experience symptoms of HAV infection (mostly adults), the symptoms include jaundice, malaise, nausea, dark urine and anorexia (Monique *et al.,* 2021). HAV infection rarely result in complications but when it does it causes fulminant hepatitis A which only affects less than 1% of patients and is characterized by worse jaundice and encephalopathy (Stephanie *et al.,* 2006).

Hepatitis A virus can be detected in stool, blood and serum. Current infection can be diagnosed in serum by detecting the presence of anti-HAV IgM antibodies which can be seen after infection and remain present for 6 months, while past infection is diagnosed by detecting the presence of anti-HAV IgG antibodies (Jacobsen, 2009). HAV infection can be prevented by vaccination and sanitation, it cannot be treated but can be managed by supportive care (Penina *et al.,* 2021).

1.2 Statement of problem

HAV infection is common mostly in developing countries due to factors like limited access to clean water, bad hygiene and sanitary practices. The prevalence of HAV in Nigeria, a developing country is high especially among children (Ikobah et al., 2015) but there has been no evidentiary support to prove this. Since there has been no documented report of HAV in Ogun State, this study would help estimate the prevalence of HAV in Ogun state, a south western region of Nigeria, and would help suggest measures to reduce the prevalence of the virus infection.

1.3 Aim and objectives of the study

The aim of this study is to estimate the prevalence of HAV among children in Ogun state, Nigeria. The objectives of the study include:

- To determine the epidemiology of HAV among children in Ogun state using reverse-transcriptase nested polymerase chain reaction, Nigeria.
- To determine the risk factors associated with HAV infection in Ogun state.

1.4 Significance of the study

In this study of using molecular methods to detect HAV in children presenting with diarrhea in Ogun State, the results obtained would help the community to be aware of the prevalence of the virus in the community and help them follow the suggested measures provided in order to help reduce the prevalence of the virus infection.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Hepatitis

Hepatitis is an inflammation of the liver that is mostly caused by viral infections (World Health Organization, 2013). There are five (5) viral forms of hepatitis caused by five (5) distinct hepatitis virus, which include hepatitis A, B, C, D, and E viruses (Charles, 2022). Hepatitis is not only caused by viral infections it can also result from non-infectious etiologies such as drugs and toxic agents. The viral forms of hepatitis can be distinguished based on their transmission mode, diagnosis, treatment and prevention. Hepatitis A can be spread via fecal oral route and is best treated with vaccination; hepatitis B spreads through contact with blood, semen, bodily fluids of an infected person and the most common mode of transmission is through sexual intercourse (Patricia, 2021), Hepatitis C and D also spread through contact with infected body fluids, and Hepatitis A and E have the same mode of transmission of fecal-oral transmission (Gotlieb *et al.,* 2019). Hepatitis A and E are vaccine preventable.

2.2 Hepatitis A Virology

Hepatitis A Virus is a positive single stranded RNA (+ssRNA) virus, it lacks a lipid envelope. It is a member of the Picornaviridae family having its RNA genome packed inside an icosahedral protein capsule containing 60 copies of three (3) major proteins – VP1, VP2 and VP3, also known as 1D, 1B, and 1C respectively (Dotzauer, 2008). HAV was formerly classified under the genus enteroviruses but is currently classified under the genus hepatoviruses chiefly because of its distinguishing features when compared to other enteroviruses. These features include: It has no intestinal replication phase, slower replication in cell culture, and resists heat inactivation more (Richerd *et al.,* 1997). HAV can also resist extraction by non-ionic detergents chloroform or ether. The structure of HAV virions are particles with spherical shape and have a diameter of 27-32 nm that contains a capsid that encapsidates an RNA genome (Bondarenko *et al.,* 2013).

There are two (2) types of HAV infectious particles, the quasi enveloped virion and the naked virion. The quasi enveloped virus helps to facilitate cell-to-cell interaction within the liver, while the naked virions which are shed in faeces are optimized for environmental transmission (Rivera-Serrano *et al.,* 2019).

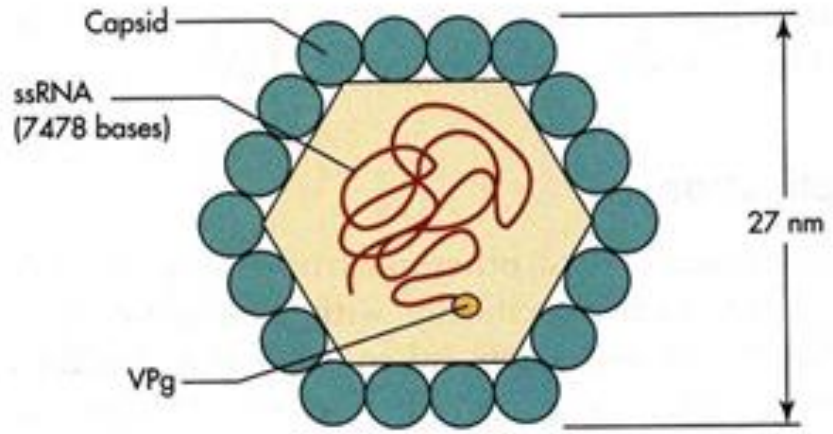

Figure 2.1: A schematic diagram of Hepatitis A Virus (HAV) (Anup, 2020).

2.2.1 Hepatitis A Virus (HAV) genomic structure

The length of the genome is approximately 7500 nucleotides and it contains an Open Reading Fragment (ORF) that encodes a polyprotein in which major proteins represent 'amino-terminal third' (P1 segment) while the other polyprotein comprises the nonstructural proteins required for the replication of HAV RNA are – 2B, 2C, 3A, 3B (also known as VPg and is covalently linked to 5'end of the genomic RNA and is the protein primer for the RNA synthesis), $3C^{pro}$ (Cysteine protease for post-translational cleavage within polyprotein) and $3D^{pol}$ (viral RNA dependent and RNA polymerase) (Stanley *et al.,* 2006; McKnight and Lemon, 2018). The role of the 2B and 2C protein are not known but some reports show that for 2B it helps to stabilize membrane and 2C may have a helicase activity due to the encoding of NTP-binding motif, and the 3A protein (preVpg) anchors 3B protein to HAV-RNA (Yokosuka, 2000). The HAV genome has a positive polarity which means that the viral RNA can serve as messenger RNA directly (Hussain *et al.,* 2011). HAV proteins are produced by translation of the single Open Reading Frame (ORF) under the direction of the internal Ribosome Entry Site (McKnight *et al.,* 2018). The 5' noncoding region (has signals important for the recognition and binding to host ribosomes) follows the single ORF which is then followed by 3' noncoding region. The ORF encodes polyprotein containing 2,227 amino acids (Cohen 1989). The open Reading Fragment (ORF) can be divided into 3 regions namely: P1 region that encodes the capsid poly peptide VP1- VP3 and VP4, P2 and P3 regions region that encode the non-structural proteins that are very important in the RNA replication (Almajhdi *et al.,* 2011). Any of the two methionine codons could be the start site for translation.

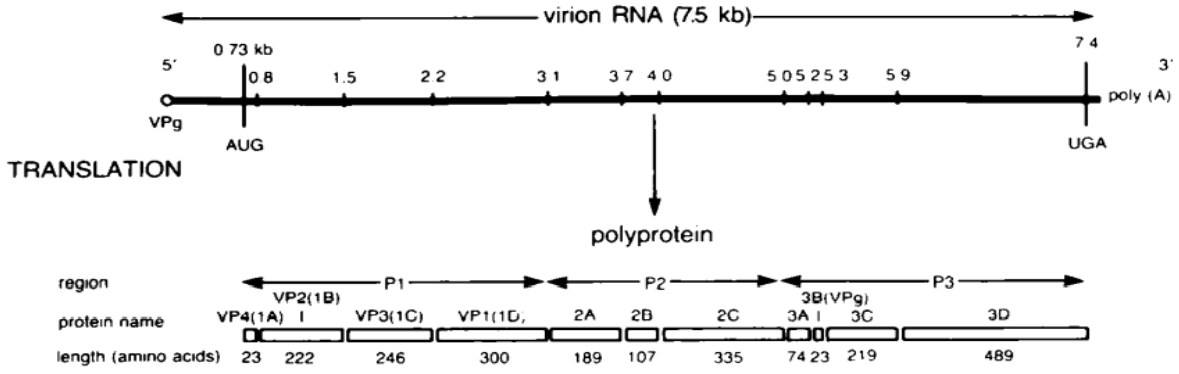

Figure 2.2: Organization of Hepatitis A viral genome (Cohen, 1989)

2.2.2 Replication of Hepatitis A Virus (HAV)

Replication of hepatitis A virus takes place in the cytoplasm of the infected cells (Yokosuka, 2000). The mechanism of entry of the two HAV virus particles (naked virion and quasi enveloped virion) are different but will later get to share the same steps in the entry process such as the interaction between the capsid and a specific cell receptor which is important for entry and uncoating for the other picornaviruses and also important in delivering RNA genome across endosomal membranes (McKnight and Lemon, 2018). Members of the Picornaviridae are known to have no envelope so they are released non-lytically from an infected cell and are found enclosed in small extracellular vesicles (EVs) (Shirasaki *et al.,* 2022). The naked virus particles are shed in the feces of an infected individual while the quasi enveloped virus particles are found circulating in the blood of an infected person (Rivera-Serrano *et al.*, 2019). HAV infection occurs as a result of ingesting the naked, non-enveloped virions.

The first step in the replication of HAV is the entry of the virus into the hepatocyte and the attachment of itself to the host cell receptor. This is then followed by the uncoating of the viral particle and the release of the positive sense RNA genome into the cell (Martin and Lemon, 2006). Then the host's ribosome will bind to the viral RNA forming polysomes. This is then followed by the translation of HAV RNA to produce a polyprotein which is then cleaved to also yield nonstructural proteins and the capsid proteins (Cohen, 1989). The IRES (Internal Ribosome Entry Site) mediates translation of viral polyprotein (Nakamura *et al.,* 2015). Polymerase and protease are some of the non-structural proteins produced (Gauss-Müller and Kusov, 2002). The viral RNA polymerase then copies +ssRNA genome to produce a replica of both +ssRNA and –ssRNA. The negative stranded RNA will be used as a template to produce additional positive stranded RNA. The next step in the replication involves the assembly of the Sixty (60) copies of the viral capsid proteins to form a protein shell that envelopes the positive stranded RNA. HAV is then assembled into the cytoplasmic membrane with the formation of viral particles inside the membrane bound vesicles. HAV will then be released from the vesicles when the particles come in contact with the bile acids in the canaliculi. After this the virus (HAV) can now be observed in the stool. The replication of HAV is slower than other picornaviruses (McKnight and Lemon, 2018).

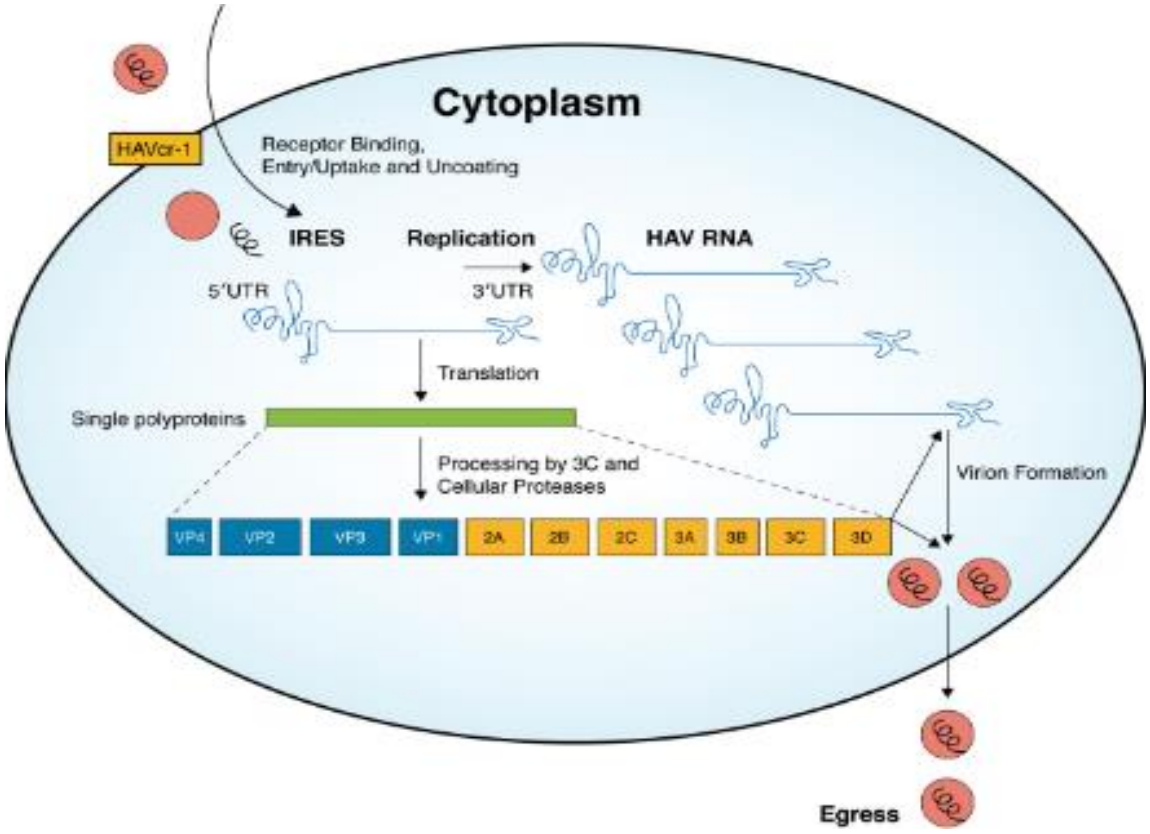

Figure 2.3: Replication cycle of Hepatitis A Virus (HAV) (Kanda *et al.*, 2015)

2.3 Hepatitis A Virus (HAV) Epidemiology

It was estimated by World Health Organization that hepatitis A occurs among 1.5 million people worldwide with the incidence rate being related mostly to socioeconomic indicators and access to safe water (Elisabetta *et al.,* 2012). Hepatitis A Virus is mostly transmitted through faecal oral route and could occur either result in sporadic or epidemic diseases (Lai and Chopra, 2019).

HAV is highly endemic among children resulting in an asymptomatic infection. Most people become infected with HAV by eating food and drinking water contaminated with HAV (Jefferies *et al.,* 2018). This is the reason why HAV infection rate is strongly linked to availability of safe drinking water and socioeconomic status. In high income regions less than 50% of the population are affected by HAV while in low income global regions more than 90% of the population are infected with HAV (Rashid *et al.,* 2018). HAV infection is also common in both developing countries and developed countries. For the case of developed countries, HAV outbreaks have been linked to consumption of contaminated food such as raw shellfish. An example is the outbreak of hepatitis A in Shanghai (1988) as a result of consumption of raw clams and it affected 292, 301 individuals (Halliday *et al.,* 1991). HAV is highly endemic in Africa, Asia, Central and South America. The risk factors associated with the spread of HAV infection in these areas include overcrowding, poor sanitation and hygienic conditions and limited access to clean water.

Large outbreaks of HAV in the US were common in 1950 but collection of data on hepatitis A cases started in 1966. The year 1971 recorded the highest number of HAV infection cases (approximately 60,000 cases) (Francisco and Noele, 2018). The studies carried out between 1990 and 1991 in various Latin American countries like in the central and Caribbean areas showed that seroprevalence rate was very high (Laura *et al.,* 2012). A study was carried out on 12,000 subjects in six Latin American countries (Dominican Republic, Mexico, Brazil, Chile, Venezuela and Argentina), Dominican Republic had the highest rate of seroprevalence (89%), and Brazil (81%), while lower rates were recorded in other regions of Brazil, Chile, Venezuela and Argentina with 64.7%, 58.1%, 55.7% and 55% respectively (Cavalcanti *et al.,* 1999).

HAV is highly endemic in Africa but information on the infection is limited. In the 1990s almost all children at the age of 12 years in South Africa were already ant-HAV positive, and before the age of 20 years, 100% of the black adults already had HAV antibody and 30-40% of the white adults were anti-HAV positive at the age of 20 years (Debora *et al.,* 2012). Recently in a report

presented by WHO, the trend of HAV seroprevalence in North Africa and Middle East Africa were similar, 100% seroprevalence was detected in Yemen in 1980 and the prevalence of anti-HAV was 28% in adults in Kuwait. The highest prevalence was shown to be among people with uneducated parents which shows the link between HAV infection and low social background (WHO, 2010).

In Asia, Hepatitis A and B are highly prevalent with Hong Kong and Urban China being the areas of intermediate endemicity, recording 30-40 cases per 100,000 population yearly (Sung, 2000). There has been a great reduction in the cases of HAV infection since the invention and license of HAV vaccines in 1995 (Penina *et al.,* 2021).

2.4 Pathogenesis of Hepatitis A Virus (HAV)

The replication of HAV occurs in the liver and it is acquired through the ingestion of food and water contaminated with the virus through faecal-oral transmission (Noele *et al.,* 2021). The quasi enveloped form of HAV can be detected in the serum and blood while the naked HAV is shed through the faeces via intestinal tract while it maintains its infectivity (Feng *et al.,* 2013). Increase in faecal shedding of the virus and viremia follow hepatocellular injury (manifested by elevated liver enzymes in serum; ALT Alanine transaminase) in acute infection of hepatitis A (Shin *et al.,* 2016a). The viral shedding in the faeces will continue for like 2-3 weeks after the initial increase in serum ALT level (Martin and Lemon, 2006). The concentration of the HAV in stool will reduce after the onset of jaundice and by the third week the excretion of virus in faeces would stop. For children, they excrete virus for a longer period of time than adults (Michael and João, 2020). HAV infection most times leads to liver injury in adults and asymptomatic infection in children (Shin and Jeong, 2018). Then Anti-HAV antibodies will first appear in serum as immunoglobulin IgM and later as IgG, virus-specific T-cell responses will then correspond with the elevation of serum ALT levels. Liver injury is not as a result of the cytopathic effect of HAV but it is caused by immune mediated mechanisms which involves innate and adaptive immune responses to HAV (Shin *et al.,* 2016a).

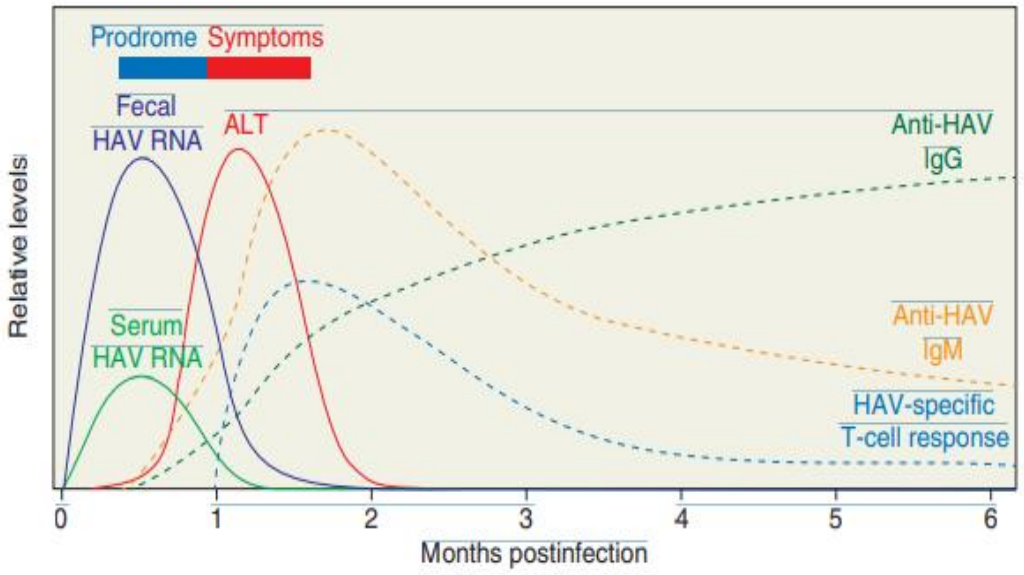

Figure 2.4: Pathogenesis of Hepatitis A Virus (HAV) (Shin and Jeong, 2018)

2.4.1 Clinical Manifestation of Hepatitis A Virus (HAV)

The clinical manifestation of HAV ranges from asymptomatic to ALF (Acute Liver Failure). The incubation period of HAV ranges from 15 – 50 days (Monique *et al.,* 2021). Development of symptoms is associated with the patient's age. HAV infection is asymptomatic in children but symptomatic in adults where it continues for 2-8 weeks (Armstrong and Bell, 2002). The symptoms of HAV infection often begins with fever, malaise, nausea or vomiting, abdominal discomfort, dark urine, jaundice and less frequently, pruritus, diarrhea, arthralgia, or skin rash may develop. During physical examination, hepatomegaly and jaundice are most times detected (Koff, 1992; Khan *et al.,* 2012).

2.5 Transmission of Hepatitis A Virus (HAV)

The main mode of HAV transmission is via faecal-oral route but can also be divided into direct transmission and indirect transmission (Acheson and Fiore, 2004). HAV transmission often occurs in areas with limited access to clean water and bad sanitary/hygiene practices. Apart from the faecal oral route of transmission, HAV can also be transmitted through sexual intercourse and rarely through parenteral mode (Aggarwal and Goel, 2015). The direct transmission involves eating food prepared by an infected person who did not properly wash his/her hands after using the bathroom, anal sexual intercourse with an infected person and not even washing hands properly after changing diaper (Michael and João, 2020). Indirect transmission is through the ingestion of food and drinking of water contaminated with the virus with seafood representing one of the major source and others involve frozen foods (Migueres *et al.,* 2021). The parenteral mode of transmission is very rare and it can occur during the manifestation of the virus during the viremia phase, during blood transfusion (Bower *et al.,* 2000).

2.6 Laboratory Diagnosis of Hepatitis A Virus (HAV)

The main standard for the diagnosis of HAV is by the detection of anti-hepatitis A virus (HAV) IgM antibodies but other methods can be used in the detection of HAV virus and they include: biochemical diagnosis, serological diagnosis (ELISA- Enzyme Linked Immunosorbent Assay), molecular diagnosis, and the use of rapid test kit (De Paula, 2012).

2.6.1 Serological Diagnosis

The serological methods used to diagnose HAV infection include the use of Enzyme Linked Immunosorbent Assay (ELISA) and the use of Immunochromatographic Assay (ICA) (De Paula, 2012). ELISA is used to detect the presence of anti-HAV IgM antibodies in the early infection phase and from the onset of symptoms while the anti-HAV IgG are detected by anti-HAV immunoassay that will detect the IgG and IgM at the same time (Desbois *et al.*, 2010). The anti-HAV IgG lasts long and provides immunity against reinfection (Dussaix *et al.*, 2010). The Immunochromatographic assay is also applicable in the detection of the HAV antibodies but only one Immunochromatographic assay (ICA rapid test) is available (Lee *et al.*, 2010). The detection of HAV antibodies is easier, simpler and even less expensive when compared to other techniques like the detection in stool (Quirós-Tejeira *et al.*, 2016).

2.6.2 Molecular Diagnosis

The molecular method used for the diagnosis of HAV infection involves amplifying the HAV RNA by reverse transcriptase which is then followed by the PCR of the cDNA and this method can be used for the detection of HAV in feces, saliva, serum and plasma (De Paula, 2012). The first step in RT-PCR is the purification of the viral RNA by RNA extraction protocol, which is then followed by the two-step RT-qPCR that starts with cDNA synthesis using random hexamers, the PCR of the cDNA (Nainan *et al.*, 2006). Agarose gel electrophoresis is then carried out for the separation, identification and purification of DNA fragments, RNA fragments and other macromolecules (Syaifudin, 2021).

2.6.3 Biochemical Diagnosis

The biochemical diagnosis of HAV infection involves the biochemical testing of the liver function. It includes measuring the serum total bilirubin, ALT (alanine transaminase), AST (Aspartate aminotransferase) and alkaline phosphate (Cacciatore, 1965).

2.7 Prevention and Treatment of Hepatitis A Virus Infection (HAV)

HAV infection does not require any specific treatment only supportive care and any patient that ends up developing fulminant hepatic failure should be given intense supportive therapy and be put up for liver transplantation (Webb *et al.,* 2020). HAV infection can be prevented by various means but the best way is through vaccination. The other means of preventing HAV infection includes good hygiene practices which involve washing of hand frequently, also avoiding uncooked food, raw shell fish, drinking and usage of clean water in carrying out activities and safe disposal of sewage (Communicable Diseases Network Australia, 2009).

2.7.1 Hepatitis A Vaccination

Hepatitis A vaccines were developed in the year 1992 but not until the year 1995 were they licenced for use by USA and Europe (Monique *et al.,* 2021). These vaccines were produced by cell culture-adapted virus which was propagated in human fibroblasts, purified from cell lysates then inactivated with formalin and adsorbed to aluminium hydroxide adjuvant (Penina *et al.,* 2021). There are two types of HAV vaccines, the single dose hepatitis A vaccine (Havrix and Vaqta) and the combined vaccine that contains both HAV and HBV antigens (Twinrix) administered intra muscularly (Bhandari *et al.,* 2021). Both Havrix and Vaqta are administered in a 2 dose schedule for children aged 6months – 4 or 5 years and the interval for the first and second those is 6-18 months (Zhang, 2020). Twinrix rapid schedule is in a 3 dose series (0, 7, 21-30 days) to adults aged 19 years or older and the booster dose given 12 months after the first administered dose (Brim *et al.,* 2007). Immunization against HAV would be beneficial to the following risk groups: international travellers, health care workers, food handlers, sewage workers, clients and employees of child care centres, institutionalized persons, illicit drug users, and male homosexuals.

(Eng *et al.,* 1993). Vaccinations against HAV have not been added to the Expanded Programme on Immunisation (EPI) in Africa (Kanyenda *et al.,* 2015).

Table 2.1: List of HAV Vaccines available (Anita *et al.*, 2019)

Vaccine	**Virus strain**	**Route of administration**	**Adjuvant**	**HAV antigen dose/ injection**		**Manufacturer**
				Paediatric	**Adult**	
HAVRIX	HM-175	Intramuscularly	Aluminium hydroxide	720 ELU	1440 ELU	GlaxoSmithKline
VAQTA	CR-326	Intramuscularly	Aluminium hydroxide	25U	50U	Merck, Sharpe and Dohme
TWINRIX	HM-175	Intramuscularly	HM-175	-	1ml (720 ELU HAV+ 20µg HBsAg)	GlaxoSmithKline
AVAXIM	GBM	Intramuscularly	Aluminium hydroxide	80U	160U	Aventis Pasteur
HEALIVE	TZ84	Intramuscularly	Aluminium hydroxide	250U	500U	Sinovac Biotech Co LTd

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study site and target population

Different primary and tertiary health care centers located in Abeokuta Local Government Area and Obafemi Owode Local Government Area of Ogun State, Nigeria (6.9980°N 3.4737°E) were selected as the sampling locations for this study. The locations of the selected primary health care centers are indicated on the map of Ogun state (Fig 3.0). The selected primary health care centers serve approximately 90% infants and children under the age of 5 in the study area.

3.2 Sample collection

Prior to sample collection, ethical approval was obtained for the study. Informed consent was also obtained from the parents of the children before sampling. With the help of the nursing staff at the health care facility, stool samples were collected with the help of a sterile plastic loop and placed in a universal stool collection sampling bottle pre-filled with Cary Blair transport medium (Cary and Blair, 1964). Samples were labelled, packed and sealed and sent to the laboratory within 24 hours for analysis.

3.3 Socio-Demographic

A Face to face interview was done with the patient's parents or guardians using a structured (closed-ended questions) questionnaire to establish the baseline demographic characteristics which included gender, age, medical history, occupation, housing and sanitary wares. The data collected were used as identifiers of associated risk factors that predispose children to diarrhea.

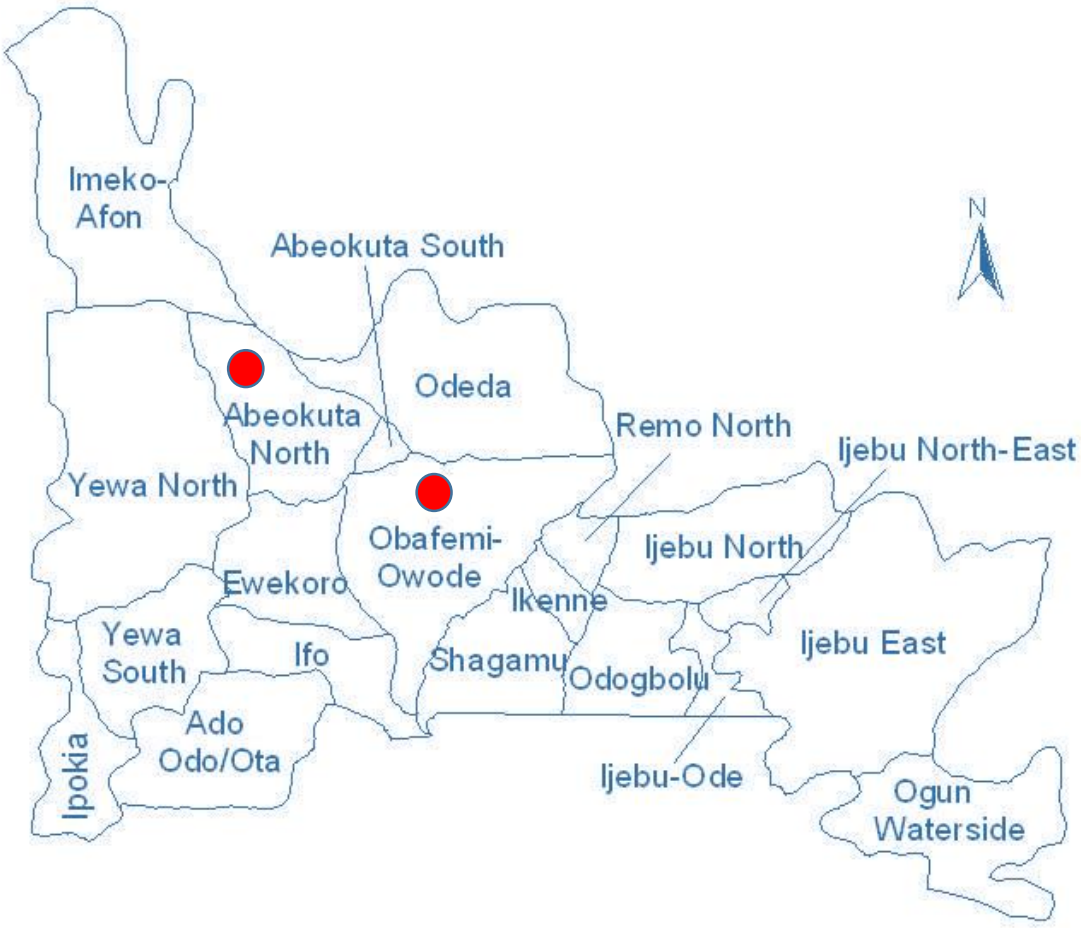

Figure 3.1: Map of the study area showing the sampling locations using red dots.

3.4 Sample Preparation Using Normal Saline

Equal volume of normal saline solution (Oxoid Limited, England) (Appendix 1) was added to the stool samples, then vortexed for 15seconds (s), after which centrifugation was carried out. The supernatant obtained after centrifugation was dispensed into a new sterile 2ml Eppendorf tube for RNA/DNA extraction

3.4.1 RNA Extraction

HAV genomic RNA was extracted using the QIAamp Viral RNA extraction method and the reagents were prepared according to the manufacturer's instruction QIAamp Viral RNA Mini Kit (QIAagen, Germany) (appendix 2). The procedure for RNA extraction is as follows:

1000 µl of viral lysis buffer AVL (QIAGEN, Germany) was pipetted 56 times into a falcon tube to make a total of 56,000 microliter of AVL buffer in a falcon tube. 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. 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, incubated at room temperature for 10 mins and pulse vortexed using the vortex mixer (Scientific Industries, United State of America) and centrifuged for few seconds. 560 µl of absolute ethanol (96-100%) was added to the sample solution and pulsed vortexed and pulsed centrifuged for 15 secs. 630 µl of the already prepared solution was dispensed into QIAamp Mini columns (QIAGEN, Germany) (in a 2ml collection tube) carefully without wetting the rims and it was centrifuged at 6000 x g (8000rpm) for 1 min using ISG centrifuge (Edulab, Japan). Each QIAamp mini column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded. 500 µl of Buffer AW1 (QIAGEN, Germany) 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 in each QIAamp Mini column and it was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3mins. 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 of the QIAamp Mini column were placed in a clean 1.5 ml micro centrifuge tube and the old collection tubes were

discarded. 60 µl of Buffer AVE (QIAGEN, Germany) was added into 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

3.5 Molecular confirmation of HAV

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).

Table 3.1: Reaction table for the cDNA generation for the 100 samples (1v-100v)

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
Script RT	200 units/ µL	100 units/ µL	0.5 µL	50.5
Script RT buffer	5X	1X	4 µL	404
dNTP mix	10 µM	500 µM	1 µL	101
RNase inhibitor	40 units/ µL	20 units/ µL	0.5 µL	50.5
RNase free water			8.5 µL	858.5
RNA template			5 µL	
Total			20 µL	

Calculations for the cDNA generation reaction table can be found in (appendix 3)

Procedure for the generation of cDNA for 100 samples (1v-100v)

The generation of cDNA for the 100 samples started with the labelling of 100 PCR strip tubes with the addition of 1 extra tube for positive control.

One step RT PCR mixture was then prepared by pipetting 858.5 µL of RNase free water (Jena bioscience, Germany) into an Eppendorf tube (labelled RT PCR master mix for easy identification), 404 µL of script Reverse Transcriptase buffer (Script RT PCR buffer) (Jena bioscience, Germany), 101 µL of dNTP mix (deoxyneucloside triphosphate) (Jena bioscience, Germany). 50.5 µL of RNase inhibitor (Jena bioscience, Germany) and then 50.5 µL of the random hexamers into the Eppendorf tube. Then the mixture was vortex ant pulse centrifuge for 8 seconds. 15 µL of the mix was then pipetted into the 100 labelled PCR strip tubes, this was then followed by the addition of 5 µL of the RNA template into each of the PCR strip tubes. 5 µL of positive control was pipetted into the 101st PCR strip tube and labelled (positive control).

The samples were then placed into the thermal cycler (Eppendorf-Nethel-Hinz GmbH, Germany) and it was set at: 42ºC for 10minutes, 50ºC for 30-60 minutes (50mins), 70ºC for 10 minutes (to inactivate the RT enzyme), and Final extension at 10ºC for ∞

Primer dilution

The primers used include: S3978, 5'GACAGATTCYACATTTGGATTGGT 3', S3979, 5'CCATYTCAAGAGTCCACACACT 3', S397A, 5' CTATTCAGATTGCAAATTAYAAT 3' and S397D, and 5' AAYTTCATYATTTCATGCTCCT 3' (Beji-Hamza *et al.,* 2015). Their working solution were prepared according to the manufacturer's instruction, Ligo (Macrogen, South Korea) (appendix 4). The working solutions were prepared as follows:

The primers were diluted with 100 µL of dH_2O, then 80µL of PCR grade water and 20 µL of the diluted primers were pipetted into labelled Eppendorf tubes (labelled according to the primers S3979 and S3978), it was pulse vortexed and pulse-centrifuged.

1ST round PCR reaction procedures

The procedure for the 1st round RT-PCR reaction is as follows:

The master mix cocktail was prepared for the first round and is shown in (Table 3.1). 1000 µl of PCR grade water (QIAGEN, Germany) was first pipetted followed by additional 111 µl of PCR grade water (total of 1,111 µl) into a new Eppendorf tube labelled mm (master mix), 404 µl of master mix, 50.5 µl each of S3978 and S3979 primers were added into the Eppendorf tube. The master mix cocktail was then pulse centrifuged. 100 PCR strip tubes were labelled (1v-100v) and then 16 µl of the master mix was pipetted into each of the 100 PCR strip tubes. 4 µl of the cDNA was then pipetted into each of the PCR strip tubes and placed into a MiniAmp thermal cycler (Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany).The thermocycler was set at 95ºC for initial denaturation for 5 minutes, 94ºC for denaturation for 30 seconds , annealing temperature at 50ºC for 30 seconds, elongation at 72ºC for 35 seconds and final elongation at 72ºC for 7 minutes and storage at -4ºC (For 35 cycles) .

The 2nd round PCR was not carried out due to absence of bands for all samples indicating negative results for all one hundred (100) samples.

3.5.1 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

1ST round PCR (POLYMERASE CHAIN REACTION) for the generated cDNA for 100 Samples (1V-100V)

The calculation for the first round PCR was done using primers: S3978 and S3979 (Beji-Hamza *et al.,* 2015).

Table 3.2: HAV 1st round reaction table for the first 100 samples (1v - 100v)

Reagents	**Initial concentration**	**Final concentration**	**Volume per reaction (V/R)**	**Number of samples n = 101**
Master mix	5X	1X	4 µL	404
S3978	20 µM	0.5 µM	0.5 µL	50.5
S3979	20 µM	0.5 µM	0.5 µL	50.5
dH20			11 µL	1,111
cDNA			4 µL	
Total			20 µL	

The calculations for the first round reaction table can be found in (appendix 5).

The absence of bands after the 1st round RT-PCR reaction showed that all samples tested negative for the presence of HAV so no 2nd round PCR was carried out for the samples.

AGAROSE GEL ELECTROPHORESIS

After amplification, the PCR products were visualized on a 1.8% agarose gel stained with ethidium bromide using the UV-trans illuminator. The agarose gel was prepared according to the manufacturer's instruction, Multi-Purpose Agarose (Cleaver Scientific, United Kingdom). The procedure for the gel electrophoresis is as follows:

- 45mls of TBE was measured and dispensed in a conical flask and 0.81g of agarose powder was added to it. It was then microwaved until the agarose powder completely dissolved.

- The hot agarose gel was dispensed into a falcon tube in which 3µl of ethidium bromide was added and the gel solution was swirled to mix and the solution was dispensed into the tank containing already fitted gel-comb and was left to solidify. One end of the box was connected to a positive electrode while the other end was connected to a negative electrode. The inner part of the gel tank contained a Tris Borate EDTA (TBE) buffer solution. The end of the gel with the well was positioned towards the negative electrode while the other end was positioned towards the positive electrode.
- Each PCR reaction was transferred into each of the wells, one well was reserved for a DNA ladder (a standard reference that contains DNA fragments of known lengths). The power to the gel box was switched on, and current began to flow through the gel. Adenovirus is a DNA virus and DNA molecules have a negative charge because of the phosphate groups in their sugar-phosphate backbone, so they start moving through the matrix of the gel towards the positive pole. The gel ran for 30minutes before being viewed using the gel documentation system which is the UV trans-illuminator.
- The required band size for the 1st round primers (S3978 & S3979) were 511bps, while that for the 2nd round primers (S397A & S397D) was 313bps.

CHAPTER FOUR

4.0 RESULTS

After the first round PCR and gel electrophoresis, no sample appeared to be positive for Hepatitis A Virus (HAV) out of the 100 samples used in this study. Below is a representative gel picture of 18 out of the 100 samples that appeared negative after the 1st round RT-PCR reaction. The gel image result was the same for the remaining 82 samples (negative) used in this study, making a total of 100 samples negative for the presence of Hepatitis A Virus (HAV). The expected band size for the first round primers was 5llbps.

Plate 4.1: Shows the representative gel plate for 1st round PCR for 18 samples using S3978 and S3979 primers.

The table showing the results for the 18 representative samples can be found in (appendix 7)

DISCUSSION

The Pattern of hepatitis A virus (HAV) infection is different world wide due to the fact that it is linked to the socio economic conditions, the hygiene and sanitary conditions of each geographic area (Shapiro and Margolis, 1993). HAV is common in developing countries and developed countries (Elisabetta *et al.*, 2012). A review done on HAV seroprevalence in children in African countries (13 out of 54) between 2008 and 2018 had an estimated average of 57.0% Sero prevalence compared to 95% for adults (Patterson *et al.,* 2019). The result of this review reported on the seroprevalence of HAV showed that Africa should not be included among the high HAV endemic region (Hussey *et al*., 2019). There is limited epidemiological data on HAV infection in Nigeria, only community based studies to detect the seroprevalence of HAV have been carried out (Ikobah *et al.*, 2015).

There was 0 prevalence of HAV in this study compared to the demographic study done on HAV infections among 300 outpatients from 6 different hospitals in Kaduna with 31 of them being aged 1-10 years and with a seroprevalence of 0.3%, HAV infection was termed to be hypo endemic in the study (Waje *et al.,* 2017). Also, the 0 prevalence of this study is however very low when compared to other studies carried out in South-west, Nigeria with high prevalence rates such as: 7.2%, 37%, and 25% (Ayoola, 1982; Afegbua *et al.*, 2013; Ikobah *et al.,* 2015). Although Ogun state is a rural area, the 0 prevalence in this study could be as a result of improved sanitation good hygiene practices and access to clean water. In another study carried out in south Africa on laboratory confirmed acute Hepatitis A cases between the years 2005-2015, 3654 cases out of 459,992 cases were children aged 5-9 years that tested positive for HAV (out of a total of 11,983 children tested). This revealed a seroprevalence of (2.3%) (Haeri Mazanderani *et al.,* 2019). Compared to the 0 prevalence in this study, the prevalence of HAV is a little bit higher in South Africa (2.3%) and this could be as a result of the high population (11,983 children) recruited in the study compared to the little population (100 children) recruited for this study. The 0 prevalence in this study is also very low compared to a regional prevalence study carried out within the years 1990-2005 were there was (72%) prevalence of HAV in children under the age of 5. The conclusion of the study was that the West sub-Saharan regions of Africa (Nigeria included) had a

high level of endemicity but with very limited evidentiary support to prove it (Jacobsen and Wiersma, 2010). The 0 prevalence of HAV in this study could also be as a result of the development of herd immunity in the area since humans are the common reservoirs of hepatitis A virus. It could be said that the population in Ogun state had developed herd immunity over time against HAV, which could be the reason for the 0 prevalence of HAV in this study.

It is apparent that HAV is more common during childhood in Africa but in present epidemiological studies there has been a shift in the infection age. HAV is more common now in early adulthood than in early childhood due to the improved sanitary condition, socio economic status and access to clean water (Melhem *et al.,* 2014). Recently, new challenges have been presented for the control of HAV especially in adults (Jacobsen, 2014; Jacobsen, 2019). Also, as HAV is said to be associated with the level of development, there is a possibility for further decline in HAV infection in African countries as strategies are being implemented to improve sanitation, and hygiene practices. (Kanyenda *et al.,* 2015).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Africa is said to be an endemic area for HAV but has experienced a transition over the past 2 decades due to improvement in some of the risk factors such as source of water and sanitation. Nigeria as one of the developing countries in West Africa is faced with the risk factors associated with HAV except for some areas where improvements have been made so its prevalence still varies in different states. A rise in the socioeconomic indicators, access to clean water, good hygiene and development of herd immunity could result in the decrease in the incidence of HAV infection and lower age-seroprevalence rates especially among the children. Currently, information on HAV in Ogun state is limited so this study is directed towards estimating the prevalence of HAV and producing epidemiological data on the prevalence of HAV among children in Ogun state and with the 0 prevalence in this study, it could be said that HAV is not endemic among children in Ogun state.

5.2 Recommendation

Indeed, HAV was not prevalent in the study population, it does not strike out the fact that HAV could still be detected in other areas in Ogun state or south west regions of Nigeria. In some studies, Africa is still included as an HAV endemic area and is more common in children aged 5-17 years. As vaccinations against HAV have not been added to the Expanded Programme on Immunisation (EPI) in Africa, there is a possibility for a future HAV epidemic if the epidemiology of HAV has not been fully understood. The following recommendations are proposed:

- HAV vaccines should be included in the Expanded Programme on Immunisation (EPI) in African countries.
- Implementation of strategies to improve the sanitation and hygiene conditions, and access to clean water should cut across all African countries with the exemption of none.
- Parents should make sure children wash their hands after toilet use, before and after eating.

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APPENDIX

Appendix 1: Materials used

Measuring cylinder, Jena bioscience Script cDNA synthesis kit, QIAamp Viral RNA Mini Kit, Micropipette, Pipette tips, Vortex mixer, Centrifuge, Eppendorf tubes, Spin column, Agarose Tank, Conical flask, Measuring scale, Falcon tube, powdered gloves, Nitrite gloves, minicamp Thermal cycler, Hot plate stirrer, Microwave, PCR strip tubes, Safety cabinet, UV Tran Illuminator.

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), Master mix, PCR grade water, Ethidium Bromide, Agarose gel powder, TBE, Primers (S3978, S3979, S397A and S397D), Template (cDNA).

Appendix 3: Sample preparation

Normal saline solution was prepared first by adding a normal saline tablet into a conical flask containing 500ml of water then stirred using a hot plate stirrer. The tablet is stirred till it dissolves. A total of 100 Eppendorf tubes were allocated to the hundred samples and were labelled (1v-100v). 700µl of normal saline was pipetted into the labelled Eppendorf tubes followed by the pipetting of 700µl of stool samples into the Eppendorf tubes already containing the normal saline. The Eppendorf tubes are then spun in a centrifuge at 21370 x g for 3 minutes.

Appendix 4: Reagent preparation for RNA extraction

n x 0.56ml **= y** ml

y ml **x** 10 µl/ml **=z** µl

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 0.56mls of buffer AVL = 56ml (buffer AVL)

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

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

n= 100, **y**=56ml and **z**=560µl

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

Appendix 5: Calculations for cDNA generation for 100 samples

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

$$C_1V_1=C_2V_2$$

C_1 – Initial concentration

V_1 – Initial Volume

C_2 – Final concentration

V_2 – Final Volume

Where,

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

According to $C_1V_1=C_2V_2$,

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

Therefore, total volume per Reaction (V/R) for the random hexamers = 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 all 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 100 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 6: Preparation of working solution for HAV primers

Micro molar concentration of primers = 100 µM

Working Solution = 20 µM

$$C_1V_1=C_2V_2,$$

C_1 – Initial concentration

V_1 – Initial Volume

C_2 – Final concentration

V_2 – Final Volume

Where,

C_1 = 100 (Micro molar 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 + 80 µL of dH_2O

Appendix 7: Calculations for the 1st and 2nd round RT-PCR reaction

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

$$C_1V_1=C_2V_2$$

C_1 – Initial concentration

V_1 – Initial Volume

C_2 – Final concentration

V_2 – Final Volume

Where,

C_1 = 5, V_1 = ?, C_2 = 1, V_2 = 20

According to $C_1V_1=C_2V_2$,

5 X V_1 = 1 X 20

V_1= 20/5 = 4 µL

To get the total volume per reaction of the primer S3978,

$$C_1V_1=C_2V_2$$

Where,

$C_1 = 20$

$V_1 = ?$

$C_2 = 0.5$

$V_2 = 20$

According to $C_1V_1=C_2V_2$,

$$20 \text{ X } V_1 = 0.5 \text{ X } 20$$

$$V_1 = 10/20 = 0.5 \text{ µL}$$

This volume is the same for the 2nd primer (S3979) because it has the same final and intial concentration with the first primer (S3978).

To get the total volume of dH_2O,

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

i.e. 20 – (4 + 0.5 +0.5 + 4) = 20 – 9 = 11 µL

Appendix 8: Preparation of agarose gel

For the preparation of 1 tank of agarose gel electrophoresis, these procedures are to be followed:

Preparation of 1.8% of agarose gel

Total volume of gel slabs = 45mls

Therefore 1.8÷100 x 45÷1=0.81g of Multipurpose Agarose

Appendix 9: Table showing the results for the 18 representative samples

SAMPLE CODE	SAMPLE ID	RESULT
SH 198	67V	Negative
SH 262	68V	Negative
SH 187	69V	Negative
SH 146	70V	Negative
SH 241	71V	Negative
SH 185	72V	Negative
SH 246	73V	Negative
SH 263	74V	Negative
SH 182	75V	Negative
SH 245	76V	Negative
SH 144	77V	Negative
SH 238	78V	Negative
SH 261	79V	Negative
SH 243	80V	Negative
SH 186	81V	Negative
SH 139	82V	Negative
SH 142	83V	Negative
SH 200	84V	Negative