PREVALENCE AND PHYLOGENETIC ANALYSIS OF HEPATITIS A VIRUS IN PATIENTS ATTENDING MPANDA GENERAL HOSPITAL IN BURUNDI

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Prevalence and Phylogenetic Analysis of Hepatitis A Virus in Patients Attending Mpanda General Hospital in Burundi

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DECLARATION

This thesis is my original work and has not been presented for an award of a degree in any other university

Signature……...…Date...……

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This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

I dedicate this thesis to my parents, sisters and brothers and to my wife, Emelyne NDAYIZEYE, for her virtue of self-dependence, motivation and humility. Further, I dedicate this thesis to my lovely daughter Carly Melker HARABANDI with honor and love.

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ABBREVIATIONS AND ACRONYMS

ACIP	Advisory Committee on Immunization Practices
ALAT	Alanine Aminotransferase
AGE	Agarose Gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
BNEC	Burundi national Ethical Committee
CD_8T	Cluster of Differentiation 8 T-cells
CNTS	Centre National de Transfusion Sanguine
CVR	Center for Virus Research
DHS	Demographic and Health Survey
DRC	Democratic Republic of Congo
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ERC	Ethical Review Committee
EPID	Enhanced Privacy Identification
GIBU	Geographic Institute of Burundi
ID	Identification
IgG	Immunoglobulin G
IGEBU	Institut Géographique du Burundi
IgM	Immunoglobulin M
ISTEEBU	Institut de Statistique et d'Etudes Economiques du Burundi
MDG	Millennium Development Goals
MEGA	Molecular Evolutionary Genetics Analysis
MMWR	Morbidity and Mortality Weekly Report

MoH	Ministry of Health
NASBA	Nucleic Acid Sequencing Based Assay
NTR	Nontranslated Region
OP	Optical Density
PEG	Polyethylene Glycol
P	Protein
PCR-SSCP	Polymerase Chain Reaction Single-Strand Conformation Polymorphism
PNDS	Programme National de Développement Sanitaire
PSCT	Peripheral Stem Cell Transplantation
RGPH	Recensement Général de la Population et de l'Habitant
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-QPCR	Reverse Transcriptase quantitative Polymerase chain reaction
SPSS	Statistical Package for Social Sciences
UNECA	United Nations Economic Commission for Africa
VP	Viral Protein
VPg	Virion Protein Genome linked

ABSTRACT

Viral hepatitis is a public health problem worldwide. Hepatitis A, transmitted by fecal-oral route, is an infectious viral disease caused by hepatitis A virus (HAV) and occurs mainly due to poor sanitation. Hepatitis A, the most common cause of acute hepatitis, is wrongly considered a benign disease despite the risk of fulminant hepatic failure. This study aimed to determine the prevalence and phylogenetic characteristics of HAV in patients attending Mpanda General Hospital in Burundi. The association between demographic factors and HAV cases was also established. A cross-sectional study was used. Participants were recruited using a consecutive sampling technique. Data and blood samples took three months (October to December 2017). Data from consented/assented participants were collected using questionnaires. Five milliliters of venous blood were collected from 385 participants and analyzed. Anti-HAV IgM and IgG antibodies were screened using Rapid test and Enzyme Linked Immunosorbent Assay Kits. Anti-HAV IgM positive samples were used for molecular analysis. A nested Reverse Transcription-PCR was performed to detect HAV genome using gene specific primers and the positive samples sequenced for HAV genotype. The prevalence of HAV infection and the 95% CI were calculated. The association between HAV cases and independent variables was assessed by a bivariate logistic regression and p-value ≤ 0.05 was considered significant. The median age was 23 and the range 72 [2-74] years. The overall prevalence of Hepatitis A virus was 60.3% (232/385). There was a significant association between age, lack of clean water, traditional latrines and Hepatitis A Virus cases. Of the 124 cases positive for anti-HAV IgM, nine were positive for HAV RNA. Phylogenetic analysis revealed that all isolates belonged to genotype IB. From findings, good hygienic practices and sanitation would reduce the incidence of the HAV infection. Further research aimed at HAV genotype characterization should be performed in other parts of Burundi to identify the genotype most common in the country. There is need for a nationwide survey of HAV infection in Burundi involving a nationwide sample to determine the overall prevalence and molecular characterization of HAV in the country.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Hepatitis A, the most common acute hepatitis caused by the hepatitis A virus (HAV), is wrongly considered a benign disease despite the risk of fulminant hepatic failure (Guenifi *et al*., 2017). It is an inflammation, usually caused by a viral infection, although it can also be caused by toxic agents or other diseases, such as autoimmune and metabolic diseases (Mudawi, 2008).

Many viruses cause hepatitis. Of these, five medically important viruses are commonly described as "hepatitis viruses" because their main site of infection is the liver (Han *et al*., 2000). These five are: Hepatitis A Virus (HAV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Hepatitis D Virus (HDV) and Hepatitis E Virus (HEV). Hepatitis A virus (HAV) is one of the most important causes of acute infectious hepatitis (Lee *et al*., 2009). These viruses can all result in acute disease with symptoms of nausea, abdominal pain, fatigue, malaise, and jaundice (Rapti; Hadziyannis, 2015). Factors known to trigger the spread of HAV have been described previously (Jacobsen; Wiersma, 2010).

Four of the seven genotypes of HAV affect humans, but only one serotype exists (Desbois *et al*., 2010). Seroprevalence of HAV infection shows great differences not only between different countries but also between regions in the same country. It is inversely related to the level of socio-economic status, sanitation and personnel hygiene (Shapiro and Margolis, 1993). It has been shown that infection rates remain high in most African countries (Kathryn, 2010). Information on HAV infection in Burundi as in many other countries of Africa is limited. Available data shows that most of Africa remains a high endemicity region, with the exception of subpopulations in some areas (Franco, 2012).

Burundi is currently scaling-up vaccines for all immunisable diseases. However, despite efforts and achievements, there is relatively little information regarding the

prevalence, risk factors and genotypes of Hepatitis A Virus, one of the most probable cause of diarrheal illness among individuals presenting at the treatment centers in the country. The diarrhea is one of the five causes of morbidity in Burundi (WHO; 2012; Burundi MoH, 2004).

1.2 Statement of the problem

Hepatitis A virus (HAV) and hepatitis E virus (HEV) are the leading causes of acute viral hepatitis in the world (Hesamizadeh *et al*., 2016). In developing countries, these infections are associated with poor hygiene and, in particular, the lack of clean drinking water and, in some areas, inadequate sanitation (Traoré *et al*., 2012).

Hepatitis A virus infection is associated with diarrhea. Although diarrhea is the third leading cause of morbidity among children under five and the fourth leading cause of mortality in Burundi, it is estimated that 25% of such episodes among these children are of unknown etiology within weeks (Diouf *et al*., 2014). It is thus not clear on the magnitude and the burden of HAV associated diarrhea among this population. It is noted that the seroprevalence of HAV infection rates in the less developed regions and in several developing countries are common in the first years of life and are approaching 100% (Franco *et al*., 2012).

In Burundi no study has been carried out to document the magnitude of Hepatitis A. There are also limited resources in most healthcare facilities in the country to carry out the diagnosis for HAV so as to know the true prevalence. Accompanied with lack of prevalence data is also the fact that the circulating strains in the country are not known, hindering the possibility of recommending chemotherapeutic or vaccination strategy for the country. At present, no HAV vaccine is available in Burundi.

Lack of a potential empirical management strategy makes treatment and control of HAV makes treatment, control and prevention of this infection more expensive for a country which has been ranked 185 out of 187 of the most poor countries in the world (Voget-Kleschin; Lieske, 2012). During the past years, factors influencing diarrhea prevalence in Burundi were largely neglected, with few exceptions

(Birmingham *et al*., 1997). This in turn hinders informed decision making as well as effective health planning and programme implementation. Mpanda district is predominantly rural with a small proportion of urban residence and experiences a lack of clean drinking water (PNDS, 2011-2015).

1.3 Justification of the study

In Burundi diarrheal related illness of unknown etiology are a major concern, posing a public health threat in the country. There is little information on the HAV virus infection because it is neglected and underestimated. Diarrheal related illnesses are commonly reported in many hospitals. However, most are unclassified etiologically, yet laboratory facilities are significantly constrained by economic considerations.

Limited resources in most healthcare facilities in the country have led to magnification of this problem. Inhabitants of Mpanda District are at large suffer from diverse diseases of the poor sanitation, particularly diarrheal related diseases and other illnesses transmitted through feacal-oral route are common cases just like in others area of the world with similar characteristics and these may be attributed to insufficient water supply and poor sanitation (CDC, 2003). HAV is one of the most suspicious agents that may be present and uncharacterized in this area because there is no routine diagnostic test done.

Limited data and information on incidence and prevalence of HAV infection has resulted to underestimation of the disease burden in the country. In Burundi, no study has been conducted to determine the prevalence and genotypes of HAV strains circulating in the country so far. As result, it is difficult to rule out the possibility of the presence of uncommon HAV genotype capable to escape from protection conferred by the exiting vaccine.

At present, there are no reports of the presence of HAV subgenotypes circulating in Burundi. Molecular characterization of HAV genotypes and subgenotypes is important for establishing evolutionary relationships and for understanding its origin or patterns of transmission. Burundi is a developing country located in sub-Saharan

Africa and, as far as we know, knowledge of the specific HAV strains circulating in this population remains unknown. However, the few serological data reported suggest that the virus is endemic in region (Forbi et al., 2013).

This study seeks to determine the prevalence and genotypes of HAV in Mpanda General Hospital. Determining the prevalence of HAV in Mpanda General Hospital could shed light on the current proportions of diarrhea cases caused by HAV infection. Furthermore, investigating on molecular characteristic of HAV in this hospital could allow the detection of uncommon HAV strains in Burundi. The results of this study will help to form a base for the surveillance of HAV infection by including it in differential diagnosis of diarrheal related illnesses in the country.

1.4 Research questions

1. What is the prevalence of HAV in patients attending Mpanda General Hospital?

2. Is there an association between HAV cases with socio-demographic factors in patients attending Mpanda General Hospital patients?

3. What are the genotypes of HAV circulating in the patients attending Mpanda General Hospital?

1.5 Objectives

1.5.1 General objective

To determine the prevalence and phylogenetic characteristics of HAV in patients attending Mpanda General Hospital in Burundi.

1.5.2 Specific objectives

1. To determine the prevalence of HAV in patients attending Mpanda General Hospital.
2. To establish the association between cases of HAV and the socio-demographic factors in Mpanda General Hospital patients.

3. To determine the genotypes of HAV circulating in the patients attending Mpanda General Hospital.

CHAPTER TWO

LITERATURE REVIEW

2.1 Virus structure

Hepatitis A virus (HAV) is a member of the *Hepatovirus* genus of *Picornaviridae* family (Poovorawan *et al*., 2005). Hepatitis A Virus is a non-enveloped, linear, single stranded RNA virus of an icosahedral symmetry measuring 27-32 nm in diameter (Feinstone *et al*., 1973). The infectious particle consists of capsid protein and RNA genome. The buoyant density of the mature particle is 1.33g/cm3 in CsCl solutions and the sedimentation coefficient is 160S in sucrose solutions (Ticehurst *et al*., 1983).

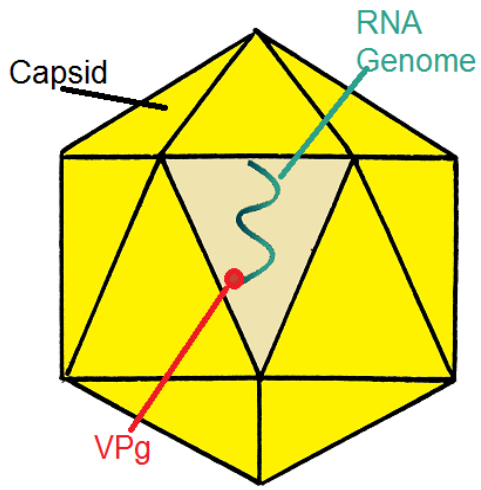


Figure 2.1: Hepatitis A Virus structure (McKnight and Lemon, 2018)

2.1.1 Hepatitis A Virus genome organization

Hepatitis A Virus has a positive-polarity, single-stranded RNA that is approximately 7.5kb in length (Nainan *et al*., 2005). HAV is known to display a high degree of antigenic and genetic conservation contrary to the high frequency of genetic changes seen in RNA viruses (Singh *et al*., 2015). Molecular epidemiology of HAV is important to understand the strains circulating in various geographical regions and

tracing the source of contamination in an outbreak situation (Chobe; Arankalle, 2009). The HAV strains isolated from various parts of the world constitute a single serotype and are divided into six genotypes (I-VI) (Singh *et al*., 2015). Genotypes I-III are most commonly associated with human infections and have a variable geographical distribution. Majority of human strains (80%) belong to genotype I (Chitambar *et al*., 2007). The molecular characterization of the infectious agents is important, as it provides the information about the circulating strains in a particular region, the invasion of new strains from different geographical areas and their role in the pathogenesis and severity of the disease (Singh *et al*., 2015). Hepatitis A Virus is classified as a Hepatovirus of Picornaviridae family. It is a positive single stranded RNA virus comprising approximately 7500 nucleotides and containing a 5′ non-translated region (NTR), both structural and non-structural protein coding regions, and a 3′NTR (Fujiwara *et al*., 2003). The 5′NTR of the virus is approximately 735 nucleotides and has high sequence identity among the various strains (Fujiwara *et al*., 2003). The VPg encoded in the P3 region, is covalently linked to the 5' genome terminus and involved in initiation of RNA synthesis. The HAV genome comprises 7.5 kb single stranded RNA and is divided into three functional regions, P1, P2 and P3 (Theamboonlers *et al*., 2009). The P1 region encodes 4 capsid polypeptides (VP1, VP2, VP3 and putative VP4) (Nainan *et al*., 2005). Since the region spanning VP1-2A has demonstrated substantial sequence heterogeneity, it is suitable for differentiating between HAV strains (Figure 2.2).

Six nonstructural proteins, each involved in replication of the HAV RNA, are derived from the remaining P2 and P3 segments of the poly-protein: 2B, 2C, 3A, 3B, 3Cpro, and 3Dpol (McKnight and Lemon, 2018). These proteins are all intimately associated with membranes. Like other picornaviruses, the 2C protein contains a heli-case motif and, when overexpressed (with or without 2B as 2BC), induces substantial mem-brane rearrangements in cells (Singh *et al*., 2015). The 3A protein provides a membrane anchor for 3B, the genome-linked protein (VPg) that likely serves as a protein primer for RNA synthesis (Nainan *et al*., 2005)**.**

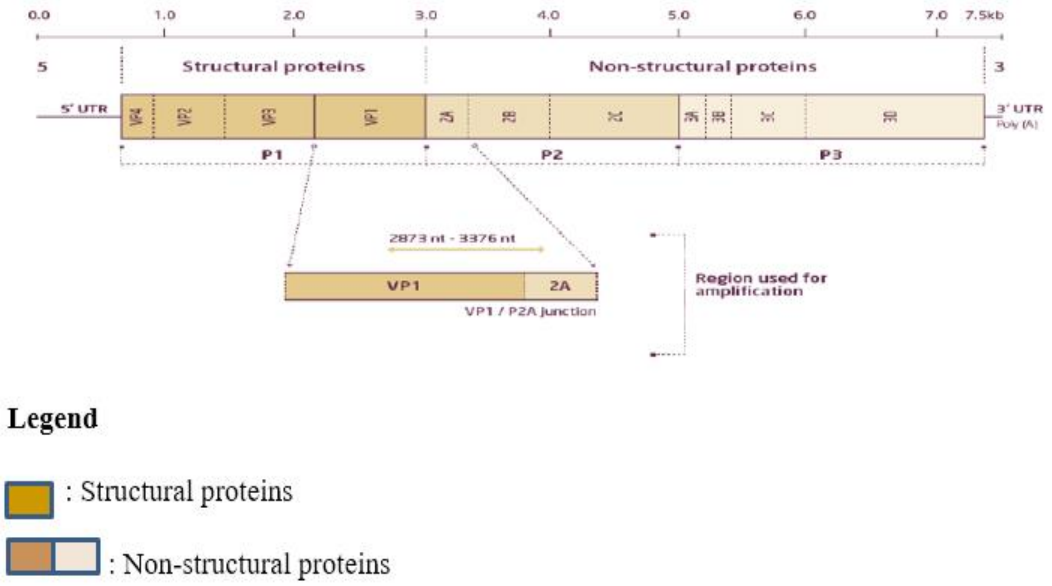


Figure 2.2: Hepatitis A Virus genome organization (Nainan *et al*., 2005).

2.1.2 HAV life cycle

Humans and vertebrates serve as the natural hosts. Transmission routes are fecal-oral and blood (Hesamizadeh *et al*., 2016). Following ingestion, HAV enters the bloodstream through the epithelium of the oropharynx or intestine (Murray *et al*., 2005).The blood carries the virus to its target, the liver, where it multiplies within hepatocytes and Kupffer cells (liver macrophages). Viral replication is cytoplasmic. Entry into the host cell is achieved by attachment of the virus to host receptors, which mediates endocytosis (Shukla *et al*., 2014). Replication follows the positive-stranded RNA virus replication model. In contrast to many other picornaviruses, including the well-studied poliovirus, HAV does not shut down cellular protein synthesis in infected cells, and generally replicates with-out cytopathic effect (McKnight and Lemon, 2018). One of the most interesting features of the virus is its recently recognized capacity for non-lytic release from infected cells as membrane-wrapped, quasi-enveloped infectious virions (eHAV) (Feng et al. 2013). The eHAV particles have a specific infectivity similar to that of the naked virion, and represent a second form of infectious virus. Their mechanism of biogenesis appears to be unique among the Picornaviridae (McKnight and Lemon, 2018). The virus exits the host cell

by lysis and viroporins and the virions are secreted into the bile and released in stool (Hirai-Yuki *et al*. , 2016).

2.1.3 Hepatitis A virus genetic diversity

The nucleotide sequence comparison based on the limited segments of the genome has been used to define seven different genotypes, which include four human clusters (genotypes I–III and VII), and three genotypes belong to simian strains (genotypes IV–VI) (Forbi *et al*., 2012). The three hepatitis A virus genotypes, I, II, and III are divided into subtypes A and B (Fujiwara *et al*., 2003). Genotype I is the most frequently reported, while genotype II is hardly ever isolated, and its genetic diversity is unknown (Desbois *et al*., 2010). Genomic characterization of HAV has been carried out mainly by sequencing of strains from different geographic regions of the world. Firstly, by using a short fragment of the VP1/2A junction region, strains were classified into seven genotypes based on >15% nucleotide variation between isolates, and the subgenotypes with >7.5% to <15% nucleotide variation (Coudray-Meunier *et al.,* 2014). Then, the complete genomic data indicated that genotypes II and VII should be considered a single genotype, based upon the complete VP1 sequence (Bruni *et al.,* 2016). By sequencing of the VP1/2A junction and the VP1 gene, three genotypes (I, II, III) divided in two subtypes (A and B) have been described.

2.1.4 HAV genome region for typing

The comparative nucleotide sequence analysis has proved to be a powerful tool for genotyping, identification of the source of infection, epidemiological tracking and for the examination of genetic divergence (Joshi *et al.,* 2008). Identification of origin of contamination and links between geographically separated outbreaks using molecular techniques has accelerated the measures that are needed to be taken for prevention and control of disease (Sánchez *et al*., 2002). The HAV genotype has been determined by sequencing the viral protein 1/non-structural protein 2A (VP1/P2A) junction region (Robertson *et al*., 1992).

2.2 Global epidemiology of Hepatitis A

Hepatitis A occurs worldwide and it is estimated that tens of millions of individuals worldwide contract HAV through fecal–oral transmission each year, usually via ingestion of contaminated food or water or through contact with an infectious person (Jacobsen, 2018; Rein *et al*., 2014). Sero-epidemiological studies show that prevalence of anti-hepatitis A antibodies varies from 15 to close 100 in different parts of the world (Barzaga, 2000). HAV occurs in all countries of the world and the incidence of infection varies with the local socioeconomic conditions and health standards (Roque-Afonso *et al*., 2006). Globally, 1.4 million cases of HAV occur annually with a majority of the cases concentrated in the less developed countries where several risk factors facilitate transmission (Kanyenda *et al*., 2015). Region when Hepatitis A is highly common include the Indian sub-continent (particularly India, Pakistan, Bangladesh and Nepal), sub-Sahara, East Africa and parts of the Far East (excluding Japan), South and Central America, and the Middle East (CDC, 2003). In many low-income countries, HAV continues to be highly endemic and most children contract the virus before their fifth birthday (Jacobsen, 2018). Most high income countries such as those in western and Northern Europe, North America and Australia, New Zealand and Japan are of low endemicity for Hepatitis A (Mohd Hanafiah *et al*, 2011). The United States generally has a low endemicity of HAV infection, although high rates of infection occur in certain populations (Figure 2. 2) (CDC, 2003).

Approximately 37% of the world's population does not have access to improved sanitation, which means that a significant proportion of the global human population lives in conditions where HAV can thrive (Koroglu *et al*., 2017). As access to water and sanitation increase and economies grow, HAV incidence rates tend to decrease (Jacobsen *et al*., 2005). However, when incidence rates decrease, the proportion of adults who remain susceptible to HAV infection begins to increase (Koroglu *et al*., 2017).

Figure 2.3: Global epidemiology and Geographical distribution of HAV infection (CDC, 2003)

Legend

2.3 Hepatitis A in Africa

Information on HAV infection in Africa is limited and available data shows that most of Africa remains a high endemicity region, with the exception of subpopulations in some areas, such as white people in South Africa (Franco *et al*., 2012). In the 1990s, almost 100% of black children in South Africa were anti-HAV-positive by the age of 12 years and almost 100% of black adults had antibodies to HAV before the age of 20 years, while only 30-40% of white adults were anti-HAV-positive by the age of 20 years, rising to about 60% by the age of 40-49 years (Tufenkeji, 2000). North Africa has an intermediate level of anti-HAV seroprevalence. More recent data shows that, in general, urban areas have experienced a decline in hepatitis A infection, while rates in rural areas remain high and the prevalence is generally lower

in higher social classes (WHO, 2010). Previous examinations of hepatitis A seroprevalence in sub-Saharan Africa have generally found very high endemicity levels, with more than half of children having serological evidence of prior infection by their fifth birthdays (Jacobsen, 2016). In DR Congo, the annual incidence was calculated to be between 96% and 100% (Jacobsen; Koopman, 2004). In some west African countries such as Senegal 100%, and Cameroun 90%, the HAV prevalence was found to be high (Weis *et al*., 2017; Ibrahim, 2015). In Tanzania, a prevalence of 3.1% was reported at Muhimbili National Hospital in Dar es Salaam among HIV-infected adults presenting for care and treatment (Nagu *et al*., 2008). It has been reported that prevalence of HAV among Kenyan patients in KNH ranges from 12 to 41.7% (Greenfield *et al*., 1984; Atina *et al*., 2004).

2.4 Mode of transmission and sources of HAV infection

Hepatitis A Virus infection is transmitted primarily by the fecal-oral route, person-to-person contact, or ingestion of contaminated food or water, transmission via blood has also been reported, but only on rare occasion (Hamza *et al*., 2017). The virus is spread through food and water contaminated by the feces of infected people (Ibrahim, 2015). Although most physicians considered that a respiratory-type droplet infection was more likely (Ford, 1943). The latest data from the Viral Hepatitis Surveillance Program (1993) indicate that contact with a person infected with hepatitis A is the most common identifiable source of infection (22%). The prolonged shedding of HAV before and after the onset of symptoms, in association with the lack of good hygienic practices and the sharing of objects in the domiciliary environment, may contribute to a more suitable scenario for person-to-person HAV transmission (Yun *et al*., 2011) (Neffatti *et al*., 2017). Hepatitis A outbreaks among homosexual men were reported in the United States and abroad (Petrignani *et al*., 2008; Henning *et al*., 1995). Higher seroprevalence rates of hepatitis A infection are associated with oral-anal contact regardless of sexual orientation not with homosexuality per se (Villano *et al*., 1997). Once considered rare outside experimental studies, parenteral transmission of hepatitis A complicating transfusion of blood and blood products has been reported many times (Sherertz *et al*., 1984).

Blood from a single donor who became ill one week after donation transmitted disease to 11 recipient neonates and thence secondarily to an additional 44 persons (Noble *et al*., 1984). Transmission associated with platelet and plasma donation processing and anticancer immunotherapy reagents has also been documented (Weisfuse *et al*., 1990). The other group at risk for HAV infection by parenteral transmission is the injection drug-using population (Grinde *et al*., 1997). Approximately 40 to 50% of injection drug users in northern Europe are anti-HAV positive (Krook *et al*., 1997). In Scandinavia and France the seropositive rate is significantly higher than in a matched control population (Holter; Siebke, 1988). Transmission of HAV from hospitalized patients with unsuspected disease to staff is well recognized as a nosocomial infection (Burkholder *et al*., 1995).

2.5 Pathogenesis of HAV infection

There is no apparent virus-mediated cytotoxicity presumably because of the virus' own requirement for an intact eIF4G (Vaughan *et al.*, 2013). Liver pathology is likely immune-mediated. The risk for symptomatic infection is directly related to age, with more than 80% of adults having symptoms of acute viral hepatitis (Hamza *et al*., 2017). Incubation period 2-6 weeks, many infections are silent particularly in young children. Clinical illness usually starts with a few days of malaise, loss of appetite, vague abdominal discomfort, and fever (McKnight and Lemon, 2018). Urine becomes dark and faeces pale followed by jaundice, a yellowing of the skin or whites of the eyes due to hyperbilirubinemia. The patient starts to feel better within the next week and the jaundice disappears within a month. Hepatitis A is self-limiting but relapses have been reported and the severity of illness is less in children than in adults (Singh *et al*., 2015). Complications as fulminant hepatitis are seen mainly in adults and the mortality is about 1% an (Vaughan *et al*., 2013).

2.6 Clinical features of HAV infection

Hepatitis A Virus is generally asymptomatic, self-limited and can produce effects that range from a lack of symptoms to death from fulminant hepatitis (Poovorawan *et al*., 2005). In children, most infections (70%) are asymptomatic (Hadler *et al*., 1980).

Among older children and adults, infection is usually symptomatic, with jaundice occurring in 70% of patients (Lednar *et al*., 1985). After an average incubation period of 28 days (range, 15 to 50 days), most HAV-infected persons develop nonspecific signs and symptoms followed by gastrointestinal symptoms (Petrignani *et al*., 2008). Characteristic prodromal symptoms, preceding the onset of jaundice, include malaise and weakness, fever, anorexia, nausea and vomiting, and abdominal discomfort (Koff, 1992). Fulminant hepatitis is a rare complication of HAV and the risk of acute liver failure ranges from 0.015 to 0.5%, with the highest rates occurring among young children and older adults with underlying chronic liver disease (Akriviadis; Redeker, 1989). Less commonly, pruritus, diarrhea, arthralgia, or skin rash develop. When the patient seeks medical advice, the fever has usually disappeared. On physical examination, hepatomegaly (78%) and jaundice (40%–80%) are frequently detected (Shin and Jeong, 2018). Reported extrahepatic complications include acute kidney injury, acalculous cholecystitis, pancreatitis, pericardial or pleural effusion, pure red-cell aplasia, hemophagocytosis, hemolysis, skin rash, acute reactive arthritis, and neurological manifestations such as mononeuritis, Guillain–Barré syndrome, and transverse myelitis.

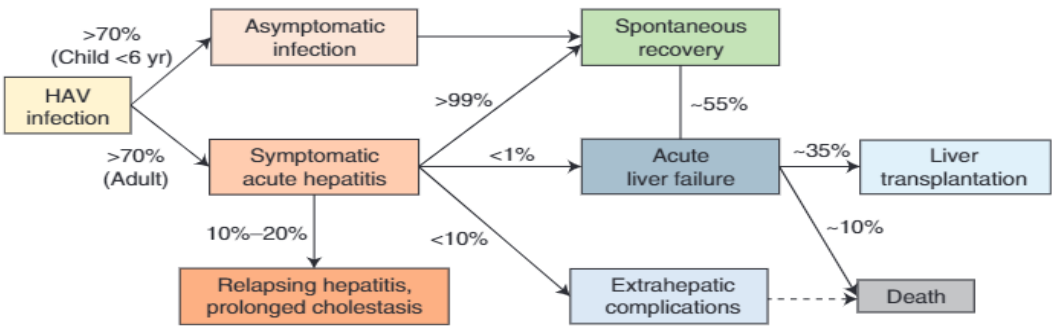


Figure 2.4: A typical course of hepatitis A Virus infection (Shin and Jeong, 2018).

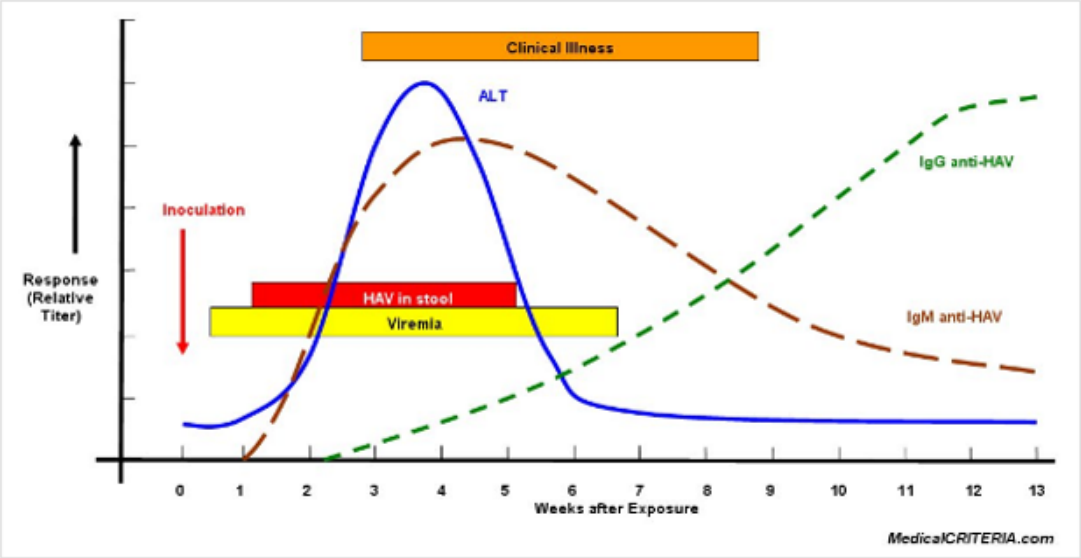
2.7 Diagnosis of HAV infection

2.7.1 Detection of anti-HAV specific antibodies

The detection of specific IgM antibodies is an important and valuable tool in the rapid and early diagnosis of parasitic and infectious diseases (Duermeyer *et al*., 1979). The humoral immune response plays the pivotal role in the diagnosis of HAV infection and the differentiation of hepatitis A from other types of viral hepatitis (Nanina *et al*., 2006). There are a number of commercially available assays for the detection of IgM and total anti-HAV (Dignani *et al*., 2003). Diagnostically, IgM anti-HAV has been used as the primary marker of acute infection; it is comprised mainly of antibodies against capsid proteins (Cuthbert, 2001). A number of methods have been used to detect this virus-specific antibody class, including enzyme-linked immunosorbent assay and dot blot immunogold filtration (Rezende *et al*., 2003) (Shao *et al*., 2003). Immunoglobulin M anti-HAV enzyme immunoassays are available commercially (Poddar, 2002).

2.7.2 Antigen detection

Hepatitis A Virus antigen can be detected in stool by Enzyme Linked Immunosorbent Assay (ELISA) test. The shedding begins after two weeks before the icteric phase of the disease and reached a peak after about one week before icterus (figure 2. 5). A positive result of Hepatitis A Virus in the stool of patients indicates a fresh and contagious infection (Coulepis *et al*., 1980). With the beginning of the icteric phase, the HAV shedding drops steeply but HAV antigen could be found in the stool of some patients two weeks after onset of icterus. The detection of HAV in specimen other than stool is also possible with the ELISA test, for example in lysates of HAV infected cells or in culture supernatants (Thomas, 1988).



Legend

: Clinical phase of the disease

: Presence of the virus in the stool

: Presence of the virus in blood

Figure 2.5: Hepatitis A infection; typical serologic course (Guillermo *et al.*, 2011)

2.7.3 Cell culture

Hepatitis A Virus has been grown in several cell types of human and nonhuman origins, including African green monkey kidney cells and fetal rhesus monkey kidney cells (Daemer *et al.*, 1981; Flehmig, 1980). In contrast to most picornaviruses, HAV of human origin requires an extensive adaptation period before it grows in cell culture, and once adapted, HAV produces a persistent infection and becomes attenuated, as shown by not producing disease in experimentally inoculated nonhuman primates (Feinstone *et al.*, 1983). Mutations in viral nucleic acid may play a major role in the adaptation of HAV in cell culture and attenuation (Emerson,

1991). Because of the lack of a cytopathic effect in cell culture, immunological assays are required to detect HAV antigen (Siegl *et al*., 1984).

2.7.4 Molecular detection methods

Nucleic acid detection techniques are more sensitive than immunoassays for viral antigen to detect HAV in samples of different origins (Nainan *et al*., 2006). Hepatitis A Virus has been detected with techniques such as restriction fragment length polymorphism, single-strand conformational polymorphism (Fujiwara *et al*., 2000). Southern blotting, nucleic acid sequencing-based amplification, nucleic acid hybridization, reverse transcription-PCR (RT-PCR) and antigen capture RT-PCR (Jean *et al*., 2004; Oba *et al*., 2000). Amplification of viral RNA by RT-PCR is currently the most sensitive and widely used method for detection of HAV RNA (Nainan *et al*., 2005; Stahlberg, 2004; Deng *et al*., 1994; Costa-Mattioli *et al*., 2002). Nucleic acid sequencing of selected genomic regions of HAV has been used to determine the genetic relatedness of isolates ((Nainan *et al*., 2005; Sjogren *et al*., 1991; Marziali; Akeson, 2001).

2.8 Risk Groups for Hepatitis A infection

Groups at increased risk for hepatitis A or its complications include international travelers, contacts of recent international adoptees from HAV endemic countries, municipal solid waste collectors, men who have sex with men, and users of illegal drugs (Rachiotis, 2016). Outbreaks of HAV have also been reported among persons working with hepatitis A–infected primates (CDC, 2006). This is the only occupational group known to be at increased risk for hepatitis A. Persons with chronic liver disease are not at increased risk of infection but are at increased risk of acquiring fulminant hepatitis A (Taylor *et al*., 2006). Persons with clotting factor disorders may be at increased risk of HAV because of administration of detergent-treated factor VIII and IX concentrates. Food handlers are not at increased risk for hepatitis A because of their occupation, but are noteworthy because of their critical role in common-source food borne HAV transmission (CDC, 2003). In areas with poor sanitary conditions and living standards, greatest exposure is expected early in life (Afegbua *et al*., 2013).

2.9 Treatment and management of HAV

Hepatitis A virus is a self-limited infectious disease and do not cause chronic liver disease (Mackinney-Novelo *et al*., 2012). There is no specific treatment for hepatitis A, most of patients recover with conservative treatment (Carrillo-Santisteve *et al*., 2017). Therapy is aimed at maintaining comfort and adequate nutritional balance, including replacement of fluids that are lost from vomiting and diarrhea (CDC, 2006). Treatment of acute hepatitis A is mainly symptomatic (Cho *et al*., 2011). Patients are at risk of dehydration and adequate fluid replacement therapy is indicated (Han *et al*., 2000).

2.10 Prevention and control of HAV infection

Hepatitis A is vaccine-preventable (Shi *et al*., 2018). It can be prevented by vaccination, good hygiene and sanitation (CDC, 2017). There are two types of vaccines: inactivated and a live but attenuated virus (Irving *et al*., 2012). Human normal immunoglobulin can be offered in addition or in preference to vaccine for contacts who are more than 7 days from onset of illness, and for those at risk of adverse outcome of HAV infection (Crowcroft *et al*., 2001).

Vaccination is the most effective means to prevent HAV infection and reduce disease incidence (Domínguez *et al*., 2007). It is effective in around 95% of cases and lasts for at least fifteen years and possibly a person's entire life (Ott *et al*., 2012). The WHO recommends vaccination for HAV prior to travel to countries with moderate or higher endemicity (Pedersini *et al*., 2016). Hepatitis A virus is spread from person-to-person by the fecal-oral route (El-dougdoug *et al*., 2017). Good hygiene, principally thorough hand washing after toilet use and before food preparation, is the cornerstone of prevention (Carrillo-Santisteve *et al*., 2017). For travelers to countries of high and intermediate endemicity care should be taken to avoid exposure to hepatitis A through contaminated food and water (Pedersini*etal*.,2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study site was Mpanda General Hospital, a level two referral hospital with a population of 228,807. The Mpanda General Hospital is located in Mpanda commune, Bubanza province in the northwestern region of Burundi in the plain of Imbo (Figure 3.1). Mpanda commune is predominantly rural (human settlement where community is more traditional and the rate of urbanization is quite slow) with a small urban area (human settlement where the rate of urbanization and industrialization is high) and a population of 58,913 (RGPH, 2008), whose main economic activity is agriculture. The rate of poverty is estimated at 72.5% of the households (ISTEEBU, 2014). The majority of households (89%) use latrines of the traditional type (pit) and only 22% of these latrines meet acceptable technical standards (Burundi MoH, 2011).

The proportion of Burundian inhabitants who have the access to clean water is estimated at 72% in 2008 (USAID, 2010). Moreover, even those that have access to drinking water generally do not receive it in sufficient quantity. The regional disparities remain important, some areas of Bugesera, Kumoso and Imbo are particularly defective (Burundi MoH, 2011).

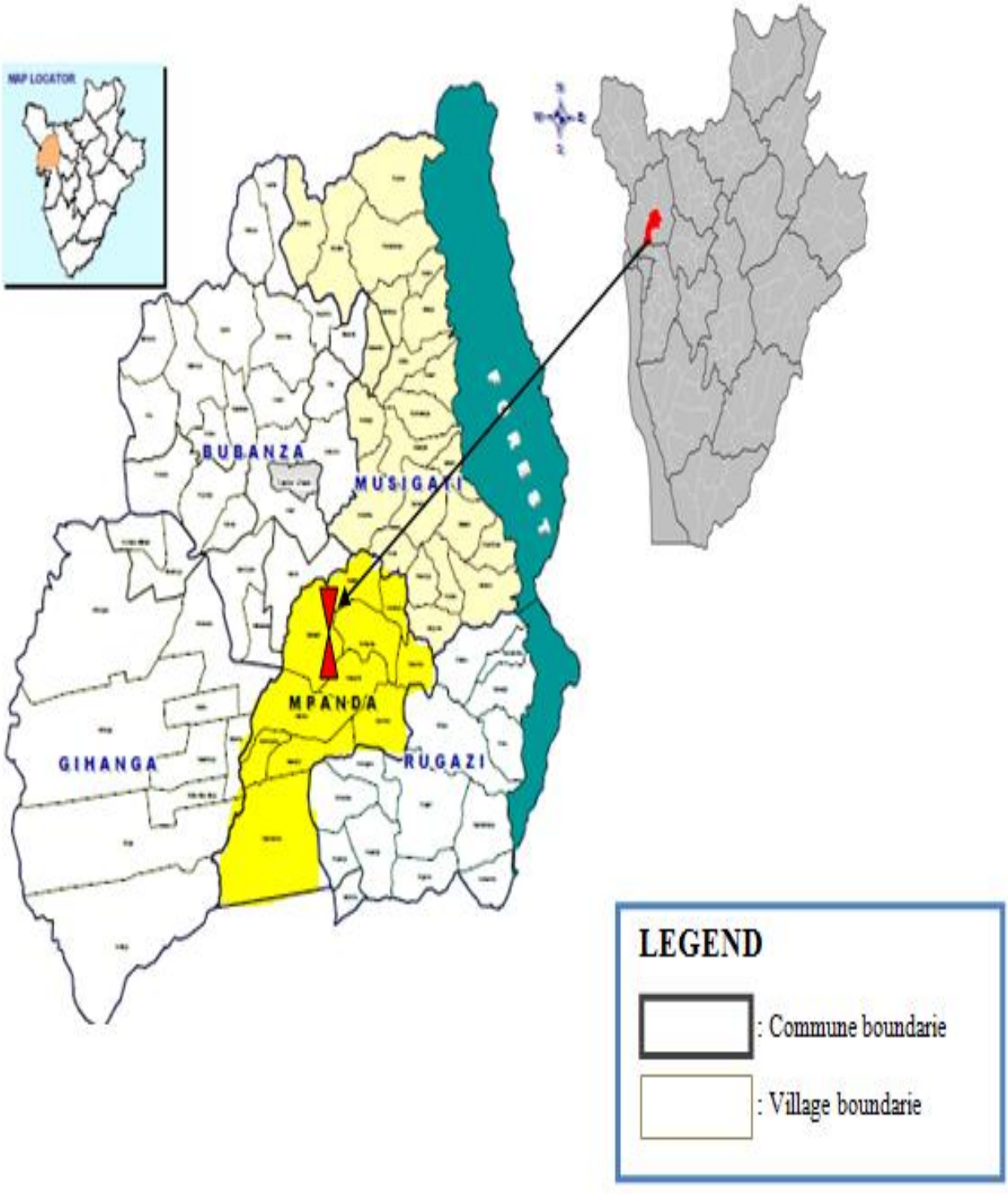


Figure 3.1: Mpanda geographic location (GIBU, 2004)

3.2 Study design

This was a cross-sectional study undertaken among the patients presenting with diarrhea at Mpanda General Hospital. The study was laboratory based and focused on patients with diarrheal of unknown etiologies. Data and blood samples were collected during three months (October to December 2017).

3.3 Study population

The population in this study was patients attending the Mpanda General Hospital with diarrhea (more than 3 stools per day) of unknown etiologies. All consenting and/or assenting participants were recruited in the study.

3. 3. 1 Inclusion criteria

i. All patients who attended Mpanda General Hospital with diarrhea of unknown etiologies during the period of the study
ii. All patients who were willing to give consent/assent

3.3.2 Exclusion criteria

i. All patients vaccinated against HAV
ii. Patients known as HAV positive
iii. Not willing to give consent

3.4 Sample size

The following formula was used to calculate the sample size (Cochran *et al*., 1977):

$$N = \frac{Z^2 \times (p) \times (1 - p)}{d^2}$$

N: minimum number of samples required; Z: z-statistics for the desired level of confidence (Z = 1.96 at 95% level of confidence). P: proportion in the target population estimated to have measured character (50%); d: is the desired level of precision which is 0.05 at 95% confidence level

$N = (1.96)^2 x 0.50 x (1- 0.50)/ (0.05)^2 =$ **384.16**

The minimum number of patients was 385.

3.5 Sampling procedure

The study participants were consecutively enrolled. Every patient who attended the hospital with diarrhea of unknown etiologies during the period of the study was selected until the sample size (385) was achieved. The recruitment of study participants was integrated within the day-to-day activities of the hospital.

3.6 Data and Specimen collection procedure

Before data and specimen collection, an informed consent/assent was signed by the participant (appendix 2). The following minimum information were included on the laboratory request form accompanying the specimen: Lab ID number, date of collection, age and gender of the subject (appendix 1). Microsoft Excel computer package was used for data entry and storage. Confidentiality of data was ensured throughout the period of the study and even after. This was achieved by making sure that patients names were not used, just ID numbers, only people involved in the study got access to the data, locking data files in a secure place and also putting pass words in the computers where data were entered.

3.6.1 Questionnaire

A standardized questionnaire was developed to record socio-demographic information (appendix 1). The questionnaire questions included residential area, age, family size, way of human waste disposal, water sources for human consumption and the clinical features. For adult subjects, the questionnaire was administered freely after seeking an informed consent (appendix 2). For children, permission was obtained from the parents or guardians.

3.6.2 Specimen collection

Five milliliters of whole blood were collected aseptically by venipuncture in EDTA tubes from all selected subjects. Before blood collection, the tube was labeled with

the subject identification code. After blood was collected, the plasma was separated from whole blood by centrifugation.

3.6.3 Transportation and storage of the blood samples

Plasma specimens in the storage bottles (cryotubes) were placed in a cool box and laboratory requisitions that accompanied the specimens were placed in the pocket of the handbag from the site of collection to Reference National Laboratory where they were stored at –80ºC until the time of laboratory investigations. Only IgM positive samples were transported under cold chain from Burundi to Center for Virus Research laboratory at KEMRI, Nairobi for molecular analysis.

3.7 Laboratory investigations

3.7.1 Serology for detection of anti HAV antibodies

The plasma samples were screened for anti-HAV (IgM and IgG) antibodies using an anti-HAV Rapid Test ″SD BIO LINE HAV IgG/IgM″ (Standard Diagnostics, Inc., Republic of Korea) and a qualitative Enzyme Immunosorbent Assay kit ″MONOLISA anti-HAV IgM/IgG″ (Bio-Rad Laboratories, Inc., Marnes-la-Coquette-France) was used for confirmation of the presence or absence of anti-HAV IgM and/or IgG antibodies according to the manufacturer's instructions as described previously (Guillermo, 2011). The results were scored as positive or negative according to the standard recommended procedures (Standard Diagnostics, Inc., Republic of Korea), (Bio-Rad Laboratories, Inc., Marnes-la-Coquette-France). Serological tests were performed in Burundi and only anti-HAV IgM positive samples were stored and then transported to Nairobi for molecular analysis.

3.7.2 Molecular analysis

3.7.2.1 Extraction of viral RNA

Nucleic acid was extracted from all samples that were screened positive for HAV IgM antibody. Hepatitis A Virus RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) as described previously (Beji-Hamza *et al*., 2015). Briefly, 560 µl of prepared Buffer AVL containing carrier RNA was pipetted

into a 1.5 ml microcentrifuge tube. A volume up to 140 µl of plasma was added to the Buffer AVL carrier RNA in the microcentrifuge tube. This was mixed by pulse-vortexing for 15 seconds. This was then incubated at room temperature (15-25°C) for 10 min. Then 560 µl of ethanol (96-100 %) was added to the sample, and mixed by pulse-vortexing for l5 seconds. At that stage, 630 µl of the extract was added to a QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 min and the supernatant discarded. The QIAamp spin column was placed into a clean 2 ml collection tube. The 500 µl of wash buffer (Buffer AW1) was added and centrifuged at 6000 x g (8000 rpm) for 1 min.

The supernatant was discarded and 500 µl of wash buffer (Buffer AW2) added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube. 60 µl of elution buffer (Buffer AVE) equilibrated to room temperature was added and incubated at room temperature for 1 min. This was then centrifuged at 6000 x g (8000 rpm) for 1 min to elute the viral RNA. This RNA was kept at -80^{o}C till use. The RNA was eluted in 60 µl of elution buffer.

3.7.2.2 HAV genome amplification

The RNA was reverse transcribed into cDNA using the FIREScript RT cDNA Synthesis KIT (Solis BioDyne, Riia 185a, 51014 Tartu, Estonia). Briefly, after incubation at 25°C for 5 min of RNA (5 µl), 1µl of primer (5µM), 0.5µl of dNTPs (500 µM), 2µl of RT Reaction Buffer (1x), 1µl of FIREScript RT (10 U/µl), 0.5µl of RiboGrip RNase inhibitor (1 U/µl), Nuclease free-H_2O up to 20µl, reverse transcription reaction was carried out at 50°C for 30 min, followed by inactivation at 85°C for 5 min. The cDNA was then amplified by nested polymerase chain reaction (PCR) of the 473-bp region of the VP1/P2A junction of the HAV genome as described in previous studies (Costafreda *et al.*, 2006; Forbi *et al.*, 2012). The primers used are presented in Table 3. 1. Both amplification reactions were performed in a Thermo Fischer Scientific thermocycler (Applied biosystems). The cycling conditions were 1 cycle of 95˚C for 5 min and 35 cycles of 95˚C for 30 s, annealing at 57˚C for 1 minute, and 72˚C for 2 minutes, and final extension of 72˚C

for 10 min (Beji-Hamza *et al*., 2015). From the first-round PCR products, 3 µL was used as a template to make 25µL for the second reaction volume with the second set of primers ; using the same cycling conditions

Table 3.1: Primers used for DNA amplification by RT-PCR (Forbi *et al*., 2012)

Primers	Sequences (5'-3')	Position
HAVF1 (outer, sense)	GGTTTCTATTCAGATTGCAAATTA	2890 -2913
HAVR1 (outer, anti-sense)	AGTAAAAACTCCAGCATCCATTTC	3397 -3374
HAVF2 (inner, sense)	TTGCAAATTACAATCATTCTG	2887-2907
HAVR2 (inner, anti-sense)	TTCAAGAGTCCACACACTTCT	3359-3339

3.7.2.3 Sequencing of PCR products

The amplicons from the nested PCR (PCR product of 7500bp) were sequenced from using the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Nieuwerkerk, Netherlands) following the manufacturer's instructions and an ABI 3730xl DNA Analyzer (Applied Biosystems). The same primers (HAVF2, HAVR2) for nested PCR were used in sequencing as previously described (Gouvea *et al*., 1990). Briefly, a reaction mixture containing the following reagents was used for sequencing PCR: Thirteen (13) µl RNase free double distilled H_2O,5x sequencing buffer,1.5 µl primers of 1 µM sense and antisense primer,1.0 µl big dye terminator and 1.0 µl PCR product making up a total reaction volume of 20 (Sane et al., 2015). The PCR tubes containing the samples were placed into the thermocycler under the following programmed conditions; Initial PCR activation step 96°C for 2 minutes; 30 cycles of: denaturation for 10 seconds at 96°C, annealing for 10 seconds at 50°C;

extension for 4 minute at 60°C and thereafter the samples were held at 4°C (Forbi *et al.*, 2013).

3.7.2.4 phylogenetic analysis

The Sequences derived in this study were subjected to BLAST and MEGA search tools to determine HAV genotypes. The sequences were aligned and gaps removed using BioEdit. The tree topology was obtained using the neighbor-joining method. A phylogenetic tree was constructed using neighbor joining method in MEGA 6 software (Singh *et al*., 2015). Alignment of DNA sequences was performed using the CLUSTALW program (http://www.clustal.org). The pairwise distance matrix was estimated using the Kimura two parameter model with the MEGA 6 software package (http://www.megasoftware.net). Bootstrap re-sampling (1000 data sets) of the multiple alignments was performed to set the statistical robustness of the tree. The tree profile was visualized with tree view PPC version 1.65 (Foley *et al*., 2015).

Genotyping was done after alignment of the sequences with reference strains using the Molecular Evolutionary Genetics Analysis (MEGA) version 6 software. Sequences generated were searched against the National Center for Biotechnology Information (NCBI) database using a Basic Local Alignment Search Tool (BLAST).

3.7.3 Discarding of specimens

After processing, the remaining specimens were discarded by first sealing the bottles and packing in biohazard bags and then incinerating after autoclaving at 121ºC for 15 minutes.

3.8 Data management

3.8.1 Data storage

Participants in the study were identified with their unique code known by only the investigator as well as the date of specimen collection. All the data obtained were recorded in a notebook, entered into a spreadsheet created in MS Excel where data were stored and protected using password known only by the principal investigator.

3.8.2 Data analysis

Data analysis was done using SPSS version 16.0 software (SPSS, Inc., Chicago, IL). The descriptive statistics was done to analyze socio-demographic factors and HAV seropositivity. The data was reported as percentages.

A bivariate logistic regression was used to adjust for odds of HAV cases associated with various demographic factors.

The results were expressed as odds ratios (OR) accompanying with p-values at 95% confidence intervals to test the statistical significance of differences observed between different proportions. A P values $\leq$ 0.05 was considered as an indicator of statistical significance.

3.9 Ethical consideration

Authorization to carry out this study was obtained from the Burundi Ministry of Health; the study protocol was approved by the Burundi National Ethical Committee (Appendix 3). Informed consent/assent was obtained from the patients before they were enrolled into the study.

CHAPTER FOUR

RESULTS

4.1 Characteristics of the participants

A total of 385 study participants were recruited. Males accounted for 142 (36.9%) while females constituted 243 (63.1%) of the study participants. Most, 332 (86.2%) of the participants were from rural areas against 53 (13.8%) from urban area.

The median age of the study participants was 23 years and the range 72 [2-74] years. Of the all collected samples, patients aged >18 years (adults) accounted for the majority 141 (36.6%), children aged 2-6 years accounted for 114 (29.6%) and children of 12-18 years account for 72 (18.7%) with the least age group being those 7-11 years accounting for 58 (15.1%).

Most of the study participants 200 (52%) used tap water for human consumption, followed by 136 (35.3%) and 49 (12.7%) who used water from streams and wells respectively. When grouped into three, 271 (70.4%) of the study participants lived in families of 5-10 members while 97 (25.2%) lived in families of less than 5 members and the rest, 17 (4.4%) lived in families of more than 10 members. The majority 336 (87.3%) of the study participants used traditional latrines while 49 (12.7%) used modern latrines for human waste disposal (Table 4.1).

Table 4.1: Characteristics of the study participants

Variables	Frequence	
Age	**N**	**n (%)**
2-6 years	114	29,6
7-11 years	58	15,1
12-18 years	72	18,7
Adults	141	36,6
Sex		
Femele	243	63,1
Male	142	36,9
Residence		
Rural	332	86,2
Urban	53	13,8
Water source		
Stream	136	35,3
Well	49	12,7
Tap	200	52
Latrine type		
Pit	336	87,3
Toilet	49	12,7
Family size		
<5 members	97	25,2
055-10 members	271	70,4
>10 members	17	4,4

4.2 Prevalence of HAV in patients attending Mpanda General Hospital

In a total of 385 samples, 232 were anti-HAV antibody positive in which anti-HAV IgM accounted for 124 and anti-HAV IgM represented 108. The overall prevalence of HAV was thus determined at 60.3%. The cases of HAV was significantly associated with age; HAV prevalence in participants with age of 2-6 years (85.1%) was significantly higher than that in participants with age of 7-11 years (70.7%) [OR = 7.22 (4.04-12.93), P <0.001] and adults (43.9%). The majority of participants (87.3%) used traditional toilets (pit) at home for human waste disposal with a high proportion of HAV seroprevalence (62.2%) compared to (46.9%) obtained from participants with modern toilets. These findings showed that HAV prevalence was significantly associated with the latrine's type used in households [OR = 1.86 (1.02-3.40), P = 0.04]. In terms of water usage, the infection was high among participants who reported using water from streams and wells accounting for 87.5% and 63.3% respectively in comparison with those who used tap water with a proportion of 41.0% [OR = 10.07 (5.63-18.01), P < 0.001]. Regarding the association between HAV and family size, Statistical analysis showed that there was no significant association between HAV prevalence and participant's family size [OR = 0.87 (0.54-1.40), P = 0.14]. In the same manner, no significant association found between the prevalence of HAV and the residence of participants [OR = 0.64(0.36-1.15), P = 0.14]. According to the sex of participants, no significant difference was observed between male and female in HAV prevalence [OR = 0.74(0.46-1.07), P = 0.10].

Table 4.2: Cases of HAV among patients attending Mpanda General Hospital in relation to socio-demographic characteristics

Variable	Frequency (%) N=385	Anti-HAV positive (%) n=232	OR	95% CI	P-Value
Age					
2-6 years	114(29.6)	97(85.1)	7.22	4.04-12.93	<0.001
7-11 years	58(15.1)	41(70.7)	3.05	1.63-5.71	<0.001
12-18 years	72(18.7)	32(44.1)	0.89	0.59-1.48	0.79
Adults	141(36,6)	62(43,9)			Ref.
Sex					
Female	243(63.1)	154(63.4)	0.74	0.46-1.07	0.10
Male	142(36.9)	78(54.9)			Ref.
Residence					
Rural	332(86.2)	205(61.7)	0.64	0.36-1.15	0.14
Urban	53(13.8)	27(50.9)			Ref.
Water source					
Stream	136(35.3)	119(87.5)	10.07	5.63-18.01	<0.001
Well	49(12.7)	31(63.3)	2.48	1.30-4.73	<0.001
Tap	200(52.0)	82(41.0)			Ref.
Latrine type					
Pit	336(87.3)	209(62.2)	1.86	1.02-3.40	0.04
Toilet	49(12.7)	23(46.9)			Ref.
Family size					
<5 members	97(25.2)	61(62.9)	0.87	0.54-1.40	0.75
5-10 members	271(70.4)	160(59.0)	1.10	0.37-3.23	0.86
>10 members	17(4.4)	11(64.7)			Ref.

N- The total number of samples, n- The number of anti-HAV positive samples, Ref. - Reference group, OR- Odds Ratio, CI- Confidence Interval.

4.3 Molecular characterization of HAV

4.3.1 Amplification of HAV genome and Sequence Analysis

In a total of 124 anti-HAV IgM positive samples, hepatitis A Virus RNA was found in nine samples which shown visible bands in agarose gel electrophoresis (plate 4.1). Phylogenetic analysis showed that all the nine samples were HAV genotype IB.

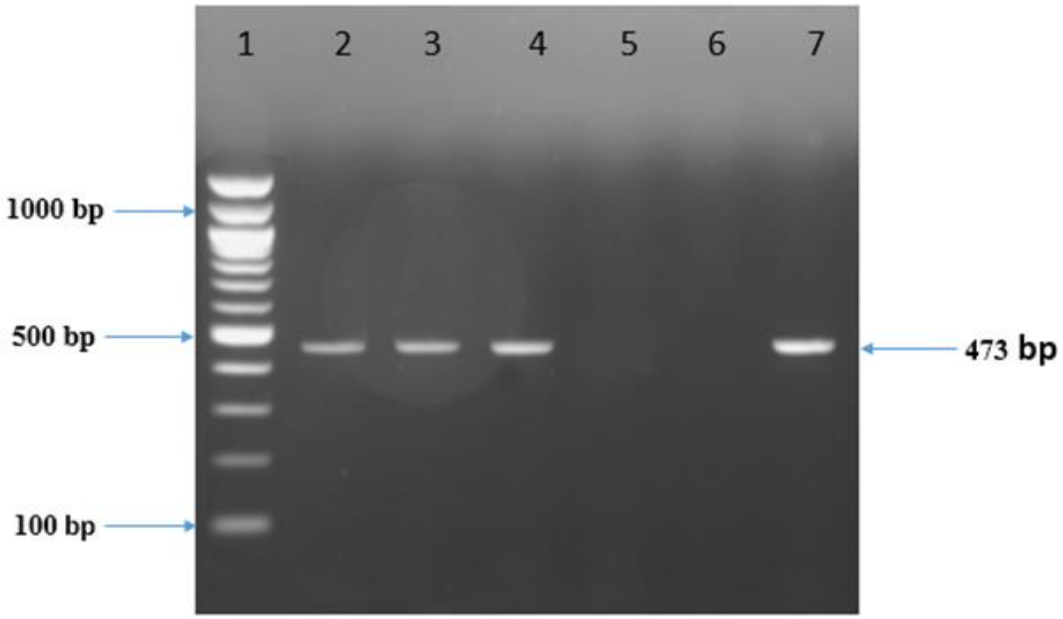


Figure 4.1: A sample of Agarose gel photo of amplified products

Line 1 represents the molecular weight marker, lanes 2, 3, 4 represent DNA positive samples with expected band of 473bp, lines 5, 6 represent DNA negative samples, and line 6 represents the positive control.

4.3.2 Phylogenetic Analysis

Genotype sequencing was performed on nine samples and all the nine were HAV IB genotype. Phylogenetic tree, illustrated with reference sequences labelled using their Gene bank accession numbers show that HAV IB clustered with those of other African countries such as Nigeria and Tunisia (figure 4. 1). The viruses from the Mpanda shared the same nucleotide sequence (≥ 95.5%) homology.

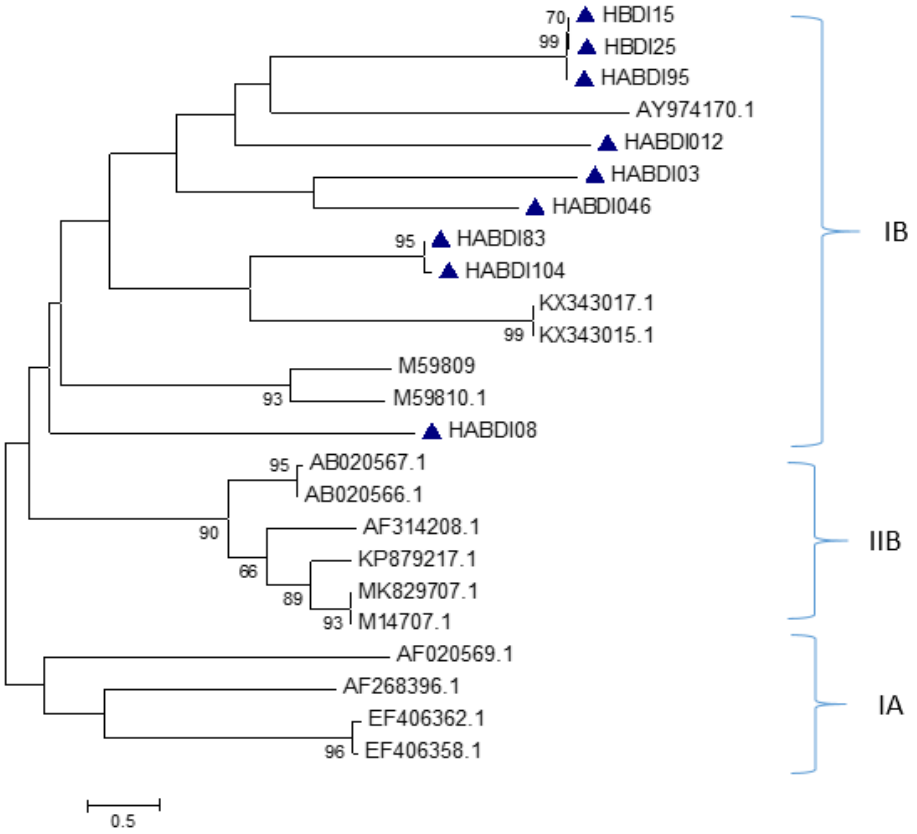


Figure 4.2: Phylogenetic tree of the nucleotide sequences of HAV circulating in Mpanda General Hospital

The diagram below depicts the degree of similarity between nucleotide sequences of HAV from Burundi and known HAV reference strains from GenBank database. HAV reference strains are indicated by accession numbers while HAV strains from this study are indicated by a filled triangle symbol followed by the sample number.

CHAPTER FIVE

DISCUSSION

5. 1 Prevalence of HAV in patients attending Mpanda General Hospital

In this study, the prevalence of Hepatitis A Virus was determined among patients attending Mpanda General Hospital in Burundi. The factors associated with HAV infection were younger age, non-availability of safe water source and traditional latrines.

The prevalence of Hepatitis A Virus infection (60.3%) was found to be high. However, it was also found to be lower than those obtained from other African countries such as Liberia 80%, Senegal 100%, and Cameroun 90% (Weis *et al*., 2017; Ibrahim, 2015). That high prevalence can be explained by the lack of clean water in that region, the majority of inhabitant use water from stream and well. Furthermore, the traditional latrines are more used than modern ones; flies can easily disseminate the viruses from fecal matter to the food or drink. It is noted that most of Africa remains a high endemicity region, with the exception of subpopulations in some areas (Franco, 2012). In developing countries, these infections are associated with poor hygiene and, in particular, the lack of clean drinking water and, in some areas, inadequate sanitation (Traoré *et al*., 2012). The findings of this study are in agreements with what was found in a study conducted in rural Amazonia (Vitral et al., 2014)

Mpanda district is predominantly rural with a small proportion of urban residence and experiences a lack of clean drinking water (PNDS, 2011-2015). Inhabitants of Mpanda District are at large suffer from diverse diseases of the poor sanitation, particularly diarrheal related diseases and other illnesses transmitted through feacal-oral route are common cases just like in others area of the world with similar characteristics and these may be attributed to insufficient water supply and poor sanitation (CDC, 2003).

5.2 Association between HAV cases and sociodemographic factors in Mpanda General Hospital patients

Age of the participants significantly affected the HAV prevalence with children of 2-6 years being more susceptible to HAV infection. The children are at a great risk of becoming exposed to the virus in their playing areas and chances of fecal oral contamination are also high in children than in adults. The initial increase in children may be due to increased contact with one another, beginning with enrolment in preschool daycare centers. These results are in agreement with what has been reported in south Tunisia in 2017 and Brazil (Neffatti *et al*., 2017) (Vitral et al., 2014). It is noted that the seroprevalence of HAV infection rates in the less developed regions and in several developing countries are common in the first years of life and are approaching 100% (Franco *et al*., 2012). Poor sanitation and lack of access to safe water usually lead to enteric infections in early childhood, consistent with the HAV age-prevalence data described in a study conducted in Brazil (Vitral et al., 2014).

A high proportion of those seropositive for HAV was obtained from subjects with traditional toilets (pit) at home (62.2%) compared to those obtained from participants with modern toilets at home (46.9%). This clearly shows that there is a very high relationship between the method of human waste disposal and HAV transmission. The relationship between sewage disposal and HAV seropositivity has been previously described (Afegbua *et al*., 2013).

In a study conducted among acute hepatitis patients at Kenyatta National Hospital in Nairobi, Kenya, it was found that all those positive for anti-HAV and anti-HEV antibodies used pit latrines in their households (Neffatti *et al*., 2017). This has been also reported previously in Northern Africa and elsewhere (Koroglu *et al*., 2017). Owing to the fact that humans are the main reservoirs for HAV and also the main transmission mode is fecal-oral route, absence of the modern toilet in some households reflects that there is likelihood of human waste being disposed poorly that, it could be possible the water sources and eventually the food.

The results of this study show that the seroprevalence of HAV infection did not differ significantly between males (54.9%) and females (63.4%), the difference in seroprevalence rates did not reach statistical significance (P>0.05). That may be due to hygienic and sanitation practices which are the same in male and female subjects. These results are in agreement with those found in a study conducted in Yemen, no gender-related difference in HAV seropositivity rate was observed (Afegbua *et al*., 2013). However, the findings of a study conducted in Nigerian in 2017 showed that HAV is more in males than in females (Okara *et al*., 2017).

Several studies have shown that the number of family members was not associated with the prevalence of HAV (Lazcano-Ponce *et al*., 2013; Mostafavi *et al*., 2016). These are in agreement with the findings of our study that may be explained by the small size of the participants who lived in families of more than 10 members.

The findings of this study demonstrated that there was no significant difference in Hepatitis A prevalence between rural (61.7%) and urban (50.9%) participants. That may be due to small sample size in participants from urban areas. Similar HAV infection trends in rural and urban residences have been reported previously in Mexico (Lazcano-Ponce *et al*., 2013). However, some studies have shown that there is a significantly higher proportion of HAV infection in rural areas than in urban areas (El-Gilany *et al*., 2010; Demiray *et al*., 2016). That has been reported previously in other studies (Miri; Alavian, 2017; Lee *et al*., 2009). The variation in the proportions obtained from the two locations definitely reflected the standards of public hygiene with particular reference to provision of clean water and efficient sewage disposal, which are deficient in rural areas.

5.3 Genotypes of HAV circulating in patients attending Mpanda General Hospital

Hepatitis A Virus RNA was detected in nine out of 124 IgM (7.3%) anti-HAV positive plasma samples. The percentage of HAV RNA positivity was lower than what one would have expected. This may have been due to collection of blood samples in most patients late in the course of the disease and this could possibly explain the disappearance of viremia. It could be due also to degradation of the

nucleic acid because the shipment of the samples took 4 days from Burundi to Nairobi in dry ice.

At present, there are no reports of the presence of HAV subgenotypes circulating in Burundi. Molecular characterization of HAV genotypes and subgenotypes is important for establishing evolutionary relationships and for understanding its origin or patterns of transmission. Burundi is a developing country located in sub-Saharan Africa and, as far as we know, knowledge of the specific HAV strains circulating in this population remains unknown. However, the few serological data reported suggest that the virus is endemic in region (Forbi et al., 2013).

In the present study, the viruses from the Mpanda shared the same nucleotide sequence homology. Phylogenetic analysis revealed that the nine sequenced samples belong to genotype IB. The VP1/2A nucleotide sequences of all Burundian isolates were 99.7% identical to each other and 96.4 to 97.6% identical to strains of the IB genotype. The presence of only a single genotype is in agreement with the study conducted in Egypt indicating that this might be the predominantly circulating genotype in the country (Sane et al., 2015).

Despite the limitations in terms of sample size, resources and time-scale, it can be said with some confidence that the results of this study provide a baseline for more research and closer work in establishing the magnitude of Hepatitis A in Burundi, if health is to be improved.

5.4 Limitation of the study

The samples used in this study were collected from October up to December 2017 and stored in Burundi before to be transported to Kenya in March 2018 for molecular analysis. Furthermore, the shipment of the samples from Burundi to Kenya took 4 days in dry ice. This implies possible degradation of the specimens during transport and storage since the percentage of HAV RNA positivity was lower compared to the screening results on field which shown higher rate of IgM anti-HAV antibodies positivity (32.21%). Furthermore, it is also possible that the primers used were not sensitive enough to detect all RNA positive samples.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. The prevalence of HAV infection in patients attending Mpanda General Hospital was determined at (60.3%).

2. The HAV infection was associated with younger age, lack of clean water and traditional latrines in both rural and urban areas while gender, residence and number of persons in the household did not significantly predict having HAV infection.

3. The all nine samples RNA positive from patients attending Mpanda General Hospital were found to belong to the genotype (IB) of HAV after gene sequencing.

6.2 Recommendations

In the view of present study findings, the following recommendations are made:

1. Initiation of the Hepatitis A Virus surveillance in the country
2. Including the Hepatitis A Virus in routine diagnosis
3. Improvements in sanitation and hygiene conditions
4. Further research involving a national sample is required to determine the overall prevalence and the genotypes of HAV in Burundi.

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APPENDICES

Appendix I: Questionnaire (In English)

a. PATIENT IDENTIFICATION

Patient code: ☐

***Age*:** ☐

Sex: ☐

Area of residence: urban ☐ rural ☐

Department: Inpatient ☐ Outpatient ☐

Complete address and contact:

……

……

b. RISK FACTORS

Water source: tape ☐ well ☐ stream ☐

***Type of latrine**:* pit ☐ toilet ☐ none ☐

Family size: ☐

c. CLINICAL FEATURES: Fever ☐ Diarrhea ☐

Jaundice ☐ Abdominal discomfort ☐

Fatigue ☐

Appendix II: Informed consent documents in English

Title: "Prevalence and Phylogenetic Analysis of Hepatitis A Virus in Patients Attending Mpanda General Hospital in Burundi."

Investigators: The study will be led by Rémy HARABANDI, Institute of Tropical Medicine and Infectious Diseases/Jomo Kenyatta University of Agriculture and Technology and will be supervised by Dr. Lihana Raphael/KEMRI and Dr. Eddy Odari/JKUAT.

Phone number: +257 79 994144/+254 799 049957

Introduction: This study is being performed as a survey to find out the victims of HAV infections and the extent of the disease in the community. You are requested to participate in this research study, and your participation is voluntary. The quality of your medical care, academic or job status will not be affected whatever by your participation in this study. This form tells you about the study, read it carefully and feel free to ask any questions you have at any time.

Purpose of the study: This study will help to establish the current situation in Mpanda commune with regards to HAV infections among patients attending Mpanda General Hospital. Also, it will help to come up with recommendations on how to reduce HAV transmission.

Procedures: The first phase of this study involves an interview, filling-in the questionnaires provided, and an anonymous blood test. This will take approximately less than 20 minutes. The interview will be confidential and names will not appear on the form. Your opinion is very valuable to the investigator. Well, you do not have to answer every question if you do not wish to. You will also not be asked to return to the hospital. However, you may voluntary come back after a given period as directed by the researcher, in case you may be interested to know your status. After the interview and filling-in the questionnaire, you will be asked to provide a 5 ml of blood sample. This blood will be tested anonymously for HAV.

Benefits: The participant may not benefit directly from this study. However, based on the study outcome, necessary recommendations will be made in order to mitigate

the problem in the area particularly with regards to diagnostic, preventive and treatment measures.

Risks: The only discomfort is a blood test; you may experience mild pain when blood sample is being obtained. However, the pain will fade away after a short moment. Information obtained on the questionnaire will be confidential and it is not easily to be traced back to the participant.

Confidentiality: The samples will be assigned a code number and the key to the code will be maintained by the principal investigator. Data will be help in folders, which will be locked in cabinets for storage throughout the study period. Computer documents will have passwords only accessible to the researcher. All the information gathered by the researcher will be used in confidence for the sole purpose of this research only.

Costs and Compensation: Your participation in the study will not take long time. You will not pay for the study procedure and you will not be compensated for the specimen.

New findings: Results will be disseminated to the Ministries of Health before being published in scientific journal.

Right to refuse or withdraw: You may withdraw your consent for you and for your child and you may withdraw from the study at any time without it affecting your rights in any way. **Questions:** If you have questions, please ask. If you have any additional question later, please contact the researcher Rémy HARABANDI on +25779994144/+254799049957, or on e-mail *remabandi@gmail.com*.

If you have any questions about the rights of you and/or your child as a research participant you may contact the chairperson of Burundi National Ethical Committee (BNEC), Dr. Jean Baptiste SINDAYIRWANYA on: +257 79 921 173 or +257 22 226 961.

English informed parental consent form for children of 2-12 years old

I have read this consent form and I have been given the opportunity to ask questions and all my questions have been responded to my satisfaction. I have received a copy of this consent form.

I ……. (Name of the parent/guardian) a parent/guardian of ……... I give permission to Mr. Rémy HARABANDI for my child to participate in this study.

Signature (or thumb print) of parent…. ……

Date …….. ……..

Researcher signature…….

Date …….. ……..

Signed, informed assent form for minors aged 13-17 years old

I have read this consent form and I have been given the opportunity to ask questions and all my questions have been responded to my satisfaction. I have received a copy of this consent form.

I……., do hereby agree to participate in the proposed research study led by Mr. Rémy HARABANDI. I have been given all the necessary information to understand the purpose and nature of this research study.

Minor's signature……..

Researcher signature…….

Date……..……..…….

Informed consent for adult to participate in the study

I have read this consent form and I have been given the opportunity to ask questions and all my questions have been responded to my satisfaction. I have received a copy of this consent form.

I……, I accept to participate in this study.

Signature (or thumb print) of participant ……..

Date ……

Signature of researcher: …….

Date …….. ……..

Appendix III: Ethical approval

BURUNDI NATIONAL ETHICAL COMMITTEE FOR THE PROTECTION OF HUMAN BEINGS PARTICIPANTING IN BIOMEDICAL AND BEHAVIORAL RESEARCH

Bujumbura, 14th October 2017

FROM: BURUNDI NATIONAL ETHICAL COMMITTEE

TO: Rémy HARABANDI (Principal Investigator)

RE: Decision of the Burundi National Ethical Committee

Dear Sir,

The Burundi National Ethical Committee analysed your research project «**prevalence and molecular characterization of Hepatitis A Virus in patients attending Mpanda General Hospital in Burundi**».

After analysis of the Ethical aspects of the project in accordance with international regulations in this field, the National Ethical Committee has approved the project. The approval is valid for a period of one year from 14th October 2017 to 13th October 2018.

However, note that any changes to the protocol must be reported to the Burundi National Ethical Committee prior to implementation. This includes changes to research design or procedures that could introduce new or more than minimum risk to human subjects.

You may embark on your study.

Sincerely

Comité National d'Ethique du Burundi

Prof. Jean Baptiste SINDAYIRWANYA

THE BURUNDI NATIONAL ETHICAL COMMITTEE PRESIDENT

Appendix IV: Publication in peer reviewed journal

International Journal of Research in Medical Sciences
Harabandi R et al. Int J Res Med Sci. 2019 Jan;7(1):135-140
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Original Research Article

Prevalence of hepatitis A virus in patients attending a referral hospital in Bubanza Province, Northwest Burundi

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ABSTRACT

Background: Viral hepatitis is a public health problem wide world. Hepatitis A, transmitted by fecal-oral route, is an infectious viral disease caused by hepatitis A virus and mainly due to poor sanitation. This study aimed to determine the prevalence of hepatitis A virus and associated factors in patients attending Mpanda referral hospital in Northwest Burundi.

Methods: A cross-sectional study was done from November 2017 to January 2018 on 385 participants aged 2 years and above. Participants were recruited using a systematic random sampling technique. Data were collected using questionnaire from consented/assented participants. Five millilitres of venous blood was collected and analyzed. Anti-hepatitis A virus antibodies were screened using Enzyme Immuno Assay. Data were analyzed using Statistical Package for the Social Sciences version 16.0 software. A descriptive analysis was followed by bivariate analysis using a Chi-square test for comparison of various sub-groups with 5% statistical significance level. Odds ratio and 95% Confidence Intervals were calculated and presented.

Results: The median age of the participants was 23 years and the range 72 years. The overall prevalence of Hepatitis A virus was 60.3%. There was a significant association between age [OR=7.22 (4.04-12.93), P <0.001], lack of clean water [OR=10.07 (5.63-18.01), P <0.001], traditional latrines [OR=1.86 (1.02-3.40), P=0.04] and Hepatitis A Virus seroprevalence.

Conclusions: Present study shows high prevalence of HAV infection in patients attending Mpanda Referral Hospital. Younger age, lack of clean water and traditional latrines play roles in increasing prevalence of HAV infection in both rural and urban areas.

Keywords: Burundi, Hepatitis A virus, Prevalence