بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Prevalence Rate of Malaria/ Hepatitis C and Hepatitis B Virus Co-infections in Saad Rashwan Health Center in Omdurman City-Khartoum State

معدل إنتشار العدوى المشتركة للملا ريا والتهاب الكبد الفيروسي ج و ب في مركز صحي سعد رشوان في مدينة امدرمان- ولاية الخرطوم

A dissertation submitted in partial fulfillment for the requirements of the degree of M.Sc. in Medical Laboratory Science (Parasitology and Medical Entomology)

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الآيـــــة

بسم الله الرّحمن الرّحيم

﴿اللّهُ لاَ إِلَـهَ إِلاّ هُوَ الْحَيُّ الْقَيُّومُ لاَ تَأْخُذُهُ سِنَةٌ وَلاَ نَوْمٌ لّهُ مَا فِي السّمَاوَاتِ وَمَا فِي الأَرْضِ مَن ذَا الّذِي يَشْفَعُ عِنْدَهُ إِلاّ بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلاَ يُحِيطُونَ بِشَيْءٍ مّنْ عِلْمِهِ إِلاّ بِمَا شَاء وَسِعَ كُرْسِيُّهُ السّمَاوَاتِ وَالأَرْضَ وَلاَ يَؤُودُهُ حِفْظُهُمَا وَهُوَ الْعَلِيُّ الْعَظِيمُ ﴾

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Dedication

To my greatest persons in my life

My mother and my father

Who always hope to see me the best in everything

To my lovely husband

Who always support me incorporeally and critically

To my brothers

Who are stand with me step by step

To my lovely son (Muaid)

I dedicate this work

Acknowledgement

Thanks firstly and finally to Allah Almighty for blessing and giving us the power to complete this research.

I would like to express my thanks and gratitude to my supervisor Dr.Tayseer Elamin for her advices, enthusiasm, help and endless guide.

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Also, greatest thanks to my aunt for her support incorporeally.

Special thanks to my best friend Marwa Mohammed Al-Hag for her help and support.

Abstract

This cross-sectional study was conducted in Saad Rashwan health center in Omdurman city during the period from June 2016 to April 2017. The aim of this study was to determine the prevalence rate of malaria/ hepatitis C and hepatitis B virus co-infections in the study area.

Two hundred subjects were included in this study; ranged between 1-90 years old, of mean age was 27.6 ± 20.9 years. Males were 102 (51%) while females were 98 (49%). Two hundred blood samples were taken from all subjects. Parasitological and Epidemiological data were obtained and recorded. Two hundred blood samples were examined to detect *Plasmodium* spp in stained thick and thin blood smears. Out of 200 blood samples, 64 (32%) were positive for *P.falciparum*.

Anti-hepatitis-C and anti-hepatitis-B viruses were detected by using immune chromatography test (ICT). Out of 200 blood samples, 5 (2.5%), 16 (8%) were positive for hepatitis C and hepatitis B respectively.

When the results were analyzed, the study showed that there is significant relationship between malaria and hepatitis-C virus (p.value= 0.020) and there is no relationship between malaria and hepatitis-B virus (p.value=0.081).

مستخلص الدراسة

أجريت هذه الدراسة المستعرضة في مركز صحي سعد رشوان في مدينة امدرمان خلال الفترة من يونيو 2016 وحتى ابريل 2017. وكان الهدف من هذه الدراسة هو تحديد معدل انتشار العدوى المشتركة للملا ريا والتهاب الكبد الفيروسي ج و ب في منطقة الدراسة.

تضمنت الدراسة 200 شخص, تراوحت أعمارهم بين 1-90سنة، ومتوسط العمر كان 27.6±20.9 سنة. الذكور كانوا 102 (51%) بينما الإناث 98 (49%). 200 عينة دم أخذت من الأشخاص الخاضعين لهذه الدراسة. البيانات الطفيلية والوبائية تم أخذها وتسجيلها.200 عينة دم تم فحصها لتحديد أنواع البلازموديوم في مسحات الدم المصبوغة السميكة والخفيفة. من أصل 200 عينة دم, 64 (32%) كانت ايجابية للبلازموديوم فالسبرم.

الأجسام المضادة لفيروس التهاب الكبد ج والأجسام المضادة لفيروس التهاب الكبد ب تم التعرف عليها باستخدام الاختبار المناعي اللوني (ICT). من أصل 200 عينة دم, 5 (2.5٪) و 16 (8٪) كانت ايجابية لفيروس التهاب الكبد ج و ب على التوالي.

عند تحليل النتائج أظهرت الدراسة أنه توجد علاقة بين الملاريا والتهاب الكبد الفيروسي ج (القيمة المعنوية=0.020) و لا توجد علاقة بين الملاريا والتهاب الكبد الفيروسي ب (القيمة المعنوية=0.081).

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Chapter one

Introduction and literature review

1.1 Introduction:

Malaria is the most important tropical disease known to man. It remains a significant problem in many tropical areas, especially in sub-Saharan Africa. Malaria is spreading as a result of environmental changes, including global warming, civil disturbances, increasing travel and drug resistance (Greenwood, 1997).

Hepatitis word is derived from the Greek hepar, meaning "liver", and itis, meaning inflammation. The most endemic regions are in sub-Saharan Africa and East Asia where as much as 10% of adults are chronic carriers (WHO, 2015). Hepatitis is a group of diseases characterized by inflammation of the liver. There are five main types of viral hepatitis type A, B, C, D, and E. Hepatitis A and E are mainly spread by contaminated food and water. While hepatitis B and C are most commonly spread by sex, during childbirth, or infected blood such as may occur during needle sharing during intravenous drug use (WHO, 2015). Worldwide in 2015 chronic hepatitis B affects about 343 million, and chronic hepatitis C about 142 million people (WHO, 2015). Endemic regions for malaria are also endemic for hepatitis B and C that might affect the malaria infection (Boraschi *et al.*, 2008). Three infections sharing an intra-hepatic stage as part of their life cycles, interactions between the three pathogens have been proposed to occur at both immunological and cellular levels (Barcus *et al.*, 2002).

1.2 Literature review:

1.2.1 Malaria:

1.2.1.1 Introduction:

Malaria represents one of the oldest documented diseases of humans, and even today, organisms in the genus *Plasmodium* kill more people than

does any other infectious disease. More than 300-500 million individuals throughout the world are infected with malaria, and 1.5-2.7 million people a year, most of whom are children, are being killed by the disease. The African countries, where 90% of the malaria deaths occur (Marshall, 1991). Human malaria is a caused by four species of genus *Plasmodium*, *P.falciparum* is the most virulent. The other species are *P.vivax*, *P.ovale*, *P.malariae* (Srinivas, 2015).

1.2.1.2 Transmission:

Malaria transmission most often occurs through the bite of an *Anopheles* mosquito. No other types of mosquitoes are known to transmit this disease. This type of mosquito becomes infected with one of the four *Plasmodium* parasites that cause malaria in humans, through a previous blood meal from an infected person. Because the malaria parasite is found in red blood cells, transmission may also occur through contact with infected blood. This can occur through a blood transfusion, an organ transplant, the shared use of needles or syringes that are contaminated with blood, or may also transmitted from a mother to her fetus, before or during delivery (congenital malaria) (Cheesbrough, 1987).

1.2.1.3 Life cycle:

The vector for malaria is the female *Anopheles* mosquito. When the vector takes a blood meal, sporozoites contained in the salivary glands of the mosquito are discharged into the puncture wound. Within an hour, these infective stages are carried via the blood to the liver, where they penetrate hepatocytes and begin to grow, thus initiating the pre-erythrocytic or primary exo-erythrocytic cycle. Detailed study of sporozoite entry into the hepatocytes indicates that the process involves parasite-encoded surface proteins and host molecules (Garnham, 1984). The sporozoites become round or oval and begin dividing repeatedly. This schizogony results in large numbers of exo-erythrocytic merozoites.

Once these merozoites leave the liver, they invade the red blood cells (RBCs), thus initiating the erythrocytes cycle. It has been reported that a secondary or dormant schizogony may occur in *P.vivax* and *P.ovale* organisms, which remain quiescent in the liver until a later time. These resting stages have been termed hypnzoites (Garnham, 1984). Delayed schizogony does not occur in *P.falciparum* and probably does not occur in *P.malariae* (Beaver *et al.,* 1984).

The situation in which the RBCs infection is not eliminated by the immune system or by therapy and the numbers in the RBCs begins to increase again with subsequent clinical symptoms is called a recrudescence. All species may cause a recrudescence. The situation in which the erythrocytic infection is eliminated and a relapse occurs later because of a new invasion of the RBCs from liver merozoites, called a recurrence or true relapse, theoretically occurs only in *P.vivax* and *P.ovale* infection (Garnham, 1984).

Once the RBCs and reticulocytes have been invaded, the parasites grow and feed on hemoglobin. Within the RBCs, the merozoite (or young trophozoite) is vacuolated, ring shaped, more or less ameboid, and uninucleate. The excess protein, an iron porphyrin, and hematin left over from the metabolism of hemoglobin combine to form malarial pigment (some workers use the term "hemozoin"). Once the nucleus begins to divide, the trophozoite is called a developing schizont. The mature schizont contains merozoites (whose number depend on the species), which are released in to the bloodstream. Many of the merozoites are destroyed by the immune system, but others invade RBCs, in which a new cycle of erythrocytic schizogony begins. After several erythrocytic generations, some of the merozoites do not become schizonts but, rather, begin to undergo development into the male and female gametocytes (Garnham, 1984).Whether this development is predetermined genetically.

It is likely that cells are committed to sexual development in the preceding round of asexual schizogony within the pre-erythrocytic schizont rather than differentiating following invasion of RBCs by uncommitted merozoites. The asexual and sexual forms just described circulate in the bloodstream during infections by three of *Plasmodium* species. However in *P.falciparum* infections, as the parasite continues to grow, the RBC membrane becomes sticky and the cells tend to adhere to the endothelial lining of the capillaries of the internal organs (Garnham, 1984). Thus, only the ring forms and the gametocytes (occasionally mature schizonts) normally appear in the peripheral blood. If gametocytes are ingested when the mosquito takes a blood meal, they mature into gametes while in the mosquito gut. The male microgametes undergo nuclear division by a process called ex-flagellation, in which the microgametes break out of the RBCs, become motile and penetrate the female macrogamete, with the fertilized stage begin called the zygote (Garnham, 1984). Thus zygote becomes elongate and motile and is called the ookinete. This stage migrates to the mosquito midgut, secretes a thin wall, and grows into the oocyst, which extends into the insects hemocele. Within a few days to 2 weeks, the oocyst matures, with the formation of hundreds of sporozoites. When the oocyst ruptures, the sporozoites are released into the hemocele and dispersed throughout the body, and some make their way into the salivary glands. When the mosquito next takes a blood meal, the sporozoites are injected with saliva into the host (Garnham, 1984) (figure 1.1).

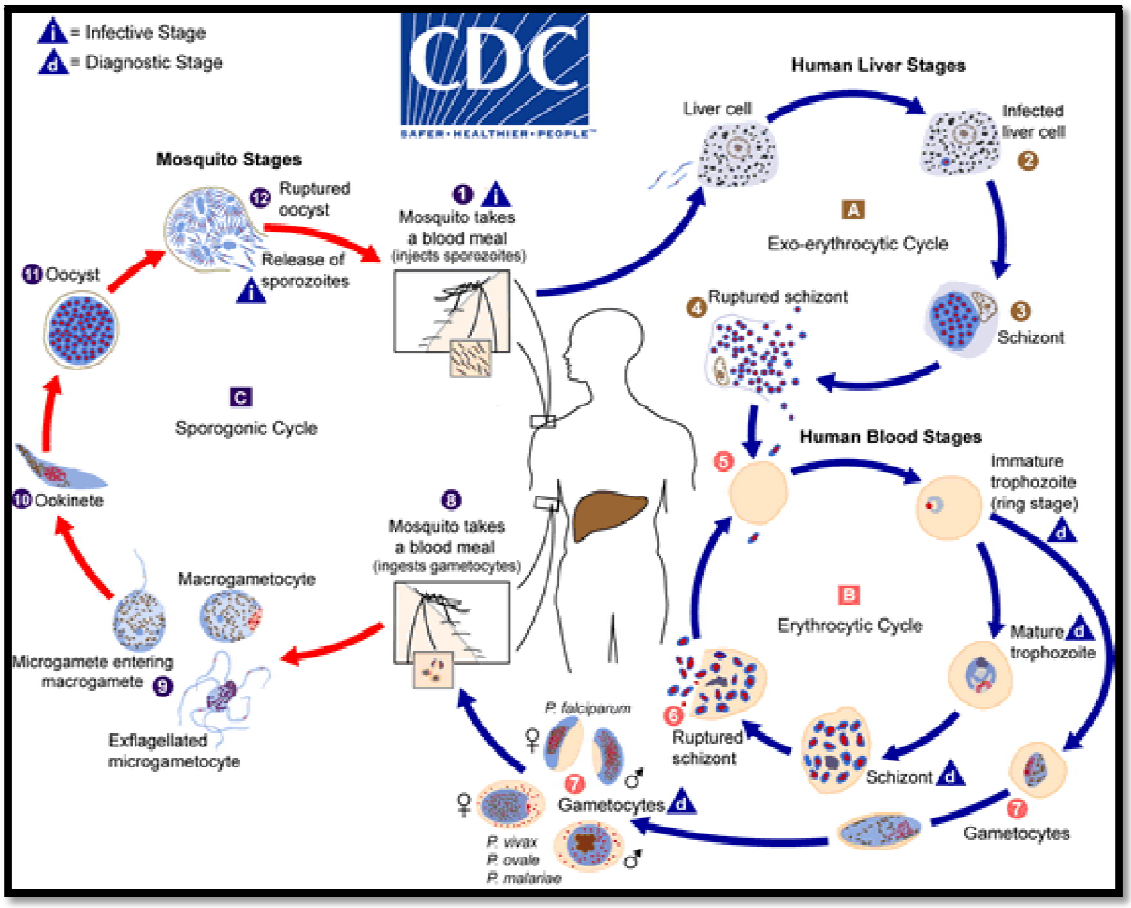

Figure (1.1): Malaria life cycle (CDC, 2016)

1.2.1.4 Pathogenesis:

The pathogenesis affect of malarial infection have been considered to be directly related to hemolysis of infected red blood cells, liberation of the metabolites of parasites, and the immunogenic pigments (Markell *et al.*, 1999). *P.vivax, P.ovale, P.malariae* and uncomplicated *P.falciparum* have similar features with fever rigors, headache, muscle aches, malaise and anorexia. Anemia may develop and liver and spleen may become enlarged. Because clinical appearance is non-specific, malaria may be misdiagnosed (Markell *et al.*, 1999). The hallmark of *P.falciparum* malaria is the sequestration of infected erythrocytes within the capillaries and post capillary venules in the brain, lung, heart, bone marrow, kidney, liver, pancreas, intestine and other organs, and the intravillous spaces of the placenta (Gillespie and Pearson, 2003 and Laveran, 2001). The slower blood flow and low oxygen tension provides a favorable environment for further parasite development. Sequestration also allows mature parasites to avoid passage through the spleen and likely clearance. *P.vivax* and *P.malariae* do not sequester, do not cause microcirculatory obstruction and are rarely fatal (Gillespie and Pearson, 2003 and Laveran, 2001). In addition to adhesion to vascular endothelium, infected erythrocytes can adhere to uninfected erythrocytes and clumps or layers of erythrocytes are sometimes observed extending into the vessel lumen in cerebral malaria. Adherence of trophozoite and schizont-infected erythrocytes in target organs appears to be a major feature of the pathophysiology of *P.falciparum* malaria. As parasites mature, the infected erythrocytes become more rigid, less deformable, and changes occur in parasite and host surface proteins (Gillespie and Pearson, 2003 and Laveran, 2001).

1.2.1.5 Clinical features:

In people with malaria, symptoms typically begin to develop 10 to 30 days after infection. They can range from mild symptoms to severe disease, and even death (Waitumbi *et al.,* 2010).

Clinical features appear as paroxysm which includes three stages:

1. Cold stage: characterized by rigor and headache. The patient feels cold and shivers even though his or her temperature is rising.

2. Fever stage: in which the temperature rises to its maximum and the back and joints and often vomiting and diarrhea.

3. Sweating stage: in which the patient perspires, the temperature falls and the headache and other pains are relived until the next rigor (Cheesbrough, 1987).

Early symptoms of malaria are generally non-specific such as fever, sweats, shaking chills, headaches, tiredness, muscle aches, nausea, vomiting, and diarrhea. More complicated diseases lead to sever malaria symptoms which include: Kidney failure, seizures, mental confusion, coma, severe anemia, fluid in the lungs (pulmonary edema), acute respiratory distress syndrome (ARDS), and bleeding due to blood clotting problems and death (Waitumbi *et al.,* 2010).

1.2.1.6 Complications:

P.vivax, *P.ovale* and *P.malariae* are relatively benign, and complications that arise during the course of infection with one of these parasites are usually due to debility or undercurrent disease (Markell *et al.,* 1999). Infection with *P.falciparum* can rapidly build up to level not obtained with other 3 species and because of physiologic characteristic of red blood cells infected with *P.falciparum*, may lead to localized capillary obstruction, decreased blood flow, tissue hypoxia, infraction and death (Markell *et al.,* 1999). Chronic *P.malariae* infection may result in immune complex deposition on glomerular walls, leading to nephrotic

syndrome (Markell *et al.,* 1999). In most cases, malaria deaths are related to one or more of these serious complications:

1.2.1.6.1 Cerebral malaria:

If infected red blood cells block small blood vessels of brain, swelling of brain or brain damage may occur. Cerebral malaria may cause coma (Waitumbi *et al.,* 2010), and it may be produced by hypoglycemia, hypoxia and lactic acidosis (Guerrant *et al.,* 2001).

1.2.1.6.2 Severe malaria:

Malaria damages red blood cells due to lysis by mature asexual intra erythrocytic parasites (schizont), which can result in severe anemia (Waitumbi *et al.,* 2010).

1.2.1.6.3 Renal failure:

Comes with urine output < 400 ml/ 24 hours in adults (< 12 ml/ kg/24 hours in children), and a serum creatinine > 265 mol/ 1(> 3.0 mg/dl) despite adequate volume repletion (Srinivas, 2015).

1.2.1.6.4 Pulmonary edema or adult respiratory distress syndrome (ARDS):

Pulmonary edema may develop rapidly in an oliguric anuric patient; it may develop without evidence of fluid retention or cardiac decompensation, possibly as the result of disseminated intravascular coagulation (DIC) (Markell *et al.,* 1999).

1.2.1.6.5 Tropical splenomegally syndrome:

Splenomegally of unknown reason appear most commonly occur in children with chronic malaria, it is characterized by elevated immunoglobulin M (IgM) level and infiltration of the liver sinusoids with B cells (Waitumbi *et al.,* 2010).

1.2.1.6.6 Black water fever "haemoglopinuria":

Macroscopic black, brown or red urine; not associated with effects of oxidant drugs or enzyme defects (like glucose-6-phosphate dehydrogenase (G6PD) deficiency) (Srinivas, 2015).

1.2.1.6.7 Hypoglycemia:

Severe forms of malaria itself can cause low blood sugar, as can `quinine, one of the most common medications used to combat malaria. Hypoglycemia due to decreased oral intake, decreased appetite from the acute malaria illness, depletion of liver glycogen and parasite consumption of glucose (Guerrant *et al.,* 2001).

1.2.1.6.8 Hyperparasitemia:

Five percent parasitized erythrocytes or > 250,000 parasites/µl (in non immune individuals) (Srinivas, 2015).

1.2.1.6.9 Metabolic (lactic) acidosis:

Metabolic acidosis is defined by an arterial blood pH of < 7.35 with a plasma bicarbonate concentration of < 22 mmol/L; and lactic acidosis is characterized a pH < 7.25 and plasma lactate >5 mmol/L (Srinivas, 2015).

1.2.1.6.10 Hypotension and shock:

Systolic blood pressure being < 50 mmHg in children between 1-5 years, or <70 mmHg in patients ≥ 5 years; cold and clammy skin or core-skin temperature difference > 100Cº (Srinivas, 2015).

1.2.1.6.11 Repeated generalized convulsions:

Three or more generalized seizures within 24 hours (Srinivas, 2015).

1.2.1.7 Epidemiology and distribution:

Malaria is one of the most important public health problems in term of morbidity and mortality, causing more than 200 million cases and 655,000 deaths every year (World Malaria Report, 2011). *P.falciparum* is the most prevalent malaria parasite on the African continent. It is

responsible for most malaria-related deaths globally. *P.vivax* has a wider distribution than *P.falciparum*, and predominates in many countries outside of Africa (World Malaria Report, 2011). Sub-Saharan Africa carries a disproportionately high share of the global malaria burden. In 2015, the region was home to 88% of malaria cases and 90% of malaria deaths. Between 2000 and 2015, malaria incidence rates (new malaria cases) fell by 37% globally, and by 42% in Africa. During this same period, malaria mortality rates fell by 60% globally and by 66% in the African Region. In that same period, malaria death rates among populations at risk fell by 60% globally among all age groups, and by 65% among children under five years (WHO, 2015).

1.2.1.8 Laboratory diagnosis:

The blood smear remains the gold standard for detection, for speciation, for parasite count and for identification of different forms of the parasites. The quantitative buffy coat (QBC) test performed by trained personnel could match a thick blood smear for detection of malaria. In thin films the red blood cells are fixed so the morphology of the parasitized cells can be seen and species identification can be made. However, malaria parasites may be missed on a thin blood film when there is a low parasitemia (Cuomo *et al.,* 2009**).** Therefore, examination of a thick blood film is recommended. With a thick blood film, the red cells are approximately 6-20 layers thick which results in a larger volume of blood being examined (Cuomo *et al.*, 2009). Films should be made without delay since morphological alteration of parasites occurs with storage of EDTA-anticoagulated blood (Cuomo *et al.*, 2009). The parasites density should be estimated after malaria parasites are detected on a blood smear, the parasite density can then be estimated from the percentage of infected RBCs, after counting 500 to 2000 RBCs (Padley *et al.*, 2003). In addition to microscopy, other laboratory diagnostic tests are available. Several

antigen detection tests (rapid diagnostic tests or RDTs) using a cassette format exist. RDTs can more rapidly determine that the patient is infected with malaria, but they cannot confirm the species or the parasitemia (Padley *et al.,* 2003). Polymerase chain reaction (PCR) using to detect parasite nucleic acid, PCR is at least 10-fold more sensitive than microscopy (Padley *et al.,* 2003) and specific than microscopy but can be performed only in reference laboratories and so results are not often available quickly enough for routine diagnosis. However, PCR is a useful tool for confirmation of species and detecting of drug resistance mutation (Padley *et al.,* 2003).

1.2.1.9 Treatment:

Malaria can be a severe potentially fatal disease (especially when caused by *Plasmodium falciparum*) and treatment should be initiated as soon as possible.

Patients who have severe *P. falciparum* malaria or who cannot take oral medications should be given the treatment by continuous intravenous infusion.

Most drugs used in treatment are active against the parasite forms in the blood (the form that causes disease) and include: chloroquine, atovaquone-proguanil (Malarone), artemether-lumefantrine (Coartem), mefloquine (Lariam), quinine, quinidine doxycycline (used in combination with quinine), clindamycin (used in combination with quinine), artesunate, and primaquine is active against the dormant parasite liver forms (hypnozoites) and prevents relapses. Primaquine should not be taken by pregnant women or by people who are deficient in G6PD (glucose-6-phosphate dehydrogenase). Patients should not take primaquine until a screening test has excluded G6PD deficiency (CDC, 2016).

Treatment of a patient with malaria depends on:

- The type (species) of the infecting parasite.
- The area where the infection was acquired and its drug-resistance status.
- The clinical status of the patient.
- Any accompanying illness or condition.
- Pregnancy.
- Drug allergies, or other medications taken by the patient (CDC, 2016).

1.2.1.10 Malaria control:

- Diagnosis and treatment.
- Prevention of infection through vector control (use of insecticide-treated mosquito nets.
- Prevention of disease by administration of antimalarial drugs to particularly vulnerable population groups such as pregnant women (World Malaria Report, 2015).

1.2.1.11 Malaria in Sudan:

There is a high burden of malaria morbidity and mortality in Sudan. Malaria incidence in Sudan was estimated to be about 9 million episodes in 2002 and the number of deaths due to malaria was about 44,000. 2,877,000 DALYs (Disability Adjusted Life Years) were lost in Sudan in 2002 due to malaria mortality, episodes, anemia and neurological sequelae. Children under 5 years of age had the highest burden. Males had the highest incidence and mortality, but females lost more DALYs (Abdalla *et al.*, 2007). According to World Malaria Report in 2015, the high transmission was in south-west of Sudan, lower transmission was in south and south-east of the country (World Malaria Report, 2015).

1.2.2 Hepatitis:

1.2.2.1 Introduction:

Hepatitis is an inflammation of the liver. The condition can be self limiting or can progress to fibrosis (scarring), cirrhosis or liver cancer.

There are 5 main hepatitis viruses, referred to as types A, B, C, D and E, these 5 types are of greatest concern because of the burden of illness and deaths they cause and the potential for outbreaks and epidemic spread. In particular, types B and C lead to chronic disease in hundreds of millions of people and together are the most common cause of liver cirrhosis and cancer (WHO, 2015).

1.2.2.2 Transmission:

The hepatitis B and C viruses are a blood borne viruses. Are most commonly transmitted through injecting drug use through the sharing of injection equipment, the reuse or inadequate sterilization of medical equipment especially syringes and needles in healthcare settings, and transfusion of unscreened blood and blood products. Hepatitis can also be transmitted sexually and can be passed from infected mother to her baby (WHO, 2015).

1.2.2.3 Life cycle:

1.2.2.3.1 Life cycle of hepatitis C virus:

Hepatitis C virus makes itself at home in a liver cell. The virus is covered with a coating that contains specific proteins. These proteins are used to locate a receptor on the surface of a liver cell. The receptor receives signals on the surface. The virus enters the liver cells outer barrier. Once it has entered the cell, the barrier surrounds the virus. The barrier swallows the virus up and brings the virus into the cell. The virus coating breaks down. Viral ribonucleic acid (RNA) carrying genetic information is released into the liver cell (Dorner *et al.*, 2013). This may happen when the outer barrier is penetrated. It could also be due to liver enzymes dissolving the cell. The viral RNA prepares to reproduce. It mimics the liver cells RNA and begins producing its RNA materials. It may also prevent the host cell from functioning properly. The replication process of the virus is not completely understood. The viral RNA is cloned over

and over to create new viruses. The virus coat is made of different protein-based coverings. These are developed by ribosomes, or cell protein builders. Protein units come together and form new particles around the viral RNA. They form a covering shaped like a sphere known as a "capsid", the capsid protect the virus genetic material. The new virus creates a bud. A protective coating surrounds the bud, and the virus is released. The process continues until the cell dies (Dorner *et al*., 2013) (figure1.2).

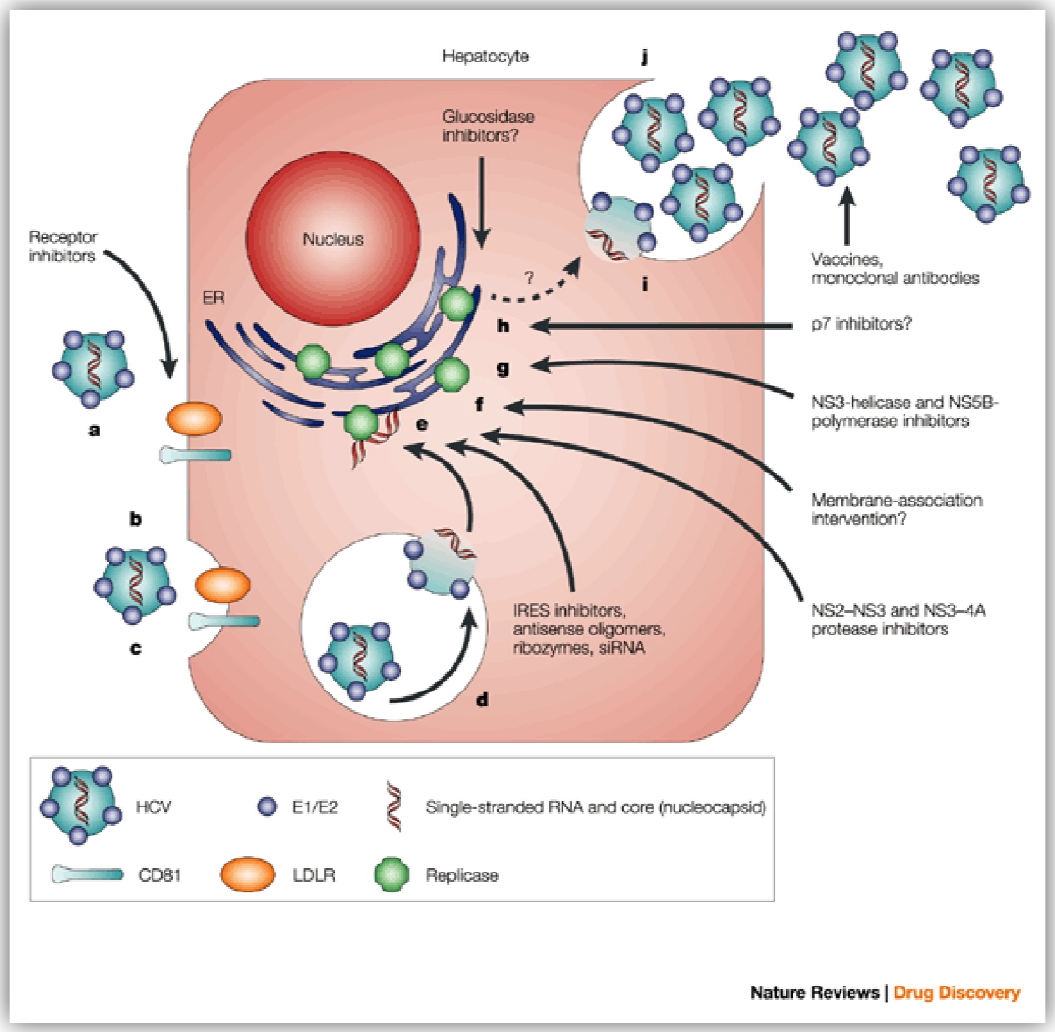

Figure (1.2): Life cycle of hepatitis C virus (CDC, 2016)

1.2.2.3.2 Life cycle of hepatitis B virus:

The virus gains entry into the cell by binding to a receptor on the surface of the cell and enters it by clathrin-dependent endocytosis. The cell surface receptor has been identified as the sodium/bile acid contransporting peptide. The virus membrane then fuses with the host cells, membrane releasing the DNA and core proteins into the cytoplasm. Because the virus multiplies via RNA made by a host enzyme (Benhenda *et al.*, 2009). The viral genomic DNA has to be transferred to the cell nucleus. The core proteins dissociate from the partially double stranded viral DNA is then made fully double stranded and transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of four viral mRNAs. The largest mRNA is used to make the new copies of the genome and to make the capsid core protein and viral DNA polymerase. These four viral transcripts go on to form progeny virions which are released from the cell or returned to the nucleus and re-cycled to produce even more copies. The long mRNA is then transported back to the cytoplasm where the virion P protein synthesizes DNA via its reverse transcriptase activity (Benhenda *et al.*, 2009) (figure 1.3).

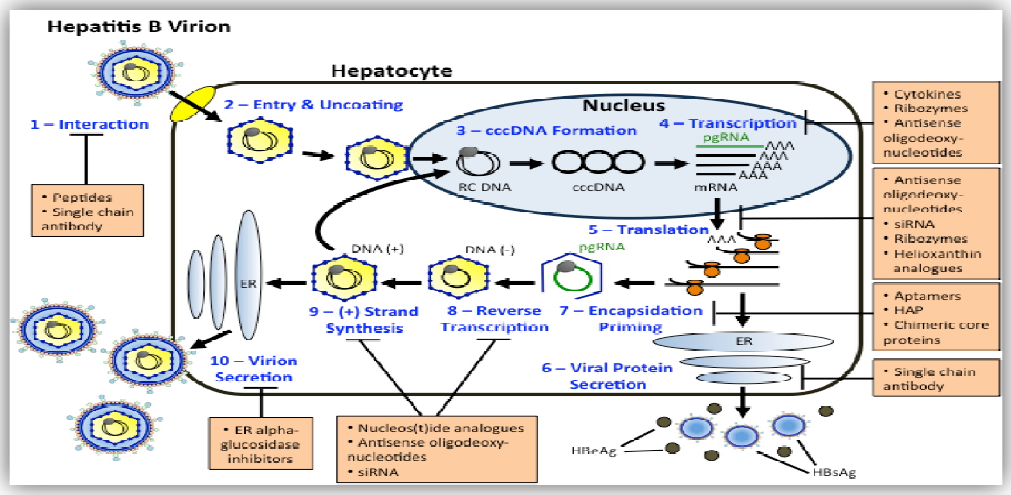

Figure (1.3): Life cycle of hepatitis B virus (CDC, 2016)

1.2.2.4 Pathogenesis:

1.2.2.4.1 Pathogenesis of hepatitis C virus:

The hepatitis C in 60-80% of patients, it is able to escape innate and adaptive immune surveillance. Thus it establishes itself as an agent of chronic hepatitis. Cytotoxic lymphocytes then contribute to liver injury in an attempt to eradicate the virus. On the other hand, strong multispecific T-lymphocyte reaction against hepatitis C virus proteins is associated with viral clearance. Both CD4+ and CD8+ lymphocyte functions are important to affect this outcome. The development of hepatocellular carcinoma is mainly restricted to patients with cirrhosis (Liang *et al.*, 2000).

1.2.2.4.2 Pathogenesis of hepatitis B virus:

Hepatitis B virus is dangerous because it attacks the liver, thus inhibiting the functions of these vital organs. The virus cause persistent infection, liver cirrhosis, hepatocellular carcinoma, and immune complex disease. Hepatitis B virus infection itself not leads to the death of infected hepatocytes. Hepatitis B virus in a non-cytolytic infection. Liver damage however, arises from cytolytic effects of the immune system cytotoxic T lymphocytes (CTL) which attempt to clear infection by killing infected cells (WHO, 2015).

1.2.2.5 Diagnosis:

It is not possible on clinical grounds to differentiate hepatitis B or C from hepatitis caused by other agents; hence laboratory confirmation of the diagnosis is essential. Laboratory diagnosis of hepatitis B and C infections focuses on the detection of the hepatitis B or C surface antigen (HBsAg, HCsAg). Acute infection is characterized by the presence of surface antigen and immunoglobulin M (IgM) antibody to the core antigen. Chronic infection is characterized by the persistence of surface antigen for at least 6 months (WHO, 2015). Any positive result by

immunochromatography test (ICT) must be confirmed by enzyme linked immune sorbent assay (ELISA), polymerase chain reaction (PCR) tests have been developed to detect and measure the amount of HBV or HCV DNA, called the viral load (WHO, 2015).

1.2.2.6 Treatment:

1.2.2.6.1 Treatment of HCV:

Hepatitis C does not always require treatment as the immune response in some people will clear the infection. Chronic infection does not develop liver damage. But recently, new antiviral drugs have been developed, these medications, called direct antiviral agents (DAA) are much more effective, safer and better-tolerated than the older therapies (WHO, 2015).

1.2.2.6.2 Treatment of HBV:

Acute hepatitis B infection does not usually require treatment and most adults clear the infection spontaneously (WHO, 2015). Early antiviral treatment may be required in less than 1% of people, whose infection takes a very aggressive course (fulminant hepatitis) or who are immunocompromised. Treatment of chronic infection may be necessary to reduce the risk of cirrhosis and liver cancer. As of 2008, there are 7 medications licensed for treatment of hepatitis B infection in United States. These include antiviral drugs lamivudine (Epivir), adefovir (Hepsera), tenofovir (Viread), telbivudine (Tyzeka) and entecavir (Baraclude), and the tow immune system modulators interferon alpha-2a and PEGylated interferon alpha-2a (WHO, 2015).

1.2.2.7 Prevention and control:

- All children should get the hepatitis B vaccine.
- Sterilization of the health care workers.
- People who use recreational, injectable drugs should be vaccinated.
- People with multiple sex partners should be vaccinated.
- There is no vaccine for hepatitis C (WHO, 2015).

1.2.2.8 Epidemiology of HCV and HBV:

HBV prevalence is highest in sub-Saharan Africa and East Asia; where between 5-10% of the adults are chronically infected. High rates of chronic infections are also found in the Amazon (WHO, 2015). HCV is found worldwide. There are multiple strains (or genotypes) of the HCV and their distribution varies by region (WHO, 2015).

1.2.3 Co-infection:

1.2.3.1 Background:

Malaria remains a major health threat worldwide. Endemic regions for malaria are also endemic for other infectious diseases that might affect the malaria infection (Boraschi *et al.,* 2008). Examples for such a common endemic infection sharing the same territory with malaria are hepatitis B virus (HBV) and hepatitis C virus (HCV) (WHO, 2012). Epidemiology of hepatitis B virus and malaria co-infection has not been well studied; some studies found that co-infection existed among 41% out of 337 blood donors (Aernan *et al.,* 2011). Malaria and hepatitis C are share some similarities in their development within the hepatocytes of the liver, co-infection of these two pathogens has largely remained unstudied, but due to epidemiological overlap, it is plausible that individuals can be afflicted with both malaria and hepatitis C. To date, it has been shown that *Plasmodium* parasites and HCV utilize four common host entry factors to gain entry into hepatocytes; heparin sulfate proteoglycans (HSPGs), scavenger receptor-B1 (SR-B1), cluster of differentiation 81 (CD-81) and apolipoprotein E (apoE) (Veltre, 2012). No protective effects for HBV vaccine against malaria were found. The interaction between malaria parasites and HCV among chronic HCV carriers has been found slow the emergence of the former, a thing that could help in determining new therapeutic approaches to defeat malaria (Gasim and Adam, 2016).

1.2.3.2 Immunology of HBV, HCV and Malaria co-infection:

Immunological features of malaria and HBV co-infection, where they described that the hepatic stage infection seemed to trigger an early T-cell-independent cytokine response along with a delayed cytokine response that was simultaneous with the infiltration of T cells. On the other hand, the T cell response appeared earlier in the blood stage than in hepatic stage infection, possibly because parasitemia was detectable earlier in those animals and a T cell-independent phase was not seen, perhaps reflecting that it was induced primarily by infected hepatocytes (Pasquetto *et al.,* 2000). Hepatitis B virus infections are common in many of the malaria endemic areas. HBV induces a robust pro-inflammatory type 1 immune response (Th1), which is important for *Plasmodium* clearance, but is also implicated in disease severity (Schofield and Grau, 2005). Whilst intriguing, little is known of the effects of HBV on the clinical presentation of malaria. Intrahepatic HBV replication is inhibited by *P.yoelii* infection in mice (Pasquetto *et al.,* 2000). Moreover, a study performed in a Vietnamese hospital showed that patients with cerebral malaria that a slightly greater risk of registering positive serology for the HBV surface antigen (HBsAg) (Barcus *et al.,* 2002). Both HCV and malaria infections use common host factors like heparin sulfate proteoglycans (HSPGs), cluster differentiation 81(CD-81), scavenger receptor B1 (SR-B1) and apolipoprotein (ApoE) (Veltre, 2012).

1.2.3.3 Impact of HBV and HCV on malaria:

Among 636 Brazilian patients that HBV infection was associated with a decreased intensity of malaria infection. They proposed that this effect is due to cytokine balance and control of inflammatory ripostes (Andrade *et al.,* 2011). In contradistinction, another group of researchers concluded that HBV and malaria do not seem to significantly affect each other and evolve independently (Freimanis *et al.,* 2012). A third opinion stated that

the immune response against *falciparum* infected red blood cells might be suppressed by HBV carrier status (Souto *et al.*, 2002). The interaction between malaria parasites and HCV among chronic HCV carriers has been found to slow the emergence of the former (Boyer *et al.*, 2011).

1.2.3.4 Future prospects:

As elimination of malaria is a global aim, supplementary tools are required, such as vaccination, in order to provide long-term prevention (El-Moamly, 2013). Strategies predict improving currently available diagnostic methods, researches dealing with therapeutic and prophylactic agents and protocols, vector control procedures, vaccine bringing up evolution, and other operational tools and approaches (El-Moamly, 2013). The interaction between malaria parasites and HCV among chronic HCV carriers has been found to slow the emergence of the former a thing that could help in determining new therapeutic approaches to defeat malaria (Boyer *et al.*, 2011).

Rationale

Endemic regions for malaria may also be endemic for other infectious diseases that might affect the malaria infection such as hepatitis-C and hepatitis-B viruses, so co-infections may occur (Boraschi *et al.,* 2008). Epidemiology of co-infections have not been well studied, and related of co-infected organisms with age, gender, occupation and blood transfused also not well studied. Therefore, this study was conducted to determine the co-infections and epidemiology of three infections in Saad Rashwan health center in Omdurman city.

Objectives

General objective:

To study prevalence rate of malaria/ hepatitis C and hepatitis B virus co-infections in Saad Rashwan Health Center in Omdurman city-Khartoum State.

Specific objectives:

- To determine relationship between intensity of malaria infection and age.
- To determine prevalence of malaria, hepatitis C, hepatitis B according to gender, age, occupation, blood transfusion and symptoms.
- To determine relationship between malaria-HCV, malaria-HBV co-infections.

Chapter Two

Materials and methods

2.1 Study design:

It is a cross-sectional study.

2.2 Study area:

This study was conducted in Saad Rashwan Health Center which is located 2 kilo miters north Omdurman Islamic University, Khartoum State.

2.3 Study population:

A total of 200 individuals who visited Saad Rashwan Health Center were included in this study. After informed consent was obtained, all individuals included have agreed to participate in the study.

2.4 Period of study:

The study was conducted during period from June 2016 to April 2017.

2.5 Sample size:

The sample size was obtained according to the following equation:

$N = (t^2*P(1-p)/M^2)$

N =Sample size

t = the normal standard deviate (t =1.96)

P = the frequency of occurrence of malaria (0.16)

M = degree of precision (0.05)

N = 1.96*1.96*0.16(1-0.16)/0.05*0.05=200

According to the above finding, the study was conducted on 200 individuals.

2.6 Sampling:

Two hundred blood samples were collected from all participants. The blood samples obtained by venipuncture and collected in EDTA anticoagulant- coated tube. Two hundred questionnaires were filled by

participants. Each sample was tested by blood films and ICT for HCV and HBV.

2.7 Data collection:

Designed questionnaire (appendix) contained the following variables: gender, age groups, occupation, blood transfusion and symptoms.

2.8 Methods:

2.8.1 Stained blood films:

2.8.1.1 Thick blood films:

Three drops of blood were added to clean and dry slide, mixed and allowed to dry. Then the slides were stained by 10% Geimsa stain, washed and air dried. Then a drop of oil was added and examined under microscope (100x oil immersion). The number of parasites was counted and reported by using the following grading as described by Cheesbrough (1987).

1-10 per 100 high power fields……+

11-100 per 100 high power fields……++

1-10 in every high power field…….+++

More than 10 in every high power field….++++

2.8.1.2 Thin blood films:

A drop of blood was added below the level of slide (2/3) and by spreader the blood was pushed forward with suitable speed, allow to air dry, then fixed with absolute methanol, allow to air dry and stained with 10% Geimsa stain. Then washed and allowed to dry, drop of oil was added and examined under microscope (100x oil immersion).

2.8.2 Immuno chromatography test (ICT) for HCV and HBV:

Five microlitres of serum were added into sample well, two drops (80µl) of assay buffer were added into the developer well. Then the results were read in 20 minutes as follow: the presence of two color bands "C" and "T", indicates a positive result for HCV or HBV. While, the presence of

only one band "C" within the result window indicates a negative result, as manufactures instructions (Biocredit).

2.9 Data analysis:

Data were analyzed using Statistical Package of Social Sciences (SPSS) version 20. Frequencies, mean and chi-squire test were used. Then data were presented in tables.

2.10 Ethical consideration:

The study adopted was approved by College of Medical Laboratory Science-Sudan University of Science and Technology. Then consent was taken from all individuals or their guardians before being included in the study. Each individual was informed on the nature of study.

Chapter three

Results

3.1 General characteristics of studied population:

This study was conducted on 200 subjects. Out of them 102 (51%) were males and 98 (49%) were females (table 3.1). The age ranged between1-90 years old with mean of age was 27.6 ±20.9 years old. The age was divided into 6 groups: 0-15, 16-30, 31-45, 46-60, 61-75 and 76-91 years old (table 3.1).

Table (3.1): Frequency of age groups among gender

Age groups (years)	Frequency		Percentage
	Males	Females	
0-15	34	35	34.5 %
16-30	31	20	25.5 %
31-45	25	22	23.5 %
46-60	4	15	9.5 %
61-75	5	5	5%
76-91	3	1	2%
Total	200		100%

3.2 Malaria:

3.2.1 Overall prevalence of malaria in the study area:

Out of 200 study subjects, 64 (32%) were positive for *P.falciparum*, while 136 (68%) were negative (table 3.2).

Table (3.2): Overall prevalence of malaria parasite in the study area

Samples	Frequency	Percentage (%)
Positive	64	32%
Negative	136	68%
Total	200	100%

3.2.2 Relation between intensity of malaria parasite and age groups:

Chi-squire test was used to determine the relation between the intensity of malaria among age groups. Out of 64 positive cases, 46 (23%) were presented as mild infection (+) and 18 (9%) as moderate infection (++) within the different age groups. The difference in rate was found to be statistically insignificant with the p.value=0.344.

Table (3.3): Intensity of malaria parasite among age groups

Malaria intensity	Age groups (years old)						
	0-15	16-30	31-45	46-60	61-75	76-91	Total
Mild infection	13(20%)	14(21.9%)	10(15.6%)	6(9.4%)	3(4.7%)	0(0.0%)	46(71.9%)
Moderate infection	8(12.5%)	3(4.7%)	6(9.4%)	1(1.6%)	0(0.0%)	0(0.0%)	18(28.1%)
Total	21(32.8%)	17(26.6%)	16(25%)	7(10.9%)	3(4.7%)	0(0.0%)	64(100%)

P=0.344

3.2.3 Prevalence of malaria parasite according to gender, age groups, occupation, blood transfusion and symptoms:

Prevalence of malaria parasite among gender, age groups, occupation, blood transfusion and symptoms was shown in (table 3.4) and (table3.5) respectively.

Table (3.4): Prevalence of malaria parasites according to gender, age groups and occupation

	Type	Malaria		Total
		Positive	Negative	
Gender	Male	37(18.5%)	65(32.5%)	102(51%)
	Female	27(13.5%)	71(35.5%)	98(49%)
Age groups (years old)	0-15	13(6.5%)	56(28%)	69(34.5%)
	16-30	19(9.5%)	32(16%)	51(25.5%)
	31-45	19(9.5%)	28(14%)	47(23.5%)
	46-60	7(3.5%)	12(6%)	19(9.5%)
	61-75	5(2.5%)	5(2.5%)	10(5%)
	76-91	1(0.5%)	3(1.5%)	4(2%)
Occupation	Student	27(13.5%)	67(33.5%)	94(47%)
	Housewife	12(6%)	19(9.5%)	31(15.5%)
	Retired	3(1.5%)	8(4%)	11(5.5%)
	Worker	22(11%)	42(21%)	64(32%)

Table (3.5): Prevalence of malaria parasite according to blood transfusion and symptoms

	Type	Malaria		Total
		Positive	Negative	
Blood transfusion	Yes	7 (3.5%)	19 (9.5%)	26 (13%)
	No	57 (28.5%)	117 (58.5%)	174(87%)
Symptoms	Fever	6 (3%)	41 (20.5%)	47(23.5%)
	Headache	0 (0.0%)	3 (1.5%)	3 (1.5%)
	Fever and headache	8 (4%)	40 (20%)	48 (24%)
	Fever, headache and other symptoms	50 (25%)	52 (26%)	102(51%)

3.3 Hepatitis:

3.3.1 Hepatitis C virus:

3.3.1.1 Overall prevalence of HCV in the study area:

Out of 200 study subjects, 5 (2.5%) were positive for HCV, while 195 (97.5%) were negative (table 3.6).

Table (3.6): Overall prevalence of HCV in study area

Samples	Frequency	Percentage (%)
Positive	5	2.5%
Negative	195	97.5%

3.3.1.2 Prevalence of HCV according to gender, age groups, occupation, blood transfusion and symptoms:

The prevalence of HCV according to gender, age groups, occupation, blood transfusion and symptoms was shown in (table 3.7) and (table 3.8) respectively.

Table (3.7): Prevalence of HCV according to gender, age groups and occupation

	Type	HCV		Total
		Positive	Negative	
Gender	Male	4(2%)	98(49%)	102(51%)
	Female	1(0.5%)	97(48.5%)	98(49%)
Age groups (years old)	0-15	0(0.0%)	69(34.5%)	69(34.5%)
	16-30	3(1.5%)	48(24%)	51(25.5%)
	31-45	2(1%)	45(22.5%)	47(23.5%)
	46-60	0(0.0%)	19(9.5%)	19(9.5%)
	61-75	0(0.0%)	10(5%)	10(5%)
	76-91	0(0.0%)	4(2%)	4(2%)
Occupation	Student	2(1%)	92 (46%)	94 (47%)
	Housewife	0 (0.0%)	31 (15.5%)	31 (15.5%)
	Retired	0 (0.0%)	11 (5.5%)	11 (5.5%)
	Worker	3 (1.5%)	61 (30.5%)	64 (32%)

Table (3.8): Prevalence of HCV according to blood transfusion and symptoms

	Type	HCV		Total
		Positive	Negative	
Blood transfusion	Yes	1 (0.5%)	25 (12.5%)	26 (13%)
	No	4 (2%)	170 (85%)	174 (87%)
Symptoms	Fever	0 (0.0%)	74 (37%)	74 (37%)
	Headache	0 (0.0%)	3 (1.5%)	3 (1.5%)
	Fever and headache	4 (2%)	44 (22%)	48 (24%)
	Fever, headache and other symptoms	1 (0.5%)	101(50.5%)	102 (51%)

3.3.2 Hepatitis B virus:

3.3.2.1 Overall prevalence of HBV in the study area:

Out of 200 study subjects, 16 (8%) were positive for HBV, while 148 (92%) were negative (table 3.9).

Table (3.9): Overall prevalence of HBV in the study area

Samples	Frequency	Percentage (%)
Positive	16	8%
Negative	148	92%
Total	200	200%

3.3.2.2 Prevalence of HBV according to gender, age groups, occupation, blood transfusion and symptoms:

The prevalence of HBV among gender, age groups, occupation, blood transfusion and symptoms was shown in (table 3.10) and (table 3.11) respectively.

Table (3.10): Prevalence of HBV according to gender, age groups and occupation

	Type	HBV		Total
		Positive	Negative	
Gender	Male	12(6%)	90(45%)	102(51%)
	Female	4(2%)	94(47%)	98(49%)
Age groups (years old)	0-15	1(0.5%)	68(34%)	69(34.5%)
	16-30	6(3%)	45(22.5%)	51(25.5%)
	31-45	5(2.5%)	42(21%)	47(23.5)
	46-60	3(1.5%)	16(8%)	19(9.5)
	61-75	1(0.5%)	9(4.5%)	10(5%)
	76-91	0(0.0%)	4(2%)	4(2%)
Occupation	Student	6(3%)	88(44%)	94(47%)
	Housewife	1(0.5%)	30(15%)	31(15.5%)
	Retired	1(0.5%)	10(5%)	11(5.5%)
	Worker	8(4%)	56(28%)	64(32%)

Table (3.11): Prevalence of HBV among blood transfusion and symptoms

	Type	HBV		Total
		Positive	Negative	
Blood transfusion	Yes	5(2.5%)	21(10.5%)	26(13%)
	No	11(5.5%)	163(81.5%)	174(87%)
Symptoms	Fever	1(0.5%)	46(23%)	47(23.5%)
	Headache	1(0.5%)	2(1%)	3(1.5%)
	Fever and Headache	2(1%)	46(23%)	48(24%)
	Fever, headache and other Symptoms	12(6%)	90(45%)	102(51%))

3.4 Co-infection:

3.4.1 Malaria- Hepatitis C virus co-infection:

The present study indicated that 4 individuals (2%) were co-infected with malaria and hepatitis C virus (table 3.12) with p.value=0.020.

Table (3.12): Prevalence of malaria-hepatitis C virus co-infection in the study area

Malaria	HCV		Total
	Positive	Negative	
Positive	4 (2%)	60 (30%)	64 (32%)
Negative	1 (0.5%)	135 (67.5%)	136 (68%)
Total	5 (2.5%)	195 (97%)	200 (100%)

P=0.020

3.4.2 Malaria- Hepatitis B virus co-infection:

The present study indicated that 2 individuals (1%) were co-infected with malaria and hepatitis B virus (table 3.13) with p.value=0.081.

Table (3.13): Prevalence of malaria-hepatitis B virus co-infection in the study area

Malaria	HBV		Total
	Positive	Negative	
Positive	2 (1%)	62 (31%)	64 (32%)
Negative	14 (7%)	122 (61%)	136 (68%)
Total	16 (8%)	184 (92%)	200 (100%)

P=0.081

Chapter Four

Discussion

This study was conducted on 200 subjects with different ages, sexes, occupations, blood transfusions and symptoms who visited the Saad Rashwan Health Center to determine the prevalence rate of malaria/ hepatitis C and hepatitis B virus co-infections. For this purpose 200 blood samples were involved and examined to detect co-infections.

This study showed that 32% of studied populations were infected with *P.falciparum*, this result disagreed with the study carried out by WHO (2012) in Kenya which showed that out of 3000 subjects, 2400 (80%) were infected with *P.falciparum*.

The prevalence of *Plasmodium* infection in this study was highest (32.8%) among age group (0-15) years old which was 32.8%. This study showed that the difference in rate between *Plasmodium* infection and age groups was found to be statistically insignificant with the p.value=0.344. These results were disagreed with study carried out in Gambia, West Africa which showed that 4% of infant deaths and 25% of deaths in age group 1-4 years old (Greenwood *et al.*, 1997).

This study showed that there was no statistically significant between prevalence of malaria and gender (p-value=0.186), occupation (p-value=0.709) and blood transfusion (p-value=0.552). While there was strong statistically significant between *Plasmodium* infection and symptoms (p.value=0.000).

This study showed that 2.5% of studied populations were infected with Hepatitis C virus infection. This finding agreed with a previous study done in Sudan by Mudawi (2008) who showed that 2.2% were positive for hepatitis C virus infection.

This study showed that there was no statistically significant between hepatitis C virus and gender (p-value=0.189), age groups (p-

value=0.347), occupation (p-value=0.496) and blood transfusion (p-value=0.637). While there was statistically significant between hepatitis C virus infection and symptoms (p.value=0.030).

This study showed that 8% of studied populations were infected with Hepatitis B virus infection. This finding disagreed with a previous study done in Sudan by Mudawi (2008) who showed that 47% was positive for hepatitis B virus infection.

This study showed that there was no statistically significant between hepatitis B virus and age groups (p-value=0.191) and occupation (p-value=0.381) While there was statistically significant between hepatitis B virus and gender (p-value=0.045), blood transfusion (p-value=0.024) and symptoms (p.value=0.052).

This study indicated that the prevalence of malaria-hepatitis C virus co-infection was 2% (p-value=0.020). This result agreed with Gasim and Adam (2016).

Also, this study indicated that the prevalence of malaria-hepatitis B virus co-infection was 1% (p-value=0.081). This result disagreed with Gasim and Adam (2016).

Chapter five

Conclusion and recommendations

5.1 Conclusion:

The study concluded that the co- prevalence of malaria/hepatitis C virus was 2% while malaria/hepatitis B virus was 1% in Saad Rashwan health center in Omdurman city.

5.2 Recommendations:

- Further studies should be done on immunological affects of hepatitis C virus and hepatitis B virus infection on malaria immune response to detect and defeat the co-infections between hepatitis C/ hepatitis B and malaria earlier.
- Further studies should be done to find epidemiology of hepatitis C, hepatitis B-malaria co-infections.
- Further studies should be done in prevalence of hepatitis C, hepatitis B-malaria co-infection in other endemic areas.
- Control activities and advices should be conducted in study area to reduce infection with malaria and hepatitis virus species.
- Advanced techniques should be used such as Enzyme Linked Immuno Sorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) to confirm the results.

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Sudan University of Science and Technology

College of Graduate Studies

M.Sc. in Medical Laboratory Science (Parasitology and Medical Entomology)

Questionnaire

Prevalence Rate of Malaria/ Hepatitis C Hepatitis B virus co-infections in Saad Rashwan health center, Omdurman city

- ID patient number …….
- Age …….years
- Gender: Male ( ) Female ( )
- Occupation: …….

-Clinical features:

- Fever: Yes ( ) No ( )
- Headache: Yes ( ) No ( )
- Other symptoms: ……

- Blood transfused: Yes ( ) No ( )

Laboratory results:

- B.F.F. M: Positive ( ) Negative ( )
- Intensity: + ( ) ++ ( ) +++ ( ) ++++ ( )
- ICT for HCV: Positive ( ) Negative ( )
- ICT for HBV: Positive ( ) Negative ( )