Sudan University of Science and Technology

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Molecular Detection of Rubella Virus among Apparently Healthy Pregnant Women in Khartoum State

الكشف الجزيئي لفيروس الحصبة الألمانية لدي النساء الحوامل السليمات ظاهريًا في ولاية الخرطوم

A thesis submitted in fulfillment for the requirements of Ph.D. degree in Medical Laboratory Science (Microbiology)

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الآيـة

قال تعالـى:

(وَقَالُوا الْحَمْدُ لِلَّهِ الَّذِي هَدَانَا لِهَٰذَا وَمَا كُنَّا لِنَهْتَدِيَ لَوْلَا أَنْ هَدَانَا اللَّهُ).

صدق الله العظيم

الأية (٤٣) من سورة الاعراف

Dedication

TO My parents

To My brothers

To soul of prof. Abdelbagi Elnagi Mohamed

Acknowledgments

Firstly the grateful thanks to ALMIGHTY ALLAH, the lord of earth and skies for giving me the power and confidence completing this research. I would like to express my thanks and appreciations to my supervisors Prof. Mohamed Abdelsalam Abdalla, College of Veterinary Medicine, Sudan University of Science and Technology and Prof. Shamson Khamis Khafi , College of Medical Laboratory Science, the National Ribat University for their unfailing patience, expert advices, supervision, guidance, efforts, suggestions and their valuable time. Extended thanks to The National Laboratory for Health especially Molecular Biology Department represented in the following persons: Mrs: Randa Ahmed and Dr. Nawal Tag Elsir for their help, cooperation and valuable advice.

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Abstract

This study was aimed to detect rubella virus antibodies (IgM and IgG) by ELISA and then was confirmed by detection viral genome by Reverse transcriptase polymerase chain reaction (RT-PCR) among apparently healthy pregnant women in Khartoum State. In this study 358 plasma and sera were collected; from which 2 (0.56%) were IgM +ve and 320 (89.39%) were IgG +ve, and there was no association between IgM and IgG results with the trimester and gravidity. The results showed that; there was no association between IgM and family members, season and socioeconomic situation while IgG had no association with miscarriage and number of miscarriages.Out of 169 there was 4(2.4%) positive by RT-PCR and there was no association between residence, trimester and season of year with the presence of RV. Also it found that primagravida had a risk in acquiring infection but family size and socioeconomic situation had no risk to get infection. The current study concludes that; rubella virus genome and recent infection were detected in few apparently healthy pregnant women, while the percentage of previous infection was high.

المستلخص

هدفت هذه الدراسة للكشف عن الأجسام المضادة من النمط (IgG) والنمط (IgM) لفيروس الحصبة الألماتية عن طريق تقنية الامتزاز المناعي المرتبط بالإنزيم. وبعدها أُكِدت النتائج بالكشف عن الحمض النووي للفيروس بإستخدام تفاعل البوليميريز المتسلسل بإستخدام إنزيم النسخ العكسي وسط النساء الحوامل السليمات ظاهرياً في ولاية الخرطوم. في هذه الدراسة جمعت ٣٥٨ بلازما وسيرم. وكانت ٢ (٠,٥٦%) إيجابية مع الجسم المضاد النمط (IgM) و٣٢٠ (٨٩,٣٩%) مع الجسم المضاد(IgG)، ووجد أن لا علاقة بين فترات الحمل وعدد مرات الحمل مع الجسم المضاد النمط (IgM) و (IgG).

وأوضحت هذه الدراسة أن لا علاقة بعدد أعضاء الأسرة و فصول السنة والوضع الإقتصادي الإجتماعي مع الجسم المضادالنمط (IgM)، بينما لم يظهر الجسم المضاد (IgG) أي علاقة مع الإجهاض وعدد مرات الإجهاض المتكرر. من بين١٦٩، هنالك ٤ (٢,٤%) أعطت نتيجة إيجابية مع تفاعل البوليميريز المتسلسل بإستخدام إنزيم النسخ العكسي ووجد أنه لا علاقة بالسكن وفترة الحمل وفصول السنة بوجود فيروس الحصبة الألمانية. ووجد أيضا أن الحمل لأول مرة تمثل خطورة لإكتساب الإصابة و ليس لعدد أفراد الأسرة والوضع الإجتماعي الإقتصادي أي خطورة لإكتساب المرض.

خلصت الدراسة الحالية أنه تم التعرف على الحمض النووي للفيروس والإصابة الحديثة للفيروس عند عدد قليل من النساء الحوامل السليمات ظاهرياً، بينما كانت نسبة الإصابة السابقة أعلى.

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Abbreviations

ACIP	Advisory Committee on Immunization Practices
AGMK	African Green Monkey Kidney
BHK	Baby Hamster Kidney
BRD-2	Bromodomain containing protein
C	Capsid protein (structural protein)
CDC	Centers for Disease Control and Prevention
CF	Complement-Fixing Antigen
CMV	Cytomegalovirus
CPE	Cytopathic effect
CRI	Congenital rubella infection
CRS	Congenital rubellasyndrome
DI	Defective Interfering
E1	Envelope protein-1(structural protein)
E2	Envelope protein-2(structural protein)
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endothelial reticulum
GVAP	Global Vaccine Action Plan
HA	Hemagglutinin Antigen
HSV-2	Herpes Simplex Virus type-2
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IUGR	Intrauterine growth restriction
LabNet	Lab network
MMR	Measles Mumps Rubella
MR	Measles Rubella
Nm	Nanometer
Nt	Nucleotide
ORF	Open reading frames
p150	Protein 150 (non-structural protein)

p90	Protein 90 (non-structural protein)
PA	Platelet-Aggregating Antigen
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase chain reaction
RCV	rubella-containing vaccine
RK-13	Rabbit Kidney
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Rubella virus
STD	Sexually transmitted disease
TBE	Tris Borate EDTA
TORCH	Toxoplasma Rubella CMV Herpes simplex viruses 1,2
VIP	Vaccine in Pregnancy
VZV	Varicella Zoster Virus
WHO	World Health Organization
WIC	Women, Infants and Children

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CHAPTER ONE
INTRODUCTION

1.1. Introduction

Rubella virus infection is a principal public health concern especially in low and middle income countries where rubella vaccination is not extensively applied. The infection is most common in children, child bearing aged women, pregnant women and young adults (Lulandala *et al*., 2017).

Rubella (German measles) is usually a mild contagious disease in children and adults that is difficult to diagnose clinically due to tenuous clinical features that are also popular to a number of other virus infections and it is asymptomatic in 25 to 50 % of cases. In some cases prodromal symptoms may be evident (South Australian Perinatal Practice Guidelines Workgroup, 2015).

Rubella infection is usually mild with non-specific symptoms or subclinical, so undiagnosed or misdiagnosed occasionally (Martínez-Quintana *et al*., 2015).

The number of rubella cases reported from 2000 to 2014 increased in the African region (from 865 cases in seven countries to 7402 cases in 44 countries). Although the rubella vaccine has been implemented in many countries since 1969, worldwide coverage is still a distant goal, particularly in Africa, where only a few countries routinely immunize against rubella (WHO, 2015; Grant, 2016).

Maternal viraemia may occur 5 to 7 days after exposure with spread of the virus throughout the body as well as trans-placental infection of the fetus (South Australian Perinatal Practice Guidelines Workgroup, 2015).

The mother is able to transmit the virus even if she is asymptomatic and fetal hurt varies according to the time of infection (Talaro and Chess, 2012).

About 10-25% of non-immunized women of childbearing age are susceptible to rubella virus infection (Adewumi *et al*., 2013).

The risk of congenital infection and defects is highest during the first 12 weeks of gestation and decreases after the 12th week of gestation with defects rare after the 20th week of gestation (McLean *et al*., 2012).

However, RV infection during pregnancy, can causes miscarriages and serious birth defects including hearing, vision, mental, and heart impairment, which are collectively known as congenital rubella syndrome (CRS) and it occurs in up to 85% of children born

to women with RV infection during the first trimester of pregnancy. In addition, CRS can lead to neonatal deaths in up to 30% of cases (Lazar *et al*., 2016).

The encumbrance of CRS in developing countries is undervalued and few reports documenting the incidence of CRS are available. In 2009, only 165 CRS cases were reported worldwide with the majority being from the World Health Organization (WHO) African and Eastern Mediterranean regions (Reef *et al.*, 2011).

It is therefore strongly recommended by the WHO that serological surveys on rubella virus infection in women of childbearing age be done (WHO, 2000).

The first report investigated and documented the presence of CRS in Sudan in cases suspected had CRS was done by Adam *et al.,* (2014).

In Sudan, national surveillance for measles and rubella was established in 2006 and many reports about rubella sero-prevalence among pregnant women are available from Khartoum State (Adam *et al*., 2013; Abdallah *et al*., 2015) and West Sudan (Hamdan *et al*., 2011).

A prenatal diagnosis of fetal infection could be proposed. Although progress has been made, the prenatal diagnosis of rubella is not always easy. The incidence of rubella has significantly decreased in many countries because of vaccination campaigns; however, rubella has not disappeared in developed countries and is a significant source of disability (Bouthry *et al*., 2014).

The only reliable proof of acute rubella infection is a positive viral culture for rubella or detection of rubella virus by polymerase chain reaction (PCR), the presence of rubella-specific IgM antibody, or demonstration of a significant rise in IgG antibody from paired acute- and convalescent-phase sera (CDC, 2015).

Despite the availability of an effective vaccine for rubella since the 1960s, the virus is still a global health concern with over 100,000 babies born with congenital rubella syndrome every year (Mangala *et al*., 2017).

1.2. Rationale

Rubella virus can be transmitted vertically from mother to fetus, causing the spectrum of congenital anomalies that define CRS (Washington State Department of Health, 2016).

Rubella infection may be present as an acute, mild or asymptomatic illness; therefore the outbreaks may occur without clinical discrimination or may be misdiagnosed as measles cases (Best *et al*., 2005; Junaid *et al*., 2011).

Persons who are asymptomatic are communicable but the period of communicability is difficult to define (Washington State Department of Health, 2016).

It recommends by World Health Organization (WHO), countries without national rubella vaccination programs should assess the burden of rubella and CRS through sero-epidemiological surveys that may be implemented in rival with measles surveillance (Mirambo *et al*., 2015).

The diagnostic RT-PCR assay should be very sensitive to reliably detect rubella virus, particularly when the patient is pregnant and at risk for congenital infection (Hübschen *et al*., 2017).

Rubella inspection is important to identify circulating viruses, path importation of new viruses, and monitor vanishing of specific wild-type RV progeny. So, Vaccine introduction may change transmission dynamics of endemic viruses; it is therefore important to monitor changes in the epidemic pattern of rubella viruses (Zhu *et al*., 2014).

Many studies concern sero-prevalence of rubella in Khartoum were done but few were published.

In 2013, a sero-prevalence study in Khartoum by Adam, *et al*., revealed that 95.1% of pregnant women were exposed to rubella infection, and Abdallah *et al*., 2015 showed that 47.8% , 8.9% were positive for IgG & IgM respectively and 50.0% were negative for both (Adam *et al*., 2013; Abdallah *et al*., 2015).

Few studies outside Khartoum were conducted by Hamdan *et al.*, (2011), in western Sudan yield the following results: IgG (65.3%) and IgM (3.4%).

Rubella vaccination is not yet included in the immunization schedule in Sudan, on top of these few reports and because there is minimal baseline information on the burden of rubella in the Sudan, we conducted this study to throwing light on this serious infection to provide information on rubella prior to routine vaccine introduction.

1.3. Objectives

1.3.1. General objective

To detect rubella virus by molecular technique among apparently healthy pregnant women in Khartoum State.

1.3.2. Specific objectives

1. To detect rubella viral IgG and IgM antibodies in the blood of apparently healthy pregnant women.
2. To confirm the diagnosis of rubella infection among pregnant women by Reverse transcriptase polymerase chain reaction (RT-PCR).
3. To find out the risk factors predisposing to the infection with rubella among apparently healthy pregnant women in Khartoum State.

CHAPTER TWO
LITERATURE REVIEW

2.1. Infection in pregnant women

Women and infants bear a considered proportion of disease morbidity because of complications associated with pregnancy and many infections have been associated with premature birth (Hollier and Wendel, 2005).

Viral infections during pregnancy have been associated with hurtful pregnancy outcomes and birth defects in the offspring; such as microcephaly or even fetal death can result. It has been well affirmed that viral infection of the cells at the maternal-fetal interface can affect placental function, which may result in pregnancy complications such as miscarriage, intrauterine growth restriction (IUGR) or, premature birth. Furthermore, a growing body of evidence suggests that viral infection of the decidua and/or placenta may result in the production of soluble immune factors that could reach the fetus and might affect fetal growth (Racicot and Mor, 2017).

Viral infections that are capable of crossing the placental barrier and reaching the fetus can have harm effects on fetal development. The association between fetal viral infection and abnormal development was described firstly by Gregg, when he discovered the association between prenatal rubella infection and cataract in 1941. There is now a large body of work demonstrating that direct infection of the fetus with CMV, HSV-2, or rubella can cause major neurosensory deficits, learning disabilities, and psychiatric disorders (Racicot and Mor, 2017).

To diagnose infection and differentiate between primary and secondary infections, laboratory testing of maternal immune status is needed. Assessment of fetal damage and prognosis requires prenatal laboratory testing primarily in those cases where a clinical decree such as drug treatment, pregnancy termination or intrauterine IgG transfusion must be taken (Mendelson *et al.*, 2006).

2.2. Rubella virus

2.2.1. Historical Preface

The name rubella is derived from Latin, meaning “little red.” Rubella was formerly considered to be a variant of measles or scarlet fever and was called “third disease”. It was not until 1814 that it was first described as a distinct disease in the German medical literature, hence the popular name “German measles” (CDC, 2015).

In 1914, Hess hypothesized a viral etiology based on his work with monkeys. Hiro and Tosaka in 1938 confirmed the viral etiology by passing the disease to children using filtered nasal washings from persons with acute cases (CDC, 2015).

Following a widespread epidemic of rubella infection in 1940, Norman Gregg, an Australian ophthalmologist, reported in 1941 the occurrence of congenital cataracts among 78 infants born following maternal rubella infection in early pregnancy. This was the first published recognition of congenital rubella syndrome (CRS) and rubella virus was first isolated in 1962 by Parkman and Weller and the first rubella vaccines were certified in 1969 (CDC, 2015).

2.2.2. Classification of RV

Rubella virus is a member of the Rubivirus genus in the family Togaviridae and the sole non-arthropod borne virus in the family which is the etiologic agent of rubella. It is a cubical, medium-sized (60 to 70 nm), lipid-enveloped virus with a positive-sense, single-stranded RNA genome (Adewumi *et al*., 2015).

2.2.3. Morphology and structure of RV

The virions have an outer thick shell that includes the glycoproteins, virus membrane and the inner virion shell consists of the capsid protein and the viral genome. The membrane and nucleocapsid are separated on average by about 70 A°. Thin strips of density run across this gap, providing continuity between the inner nucleocapsid shell and the outer glycoprotein shell (Prasad *et al*., 2017).

Variable arrays of surface glycoprotein "spikes" project away from the rubella virus membrane. The glycoproteins form rows on the virion surface with a separation of 65 A° to 90 A° between rows. The average separation between glycoprotein spikes is 50 to 55 A° along each row and the crucial characteristic of the rubella virus surface is the tendency to form sets of four to six parallel rows of glycoproteins (Prasad *et al*., 2017).

Virions are inactivated by mild heat (56°C), detergents, or lipid solvents rapidly (Murray *et al*., 2007).

2.2.4. Genome organization

The single plus-stranded RNA genome contains two open reading frames (ORF) located in the same translational frame (5’-p150-p90-3’ for the non-structural ORF, and 5’- C-E2-E1-3’ for the structural ORF) as elucidated in figure-1.

RV has only one serotype and two phylogenetic clades, which differ by 8 to 10% at the nucleotide (nt) level. A 739 nt fragment within the E1 region (nts 8,731 to 9,469) is recommended and sufficient for epidemiological analysis of RV. Clade 1 is composed of

nine recognized genotypes (1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, and 1J) and one provisional genotype 1a for strains circulating before 1984, such as the early vaccine strains, the RA 27/3 vaccine strain, and the laboratory-adapted strain F-Therien. Clade 2 comprises three recognized genotypes (2A, 2B, and 2C). While most RV genotypes have a more restricted geographic distribution, genotypes 1E and 1G, as well as 2B, are found worldwide (Claus *et al*., 2017).

In Sudan for the first time, the rubella virus genome was directly detected in clinical specimens of six CRS cases and two viruses were isolated in cell culture and phylogenetic analysis suggested that three genotypes of rubella virus (RV; 1E, 2B and 1G) were co-circulating in Sudan (Adam *et al*., 2014).

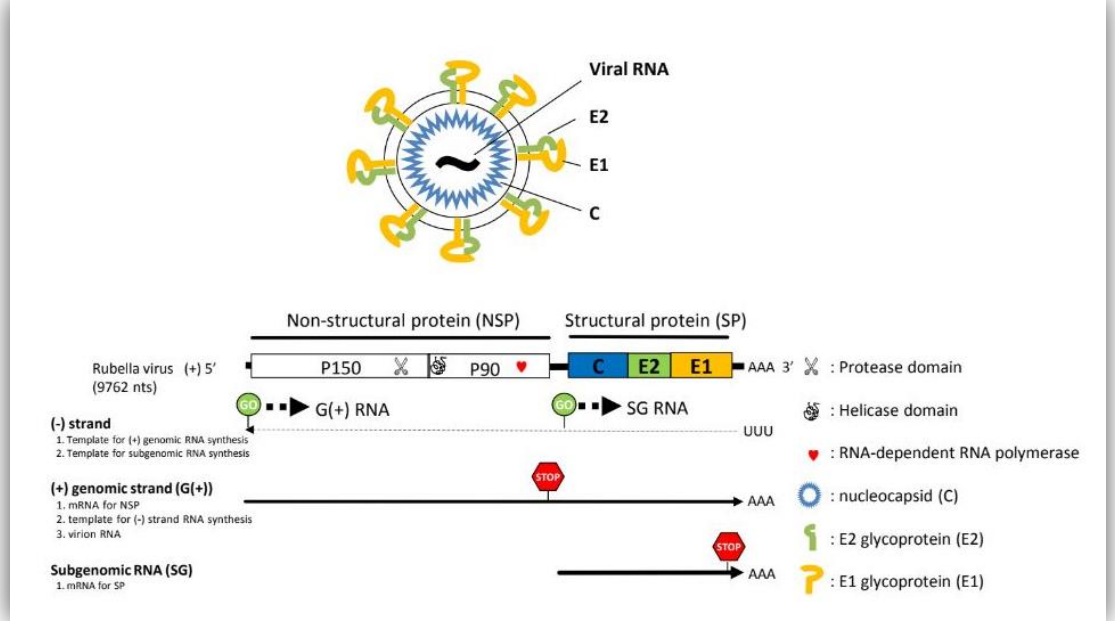

Figure 2. 1: Structure and genome organization of rubella virus (WHO, 2017)

2.2.5. Antigenic properties

Only one serotype of Rubella virus has been described, and the virus is serologically different from other togaviruses.

Early work on RV identified hemagglutinin (HA), complement-fixing (CF), and platelet-aggregating (PA) antigens. RV also has hemolytic activity (Best *et al*., 2004).

Three CF antigens have been described: (i) a large particle antigen with a density of 1.19–1.23 g/ml in sucrose gradients and associated with the infectivity and HA activity; (ii) a small particle (‘soluble’) antigen, which is possibly a subunit of the protein coat; and (iii) a 150S particle, which appears to be associated with the ribonucleoprotein core of the

virus. High-titer preparations of RV can be used as CF antigens. These are usually prepared either by concentration of infected cell culture fluids or by alkaline extraction of infected cells. BHK-21 and Vero cells are used for the production of suitable high-titer virus (Best *et al*., 2004).

2.2.6. Replication of RV

RV replication is less efficient and much slower than that of alpha viruses. In addition, it is not possible to obtain a homogeneously infected population of cells within 24 hours, even at high multiplicities of infection (Knipe and Hawley, 2007).

The host cell receptor for RV is not known yet and virus attaches to cells rapidly. Following binding to the host cell, the virus is internalized by endocytosis and at low pH, the envelope glycoproteins become fusogenic, and capsid undertakes a conformational change and becomes hydrophobic which indicates that the acidic environment within the endosome or lysosome induces fusion of the viral envelope with cellular membranes and release of genomic RNA from the nucleocapsid (Knipe and Hawley, 2007).

Sites of viral replication for RV in association with membranes. Cytoplasmic vacuoles with regularly shaped invaginations or spherules (60 nm in diameter) are connected to the vacuolar membranes by thin membranous necks, suggesting that they are the sites of viral RNA synthesis (Knipe and Hawley, 2007).

Following synthesis of the sub-genomic RNA, the RV structural proteins are translated in association with ER membranes and the structural polyprotein precursor is cleaved by signal peptidase at two sites to generate the three structural proteins. The structural proteins are then post-translationally modified and transported to the Golgi complex, which is the primary site of virus budding which occurs at both the Golgi complex and the plasma membrane, depending on cell type and the time post-infection (Knipe and Hawley, 2007).

RV nucleocapsids form in association with cellular membranes synchronistic with budding and are only rarely observed in the cytoplasm of infected cells and this may be in part because a large pool of capsid remains stably associated with membranes and the mechanisms that regulate interactions between the nucleocapsid and spike glycoproteins during virus budding are largely unknown.

The process of RV release by budding from the Golgi into vacuoles that transport virus to the cell surface prior to exocytosis occurs over several days while the cells remain viable (Knipe and Hawley, 2007).

2.2.7. RV Persistence

Studies on persistently infected cells have shown that both temperature-labile mutants and defective interfering (DI) particles are generated and are supposed to play a role in controlling the degree of viral replication, but neither is required to establish persistent infection. During long-term persistence of Vero cells, DI RNAs become the dominant species of viral RNA with genomic RNA reducing to low levels. RV persistence in cultured cells is thus a chronic infection with the majority of cells expressing viral antigen and RNA, much of which is DI RNA. These cultures release low levels of temperature-sensitive progeny virus and DI particles (Knipe and Hawley, 2007).

The interferons may play a role in viral persistence and are induced in a number of chronically infected cell lines and in human PBMC, both *in vitro* and *in vivo*. Both interferons alpha and gamma restrict RV replication, reducing the levels of viral RNA in the cell and inhibiting viral translation and this could promote both establishment and maintenance of the carrier state. However, interferon is clearly not essential because persistent infections can be established in Vero and BHK-/21 cells, which are unable to produce interferon, and exogenous interferon was not found to have an effect on RV persistence in these cells (Knipe and Hawley, 2007).

2.2.8. Epidemiology

Humans are the only known reservoir of infection, and the rubella virus is transmitted through direct inter-human contact through the aerosol route.

Rubella has a global distribution and the incidence of rubella varies according to age and the geographical zone and in industrialized countries, rubella epidemics have occurred every 5 to 9 years. Before the introduction of vaccination programs in 1968, infection principally affected the 5- to 9-year-old group, corresponding to the early school years (Bouthry *et al*., 2014).

The incidence of rubella has progressively decreased in many countries, and in industrialized countries, it is estimated to occur at a rate of 1.30/100 000 in the general population and 0.00/100 000 in the United States because it has been eliminated, although occasional imported cases are reported. Most sporadic cases occur in the immigrant population, originating in countries where rubella vaccination is not routine (Bouthry *et al*., 2014).

In temperate weathers, the incidence is highest in late winter and early spring (McIntyre *et al.*, 2002).

In the absence of vaccination, rubella is an endemic disease with epidemics every 6 to 9 years. If rubella infections occurred among non-immune pregnant women, CRS cases can occur. During the 1962–1965 global rubella pandemic, an estimated 12.5 million rubella cases occurred in the United States, resulting in 2,000 cases of encephalitis, 11,250 therapeutic or spontaneous abortions, 2,100 neonatal deaths, and 20,000 infants born with CRS (McLean *et al*., 2012).

2.2.9. Pathogenesis

In acquired infection, the virus enters the host through the upper respiratory tract, replicates, and then spreads by the bloodstream to distant sites, including lymphoid tissues, skin, and organs. Viremia in these infections has been detected for as long as 8 days before to 2 days after onset of the rash, and virus shedding from the oropharynx can be detected up to 8 days after onset. Cellular immune responses and circulating virus-antibody immune complexes are thought to play a role in mediating the inflammatory responses to infection, such as rash and arthritis (Ryan *et al*., 2010).

Maternal viremia producing congenital infection that leads to placental infection and then trans-placental spread to the fetus. Once fetal infection occurs, it persists chronically. Such persistence is probably related to an inability to eradicate the virus by immune or interferon-mediated mechanisms. There is too little inflammatory change in the fetal tissues to clarify the pathogenesis of the congenital defects and likelihoods include placental and fetal vasculitis with compromise of fetal oxygenation, chronic viral infection of cells leading to impaired mitosis, cellular necrosis, and induction of chromosomal fracture; any or all of these factors may operate at a critical stage of organogenesis to induce permanent defects. Viral persistence with circulating virus–antibody immune complexes may evoke inflammatory changes postnatally and produce continuing tissue damage (Ryan *et al*., 2010).

After birth, infants affected with rubella continue to expel the virus in the throat, urine, and intestinal tract. Virus may be isolated from virtually all tissues in the first few weeks of life and shedding of virus in the throat and urine, which persists for at least 6 months in most cases, has been known to continue for 30 months. Rubella virus has also been isolated from lens tissue removed 3 to 4 years later and these observations emphasize the fact that such infants are important reservoirs in maintaining virus transmission (Ryan *et al*., 2010).

The prolonged virus shedding is somewhat mystifying; it does not represent a typical example of immunologic tolerance. The affected infants are usually able to produce

circulating IgM and IgG antibodies to the virus, although antibodies may decrease to undetectable levels after 3 to 4 years and many infants have evidence of depressed rubella virus-specific cell-mediated immunity during the first year of life (Ryan *et al*., 2010).

2.2.10. Clinical features

Rubella is an acute, usually mild, infectious disease and the transmission of postnatal rubella is mainly through the respiratory route and commonly occurs in children and young adults. The infection may remain subclinical or cause self-limiting illness with clinical features such as low-grade fever, lymphadenopathy and skin rash (Tamirat *et al*., 2017).

2.2.10.1. Acquired Rubella

Rubella is principally a human disease that occurs between infancy and puberty. As a mild exanthematous disease, it is associated with low-grade fever, lymphadenopathy, headache, malaise, mild coryza, and conjunctivitis with a short-lived (acute) maculopapular rash (Obijimi *et al*., 2013).

Up to 50% of persons with rubella have either subclinical infections or mild symptoms without a rash. Those with symptoms usually experience a mild febrile rash illness. Young children generally have little or no prodrome, while teenagers and adults often report 1–5 days of low grade fever, malaise, and anorexia. Mild coryza and conjunctivitis may also occur. Lymphadenopathy (usually sub occipital, post-auricular, and posterior cervical) is a major clinical manifestation and may last several weeks and fever rarely persists beyond the first day of rash (Washington State Department of Health, 2016).

The maculopapular rash appears first on the face and spreads down the body and lesions are pink and rarely coalesce. The rash of acquired rubella typically lasts 3 days and is occasionally pruritic, spreading and fading more rapidly than the rash caused by measles.

Arthralgia and arthritis occur frequently in adults and up to 70% of adult females with infections experience rubella joint symptoms which appear about the same time as the rash and may persist for up to one month. The most commonly affected areas are fingers, wrists, and knees (Washington State Department of Health, 2016).

2.2.10.2. Congenital rubella syndrome (CRS)

Congenital rubella syndrome (CRS) is an illness resulting from rubella virus infection during pregnancy. When it occurs during early pregnancy, serious sequels such as miscarriages, stillbirths, and assemblage of severe birth defects in infants can result. The risk of congenital infection and defects is highest during the first 12 weeks of gestation

and decreases after the 12th week of gestation with defects rare after the 20th week of gestation (McLean *et al*., 2012).

Common congenital defects of CRS include cataracts, congenital heart disease, hearing impairment, and developmental delay. Infants with CRS usually present with more than one sign or symptom consistent with congenital rubella infection. However, infants may present with a single defect and hearing impairment is the most common single defect (McLean *et al*., 2012).

2.2.10.3. Rubella reinfection

Defined as rubella infection in someone who has previously had either documented natural rubella virus infection or successful rubella immunization.

Maternal re-infection is usually subclinical and diagnosed by changes in antibody concentration (IgG / IgM) only (Davidson and McEwan, 2014).

Rubella reinfection can occur and has been reported after both wild type rubella infection and after receiving 1 dose of rubella vaccine. Asymptomatic maternal reinfection in pregnancy has been considered to present minimal risk to the fetus (congenital infection in <10%) , but several isolated reports have been made of fetal infection and CRS among infants born to mothers who had documented serologic evidence of rubella immunity before they became pregnant and had reinfection during the first 12 weeks of gestation. CRS was not reported when reinfection occurred after 12 weeks gestation (McLean *et al*., 2013).

2.2.10.4. Complications

Complications are rare, occurring more often in adults and they can include encephalitis, neuritis, orchitis, and thrombocytopenia. Hemorrhagic manifestations can occur and are usually secondary to low platelets and vascular damage. Thrombocytopenic purpura is the most common of these, and this manifestation is seen more often in children than adults (Washington State Department of Health, 2016).

2.2.11. Laboratory diagnosis

Number of infections can present with signs and symptoms compatible with rubella. In addition, up to 50% of infected persons may have minimal or no clinical symptoms.

Therefore, a laboratory valuation is critical for confirmation of a clinical rubella diagnosis. Humoral and cell-mediated immunity develop following natural infection and with immunization (Lulandala *et al*., 2017).

2.2.11.1. Serological diagnosis

Serological detection of specific rubella antibodies in suspects remains to be crucial in diagnosis of rubella virus infection since clinical diagnosis is difficult due to non-specific symptoms (Lulandala *et al*., 2017).

With natural infection, IgM antibodies become detectable within 3–4 days and IgG antibodies within one week of the onset of rash. Rubella-specific IgM can often be detected in individuals up to two months after illness and, in a decreasing percentage of individuals, up to six or seven months after natural infection, vaccination and reinfection (WHO, 2009).

In addition, false-positive IgM test results may occur because of cross-reacting IgM antibodies (e.g. to EBV, human parvovirus B19, etc.), rheumatoid factor or other autoantibodies, and polyclonal immune stimulation by EBV (WHO, 2009).

2.2.11.2. Isolation of RV

Direct detection of virus is more sensitive than sero-diagnosis in diagnosis of RUB infection on the days immediately after presentation of symptoms (Tzeng *et al*., 2005).

Growth of rubella virus from clinical specimens can be used to diagnose postnatal rubella, CRS and throat swabs taken on the day of rash, typically a suitable time for sample collection, are usually positive for rubella virus, even although a lightly higher percentage of cases are positive 2 days before rash onset (Murray *et al*., 2007).

Virus shedding in the throat drops rapidly, and by 4 days after rash onset, only about 50% of cases are positive. In addition, viral culture is used to monitor virus in CRS and CRI patients for the purpose of determining when isolation of these patients from susceptible contacts can be stopped. The virus will grow in a variety of cell types, including Vero, BHK21, AGMK, and RK-13 cells and the primary problem encountered with tissue culture is the lack of a cell type that produces CPE in a single passage of wild-type viruses (Murray *et al*., 2007).

2.2.11.3. Detection of viral genome

Virus growth can now be identified in the absence of CPE using methods such as RT-PCR (Reverse Transcriptase - Polymerase Chain Reaction) and IFA (Immunofluorescence Assay) to detect viral RNA and proteins, respectively (Murray *et al*., 2007).

The detection of viral RNA by RT-PCR may be possible for 3-4 days longer and the optimal time to collect specimens is within four days of the onset of symptoms (WHO, 2009).

However, the only trustworthy evidence of acute rubella infection is a positive viral culture for rubella or detection of rubella virus by PCR, the presence of rubella-specific IgM antibody, or demonstration of a significant rise in IgG antibody from paired acute- and convalescent-phase sera (CDC, 2015).

2.2.11.4. Diagnosis of congenital rubella infection

2.2.11.4.1. Prenatal diagnosis

When a maternal infection is diagnosed, a prenatal diagnosis of congenital infection is recommended and it is based on the detection of RV-IgM in fetal blood or on the detection of the viral genome in amniotic fluid, fetal blood or chorionic villus biopsies.

The detection of rubella virus in chorionic villus biopsies reveals an infection of the villi, not a fetal infection (Bouthry *et al*., 2014).

To achieve approximately 100% specificity of a prenatal diagnosis and greater than 90% sensitivity; the following conditions must met: (i) at least a 6-week period passes between the infection and sampling; (ii) a sample collection is performed after 21 WG; and (iii) the samples for RT-PCR are stored and transported frozen (fetal blood for RV-IgM detection is stored and transported at 4 °C) (Bouthry *et al*., 2014).

2.2.11.4.2. Postnatal diagnosis of congenital infection

A postnatal diagnosis of congenital infection is based on the detection of a specific RV-IgM by immune-capture ELISA, which has sensitivity and specificity that approach 100% in infected newborns (<3month`s of age). In cases in which the RV-IgM test is positive, a congenital infection might be confirmed by isolating the rubella virus or by detecting the viral genome in nasopharyngeal swabs, urine and oral fluid using RT-PCR.

Performing a postnatal diagnosis of a congenital infection is important, regardless of whether a clinical manifestation of CRS is observed, to provide a specific follow-up care plan if an infection is discovered; including neurological and hearing monitoring.

However a child infected in utero could excrete the virus in saliva and urine for several months or years (Bouthry *et al*., 2014).

2.2.12. Laboratory Network

Rapid and accurate diagnosis of measles and rubella remains essential for monitoring progress and detecting outbreaks. So, the WHO Global Measles and Rubella Laboratory Network (LabNet) provides valuable worldwide information about the circulation of measles and rubella infections. As of October 2011, the LabNet involved 690 national, sub-national and regional laboratories, serving 183 countries (WHO, 2012).

All laboratories follow a standardized set of testing protocols and reporting techniques that are continually reviewed and improved as technological advances occur.

Whereas, the LabNet relies on a strong quality assurance program that monitors the performance of all laboratories through annual proficiency testing and continuous assessment. The LabNet is a vital source for immunization program as it documents the successes of vaccination efforts to interrupt measles and rubella transmission nationally and internationally and is able to monitor virus transmission patterns and help document successful elimination strategies (WHO, 2012).

2.2.13. Immune response and immunity

The disease caused by RV gives lifelong immunity and a single dose of a rubella vaccine is assumed to provide lifelong immunity; but persistent immunity may require contact with endemic cases. Infants born to immune mothers are usually protected for 6– 9 months, depending on the amount of maternal antibodies acquired (Kansas Disease Investigation Guidelines, 2013).

Administering immunoglobulin to pregnant women with acute infection is divisive. And no data suggest that immunoglobulin prevents fetal anomalies (Davidson and McEwan, 2014).

2.2.14. Treatment

No specific treatment is available and the treatment of acute rubella infection is supportive. The prognosis is generally excellent for pregnant women with rubella infection (JOGC, 2008; Kansas Disease Investigation Guidelines, 2013).

2.2.15. Rubella vaccination

In 1969, live attenuated rubella vaccines were licensed in the United States and the goal of the rubella vaccination program was and continues to be to prevent congenital rubella infections, including CRS. Following vaccine licensure, the number of reported cases of CRS in the United States declined dramatically to <1 case per year or 4 cases total during 2005–2011(McLean *et al*., 2012).

A combined formulation (MR or MMR) was begun in the 1970s. and the combined vaccine products, national and global health leaders have increasingly focused on simultaneous management of both diseases (WHO, 2012). Also there is combination with VZV in the MMR and known as MMRV (proQuad) (CDC, 2015).

The most commonly used rubella vaccines are based on the live, attenuated RA 27/3 (Rudivax) strain grown in human diploid cells; Japan and China use the TO-336 and

BRD-2 strains, respectively and vaccination after exposure is not harmful and may possibly prevent later disease (CDC, 2015).

Rubella vaccines exist as monovalent preparations or are associated with vaccines against measles, measles and mumps or measles, mumps and varicella and the antibody response rate to a single dose is higher than 95%. While after two doses, the response rate approaches 100%, and immunity is detectable at over 21 years of age, despite fading rubella virus-specific immunoglobulin G (RV-IgG) titers.

In most countries, the schedule for rubella vaccination is two doses before 24months, which is same to the schedule for measles vaccination (Bouthry *et al*., 2014).

Unfortunately, Rubella vaccine coverage in Africa only about 10% (Cohen, 2017).

2.2.15.1. Rubella Vaccination of Women of Childbearing Age

Rubella vaccine should not receive to pregnant or who intend to become pregnant within 4 weeks and all other women should be vaccinated after being informed of the theoretical risks of vaccination during pregnancy and the importance of not becoming pregnant during the 4 weeks following vaccination. ACIP does not recommend routine pregnancy screening of women before rubella vaccination (CDC, 2015).

If a pregnant woman is unintentionally vaccinated or if she becomes pregnant within 4 weeks after vaccination, she should be advised about the concern for the fetus, but MMR vaccination during pregnancy should not ordinarily be a reason to consider termination of the pregnancy (CDC, 2015).

When rubella vaccine was licensed, concern existed about women being inadvertently vaccinated while they were pregnant or shortly before conception and this concern came from the known teratogenicity of the wild-virus strain. To determine whether CRS would occur in infants of such mothers, CDC maintained a registry from 1971 to 1989 of women vaccinated during pregnancy. This was called the Vaccine in Pregnancy (VIP) Registry (CDC, 2015).

Although subclinical fetal infection has been detected serologically in approximately 1%–2% of infants born to susceptible vaccines, regardless of the vaccine strain, the data collected by CDC in the VIP Registry showed no evidence of CRS occurring in offspring of the 321 susceptible women who received rubella vaccine and who continued pregnancy to term. The observed risk of vaccine-induced malformation was 0%, with a maximum theoretical risk of 1.6%, based on 95% confidence limits (1.2% for all types of rubella vaccine). Since the risk of the vaccine to the fetus appears to be extremely low, if it exists at all, routine termination of pregnancy is not recommended and individual counseling for

these women is recommended. The ACIP continues to state that because of the small theoretical risk to the fetus of a vaccinated woman, pregnant women should not be vaccinated (CDC, 2015).

2.2.16. Current WHO Global and Regional Targets

All six WHO regions have committed to measles elimination and five regions have set target dates. The WHO Region of the Americas achieved the goal in 2002; the Western Pacific Region aims to eliminate measles by end of 2012; and the European and Eastern Mediterranean Regions are accelerating their measles control activities in order to eliminate measles by 2015.

In 2011, countries in the African Region took on the goal to eliminate measles by 2020, and in 2010 the South-East Asia Region adopted a resolution urging countries to mobilize resources to support the elimination of measles, the target date for which was under discussion. As of the publication of this plan, three of the six WHO regions had set control or elimination targets for rubella. The Americas and Europe targeted rubella and CRS elimination by 2010 and 2015, respectively. The Western Pacific Region aims to have significantly accelerated rubella and CRS prevention by 2015, and the Eastern Mediterranean Region is currently discussing the establishment of a target date for rubella elimination. The African and South-East Asia Regions have yet to establish rubella elimination, control or prevention goals (WHO, 2012).

The large-scale vaccination program in Americas and Europe has achieved a harsh reduction or elimination of both the virus and CRS. In contrast, the highest risk of CRS is found in countries where the rubella-containing vaccine (RCV) has not been introduced to the national immunization program or the vaccine coverage is low. Africa and South East Asia regions, with the respective estimated incidence of 116 and 211 per 100 000 live births in 2010, have the highest rates of CRS (Tamirat *et al*., 2017).

With the goal of extending the full benefits of immunization to all persons, the Global Vaccine Action Plan (GVAP) 2011–2020 was outlined and certified by the World Health Assembly in 2012. Towards realizing GAVP goals, rubella vaccine had been introduced in 149 (77%) of 194 WHO member countries as of September 2016.

Whilst Ethiopia has planned to introduce measles-rubella vaccine into the routine expanded program for immunization schedule for children under 1 year of age in 2019 (Tamirat *et al*., 2017).

2.2.17. Strategies to Decrease Rubella and CRS

2.2.17.1. Vaccination of Susceptible Post-pubertal Females

Elimination of original rubella and CRS can be maintained by continuing efforts to vaccinate susceptible adolescents and young adults of childbearing age, particularly those born outside the United States. So, these efforts should include vaccinating in family planning clinics, sexually transmitted disease (STD) clinics, and as part of routine gynecologic care; maximizing use of premarital serology results; emphasizing immunization for college students; vaccinating women postpartum and post-abortion; immunizing prison staff and, when possible, prison inmates, especially women inmates; offering vaccination to at-risk women through the special supplemental program for Women, Infants and Children (WIC); and implementing vaccination programs in the workplace, particularly those employing persons born outside the United States (CDC, 2015).

2.2.17.2. Prevent Transmission from Infants with CRS

Cases of U.S acquired rubella have occurred among susceptible persons providing care for infants with CRS. Because infants can shed the virus for prolonged periods, (up to 1 year of age or longer) and infants with CRS should be considered infectious until they are at least 1 year old or until two cultures of clinical specimens obtained one month apart after the infant is older than three months of age are negative for rubella virus. Infants with CRS should be placed in contact isolation during any hospital admission before age one year or until the infant is no longer considered infectious.

In addition, health officials should consider excluding infants with CRS from child care facilities until he or she is no longer considered infectious. Persons having contact with infants with CRS should have documented evidence of immunity to rubella and caregivers of infants with CRS should be aware of the potential hazard of the infants to susceptible pregnant contacts (McLean *et al*., 2012).

2.2.17.3. Hospital Rubella Programs

Emphasis should be placed on vaccinating susceptible hospital personnel, both male and female (e.g., volunteers, trainees, nurses, physicians). Ideally, all hospital employees should be immune and it is important to note that screening programs alone are not adequate. Vaccination of susceptible staff must follow (CDC, 2015).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study approach

Qualitative approach

3.2. Study design

This is an observational, cross-sectional, analytical, case study, hospital based. This aimed to detect rubella virus among apparently healthy pregnant women.

3.3. Study areas

This study was conducted as a hospital-based study in different hospitals of different geographical locations in Khartoum State (Omdurman Maternity Hospital, China Friendship Hospital, Alsaudi Hospital, Turkish Hospital, Saad Aboalula Hospital, Haj Elsafi Teaching Hospital, Ibrahim Malik Teaching Hospital and East Nile Model Hospital).

3.4. Study duration

This study was carried out in 4 years, in the period from 2014-2018.

3.5. Study population

The study was targeted all pregnant women attending the study areas were considered eligible to participate irrespective of race, age, residence and parity.

3.5.1. Inclusion criteria

Attending apparently healthy pregnant women in study areas during study period, who agreed to participate in this study, were included in this study.

3.5.2. Exclusion criteria

Non-pregnant and pregnant with rash were excluded

3.6. Sample size

The sample size was calculated using Kish Lisle formula for cross-sectional study **(**Kish, 1965).

The sample size was calculated according to following formula: $n= t^2 *P (1-P)/\mu^2$

In which t= 1.96, P= prevalence (= 50%) and $\mu= 0.05$.

The actual size was 384.

A total of three hundred and fifty eight (n=358) pregnant women were randomly selected from those attending the study areas.

3.7. Sampling technique

This study was based on non-probability convenience sampling technique.

Samples were taken from attended agreed women.

3.8. Method of data collection

Data were collected through direct interview with pregnant women. The interview instrument (Questionnaire) consists of 14 questions (appendix-A). It consists of three parts; including general information on women.

3.9. Ethical considerations

Permission to conduct the study was taken from Research Committee of College of Medical Laboratory Science; Sudan University of Science and Technology and then from Research Committee of Ministry of Health. A written informed consent was obtained from each participant (appendix-B).

3.10. Specimen collection

Five ml of blood were collected and separated into 2 aliquots; EDTA containers and plain containers. Blood containers with EDTA were centrifuged at 3000 rpm for 5 minutes, and plasma were collected in sterile cryogenic tube containers. For serum, the blood specimens were left to clot for minutes and then centrifuged at 3000 rpm for 5 minutes. Sera and plasma were stored at -70º C till the time of analysis.

3.11. Laboratory tests

An Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect the specific RV IgG and IgM antibodies.

3.11.1. ELISA for detection of RV IgM and IgG antibodies:

The rubella Virus IgM test is the initial assay for the detection of acute infections and recommended for the determination of the immune status. While the rubella Virus IgG tests detect previous exposure and recommended to detect past infection and immunization.

Commercial ELISA Kits (EUROIMMUN, Medizinischelabordiagnostika AG, Germany) were used for each immunoglobulin separately as described by the manufactures (appendix-C). In brief, the sample diluents X100 concentrate was diluted 1:101 in sample buffer for the assay run. According to the plan of working, 100 µl of the negative control, 100 µl of calibrator, 100 µl of positive control and 100 µl of diluted samples (1:101) were incubated in microplate well coated with rubella virus glycoprotein antigen at room temperature for 30 minutes. The wells were washed three times manually by washing buffer (which diluted firstly by distilled water 1:9) to remove residual plasma. Then 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgM and IgG) were added to each well and incubated at room temperature for 30 minutes.

After another washing step to eliminate unbound material, an enzyme substrate solution (TMB Substrate) was added (100 µl /well) and the plate was incubated for 15 minutes. The blue color changed to yellow after adding of the stop solution (100 µl). The optical density (O.D) in a microplate reader was read within 10 minutes at 450 – 630 nm.

3.11.1.1. Calculation and interpretation of the results:

The following formula was used to calculate the ratio:

Extinction of the control or patient sample/ extinction of the calibrator = ratio

Interpretation of results was done as follows:

Ratio < 0.8: negative

Ratio ≥ 0.8 to < 1.1: borderline

Ratio ≥ 1.1: positive

3.11.2. RNA Extraction

Extraction of RNA was done by RNA extraction kit (analytikJena) according to the manufacturer's instructions (appendix D).

3.11.3. Target amplification by Reverse Transcriptase polymerase chain reaction (RT-PCR)

Extracted RNA was converted into cDNA by reverse transcriptase enzyme by RT kit (Intron Biotechnology, Korea). In which 10µl of RNA was added to 10µl of D.D.W and cDNA synthesis reactions was performed by incubation of the reaction mixture for 1 hour at 45°C, followed by 5 minutes at 95°C using PCR machine (Applied Biosystems).

PCR was done for 169 specimens that include: all IgM+ve, all results negative by ELISA and some of IgG+ve which were selected randomly.

After cDNA was synthesized; it was amplified by PCR machine (Applied Biosystems, Roche Diagnostic Systems) by using ready premix (Intron Biotechnology, Korea). The primers were used to amplify the entire E1 gene region of 1,446 nucleotides (nt) (nt 8258 to 9703), which includes the 739 nt (8731 to 9469) corresponding to the minimum acceptable window defined by WHO for routine molecular epidemiology (WHO, 2005).

The primers design as follow: E1.1n (forward primer) 5´CTAGCTACGTCCAGCACCCT 3` (8691–8710 position) and E1.2Ra (reverse primer) 3´ACTGGTAGCACCCGGTCACA 5` (9292–9311position) (Cooray *et al*., 2006).

The reaction conditions were modified from Cooray *et al*. (2006) as following: 95°C for 3 minutes; 35 cycles of 95°C for 30seconds, 57°C for 30seconds, and 72°C for 45seconds; and finally 72°C for 5 minutes, followed by 4°C for 10 minutes.

3.11.4 Gel electrophoresis

RT-PCR (reverse transcription polymerase chain reaction) amplification was verified by gel electrophoresis. Agarose gel powder (1.5 g) was weighted by sensitive balanced and was dissolved in 100 ml1x TBE buffer, then was dissolved by microwave for 2 minutes ; after cooling ethidium bromide was added and then poured in a gel tank contains comb which was removed after polymerization of gel. In first lane marker (100 bp) was added, negative control and the samples. Then the gel was submersed by 1x TBE buffer and run for 45 minutes at voltage 75. The gel was visualized by trans-illuminator. The length of band was 621 bp.

3.12. Quality Control

Control negative and control positives were used in ELISA tests to assure the accuracy and validity of results. Also in PCR technique, positives and negative controls were used to verify no contamination; and to achieve standardized conditions.

3.13. Statistical analysis

Data were computed and analyzed by SPSS software program version 16.0 and graph pad prism version 6. Significance of differences was determined using Chi-square test and Odd ratio, and statistical significance was set at p-value ≤ 0.05. Data were presented in form of tables and figures.

CHAPTER FOUR

RESULTS

In this study 358 sera and plasma were collected from apparently healthy pregnant women, mostly were house wives and with age group range from 20 to 30 years.

The results of ELISA were showed that: 2(0.56%) were IgM positive, 356 (99.44%) were negative, 320 (89.39%) were IgG positive and 38(10.61%) were IgG negative (figure 4.2 and 4.3).

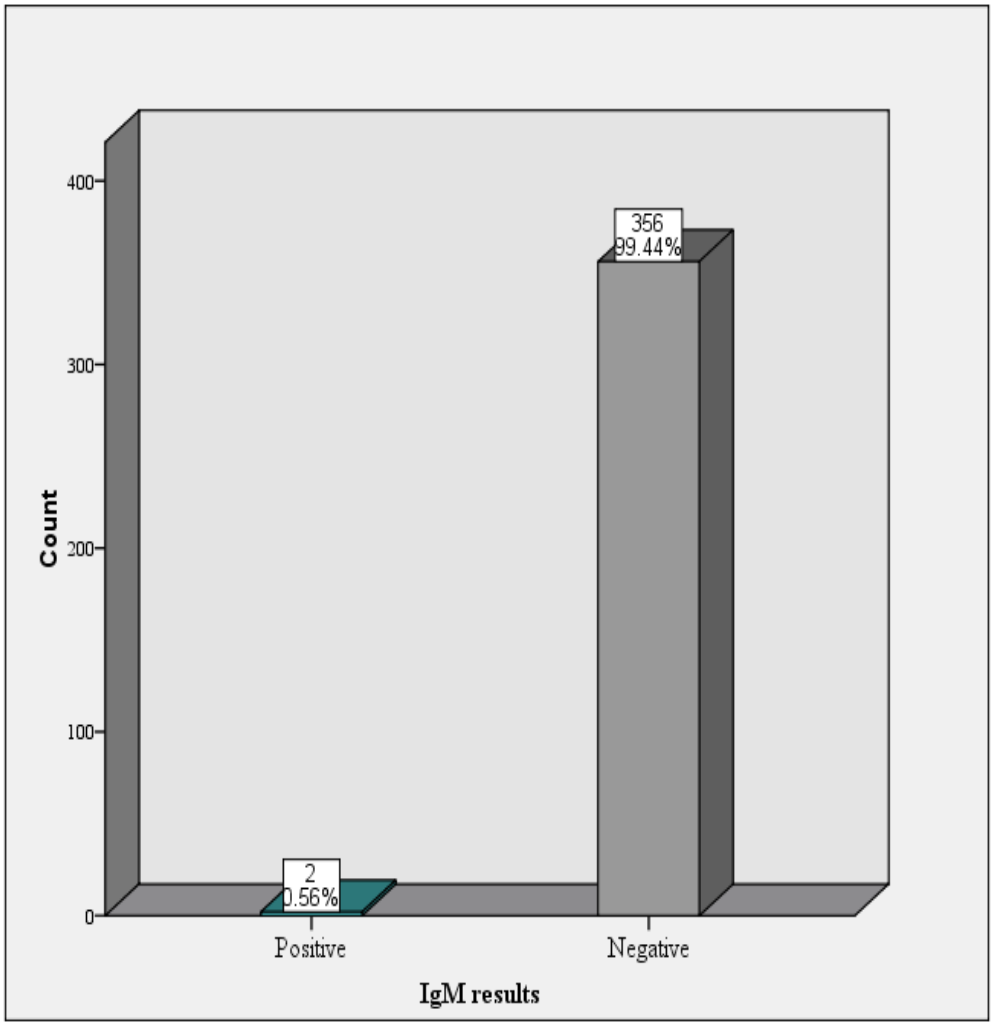

Figure 4.2: The frequency of IgM among apparently healthy pregnant women

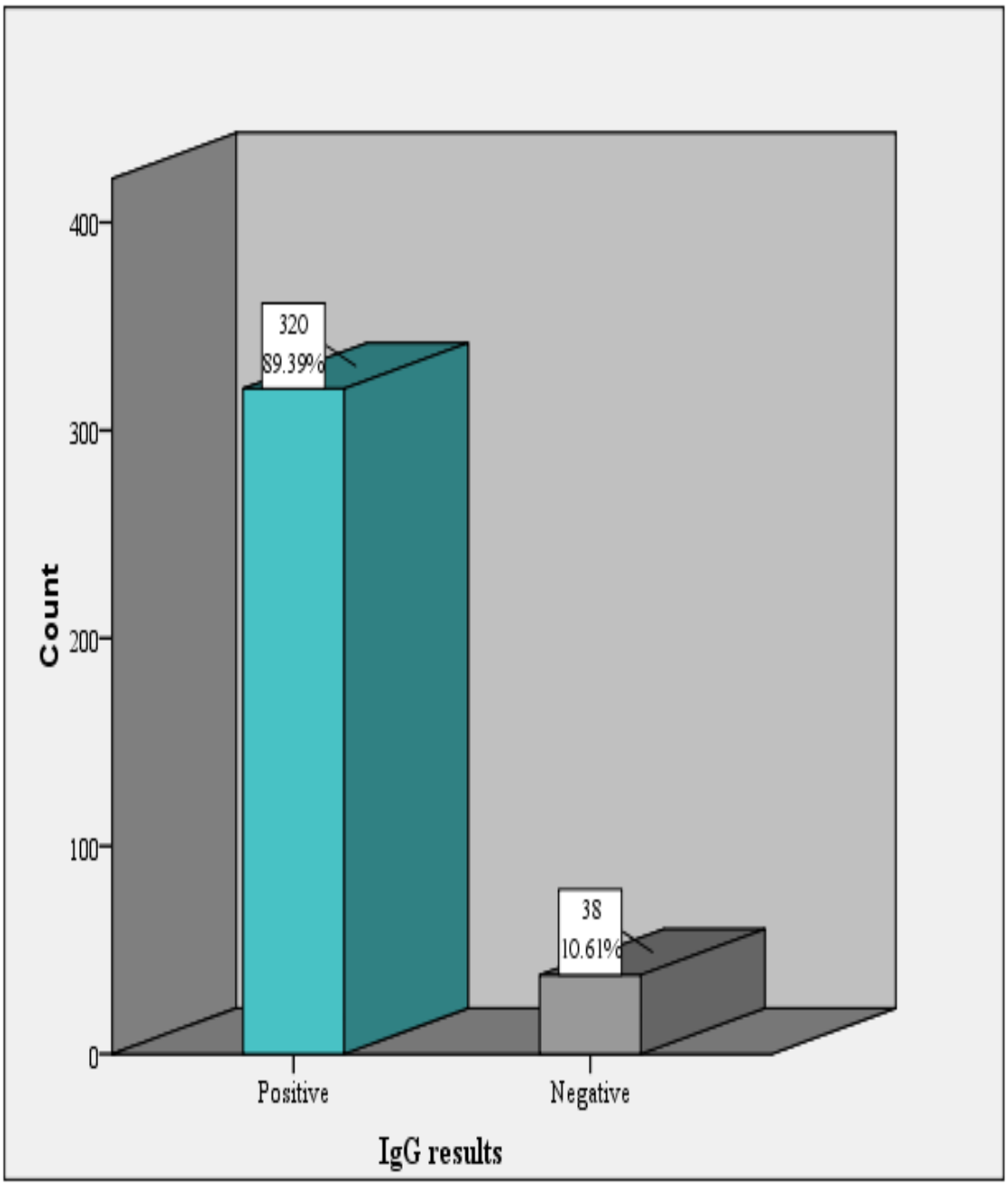

Figure 4.3: The frequency of IgG among apparently healthy pregnant women

IgM was found in one case in first trimester and in second trimester and no cases in third trimester. IgG was high in second trimester (128 cases) compared to the first trimester (77 cases); while there was no significant relation between trimesters IgM and IgG results (table 4.1).

Table 4.1: The association between trimester and IgM, IgG results among apparently healthy pregnant women

Serological tests		Trimester						Total		Significant differences
		First trimester	%	Second trimester	%	Third trimester	%	No.	%	
IgM result	Positive	1	.3%	1	.3%	0	.0%	2	.6%	0.503 N.S
	Negative	83	23.2%	144	40.2%	129	36.0%	356	99.4%	
Total		84	23.5%	145	40.5%	129	36.0%	358	100.0%	
IgG result	Positive	77	21.5%	128	35.8%	115	32.1%	320	89.4%	0.720 N.S
	Negative	7	2.0%	17	4.7%	14	3.9%	38	10.6%	
Total		84	23.5%	145	40.5%	129	36.0%	358	100.0%	

P-value = ≤ 0.05

N.S: Non significant

IgM was found only in multigravida (2 cases) while IgG was very high in multigravida (234 cases) and there was no significant association between gravidity and IgM and IgG results (table 4.2).

Table 4.2: The association between gravidity and IgM, IgG results among apparently healthy pregnant women

Serological tests		Gravidity				Total		Significant differences
		Primigravida	%	Multigravida	%	No.	%	
IgM result	Positive	0	.0%	2	.6%	2	.6%	0.384 N.S
	Negative	98	27.4%	256	72.1%	356	99.4%	
Total		98	27.4%	260	72.6%	358	100.0%	
IgG result	Positive	86	24.0%	234	65.4%	320	89.4%	0.539 N.S
	Negative	12	3.4%	26	7.3%	38	10.6%	
Total		98	27.4%	260	72.6%	358	100.0%	

P-value = ≤ 0.05

N.S: Non significant

Out of 358 specimens, IgM was found in two cases according to family members; one in each group: 2-5 members and > 5 members (0.6%). There was no association between number of family members and IgM result (table 4.3).

Table 4.3: The association between number of family members and IgM result among apparently healthy pregnant women

IgM result	Family members				Total		Significant differences
	2-5 members	%	>5 members	%	No.	%	
Positive	1	.3%	1	.3%	2	.6%	0.745 N.S
Negative	218	60.9%	138	38.5%	358	99.4%	
Total	219	61.2%	139	38.8%	358	100.0%	

P-value = ≤0.05

N.S: Non significant

Similar result was obtained for the frequency of IgM in relation to season (one case in summer and one in winter). There was no significant association between season and IgM results (table 4.4).

Table 4.4: The association between season and IgM results among apparently healthy pregnant women

IgM result	Season				Total		Significant differences
	Summer	%	Winter	%	No.	%	
Positive	1	.3%	1	.3%	2	.6%	
Negative	131	36.6%	225	62.8%	356	99.4%	0.700
Total	132	36.9%	226	63.1%	358	100.0%	N.S

P-value = ≤0.05

N.S: Non significant

The socioeconomic situation designed the same results according to the moderate, where only two cases were positive for IgM (0.6%).there was no significant association between socioeconomic situation and IgM results (table 4.5).

Table 4.5: The association between socioeconomic situation and IgM results among apparently healthy pregnant women

IgM result	Socioeconomic situation				Total		Significant differences
	Moderate	%	Low	%	No.	%	
Positive	2	.6%	0	.0%	2	.6%	0.572 N.S
Negative	307	85.8%	49	13.7%	356	99.4%	
Total	309	86.3%	49	13.7%	358	100.0%	

P-value = ≤0.05

N.S: Non significant

IgG was found in 92 cases (25.7%) from 358 specimens and there was no relation between miscarriage and IgG result (table 4.6).

Table 4.6: The relation between miscarriage and IgG result among apparently healthy pregnant women

IgG result	Miscarriage				Total		Significant differences
	Yes	%	No	%	No.	%	
Positive	92	25.7%	228	63.7%	320	89.4%	0.178 N.S
Negative	7	2.0%	31	8.7%	38	10.6%	
Total	99	27.7%	259	72.3%	358	100.0%	

P-value = ≤0.05

N.S: Non significant

The number of miscarriages has substantial effect on the frequency of IgG result, 57 out of 97 (58.8%) was positive in the once miscarriage followed by twice and then more than twice. but no significant relation number of miscarriage and IgG results (table 4.7).

Table 4.7: The relation number of miscarriage and IgG results among apparently healthy pregnant women

IgG result	No. of miscarriages						Total		Significant differences
	Once	%	Twice	%	More than twice	%	No.	%	
Positive	57	58.8%	20	20.6%	13	13.4%	90	92.8%	0.358 N.S
Negative	6	6.2%	0	.0%	1	1.0%	7	7.2%	
Total	63	64.9%	20	20.6%	14	14.4%	97	100.0%	

P-value = ≤0.05

N.S: Non significant

Out of 358 specimen, 6 cases (1.7%) had previous skin rash gave positive result with IgG, and there was no meaning relation between history skin rash and IgG results (table 4.8).

Table 4.8: The relation of history skin rash and IgG results among apparently healthy pregnant women

IgG result	History of skin rash				Total		Significant differences
	Yes	%	No	%	No.	%	
Positive	6	1.7%	314	87.7%	320	89.4%	0.750 N.S
Negative	1	.3%	37	10.3%	38	10.6%	
Total	7	2.0%	351	98.0%	358	100.0%	

P-value = ≤ 0.05

N.S: Non significant

IgG was found positive in 10 cases (2.8%) had child with congenital abnormalities and there was no significant association between Birth child with congenital abnormalities and IgG results (table 4.9).

Table 4.9: The association between Birth child with congenital abnormalities and IgG results among apparently healthy pregnant women

IgG result	Birth child with congenital abnormalities				Total		Significant differences
	Yes	%	No	%	No.	%	
Positive	10	2.8%	310	86.6%	320	89.4%	0.269 N.S
Negative	0	.0%	38	10.6%	38	10.6%	
Total	10	2.8%	348	97.2%	358	100. 0%	

P-value = ≤ 0.05

N.S: Non significant

PCR results was showed 4 (2.4%) were positive and 165(97.6) were negative as explained in figure 4.4.

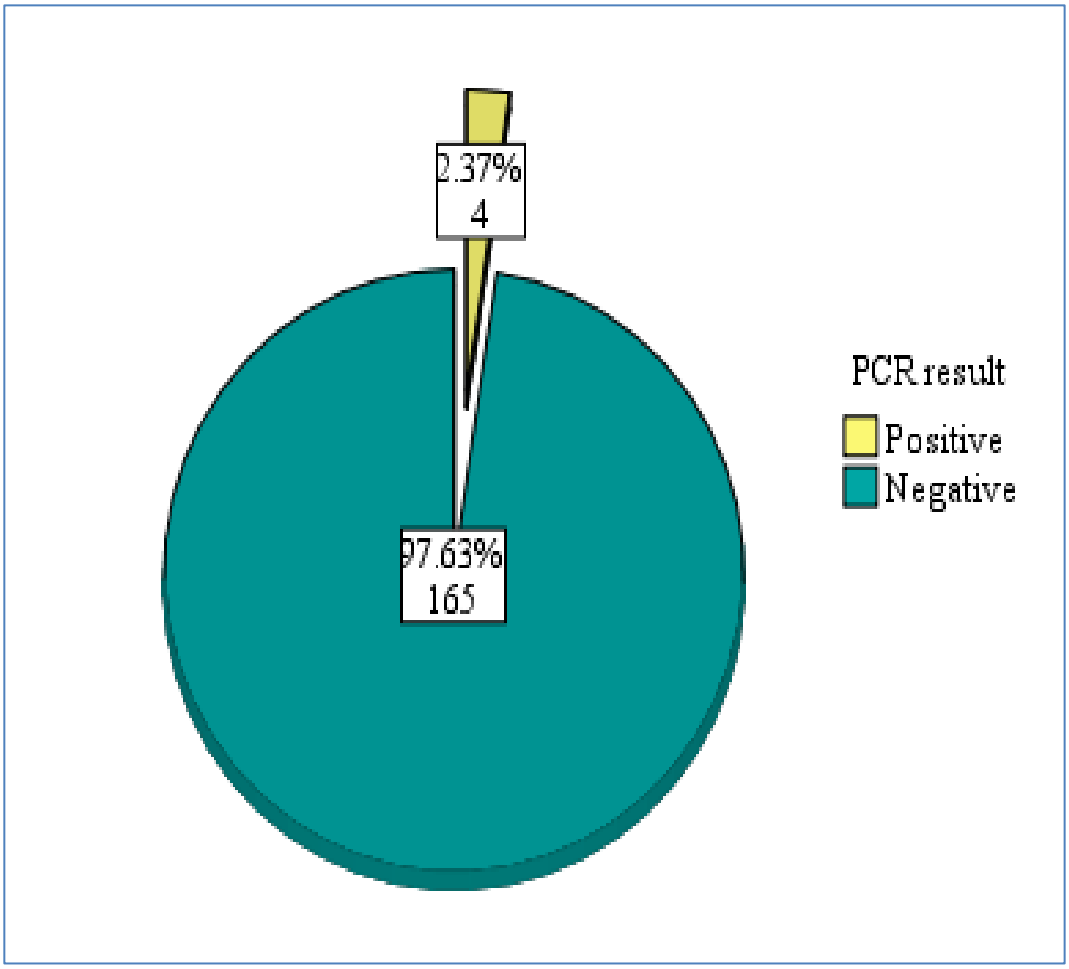

Figure 4 .4: The percentage of PCR result among apparently healthy pregnant women

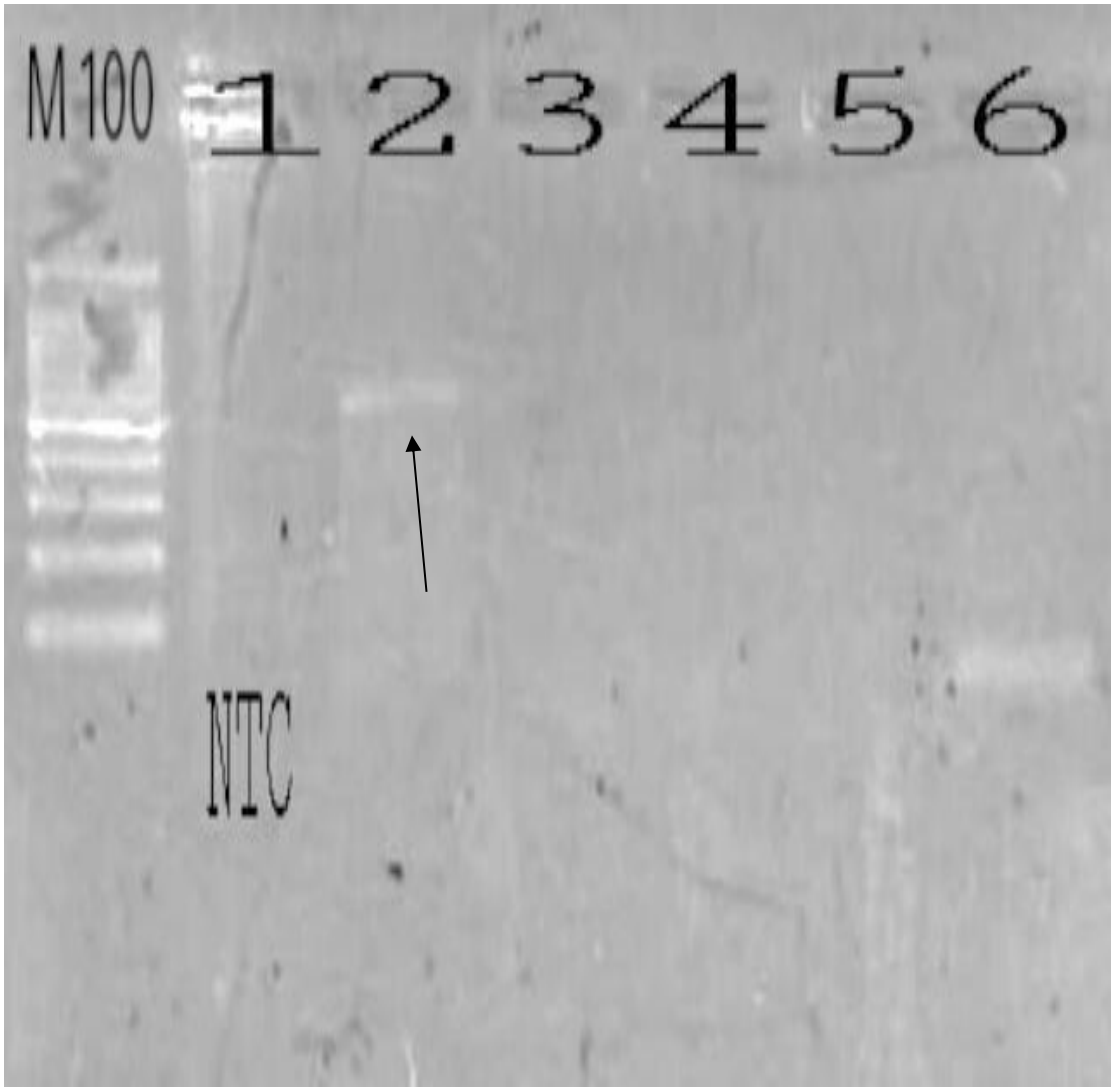

Figure 4. 5: PCR product for E1 gene of rubella virus on 1.5% agarose gel

M= DNA marker 100 bp NTC= negative test control (lane 1)

Lanes 2,3,4,5 and 6 are tested samples

Lane 2= positive with band length 621 bp

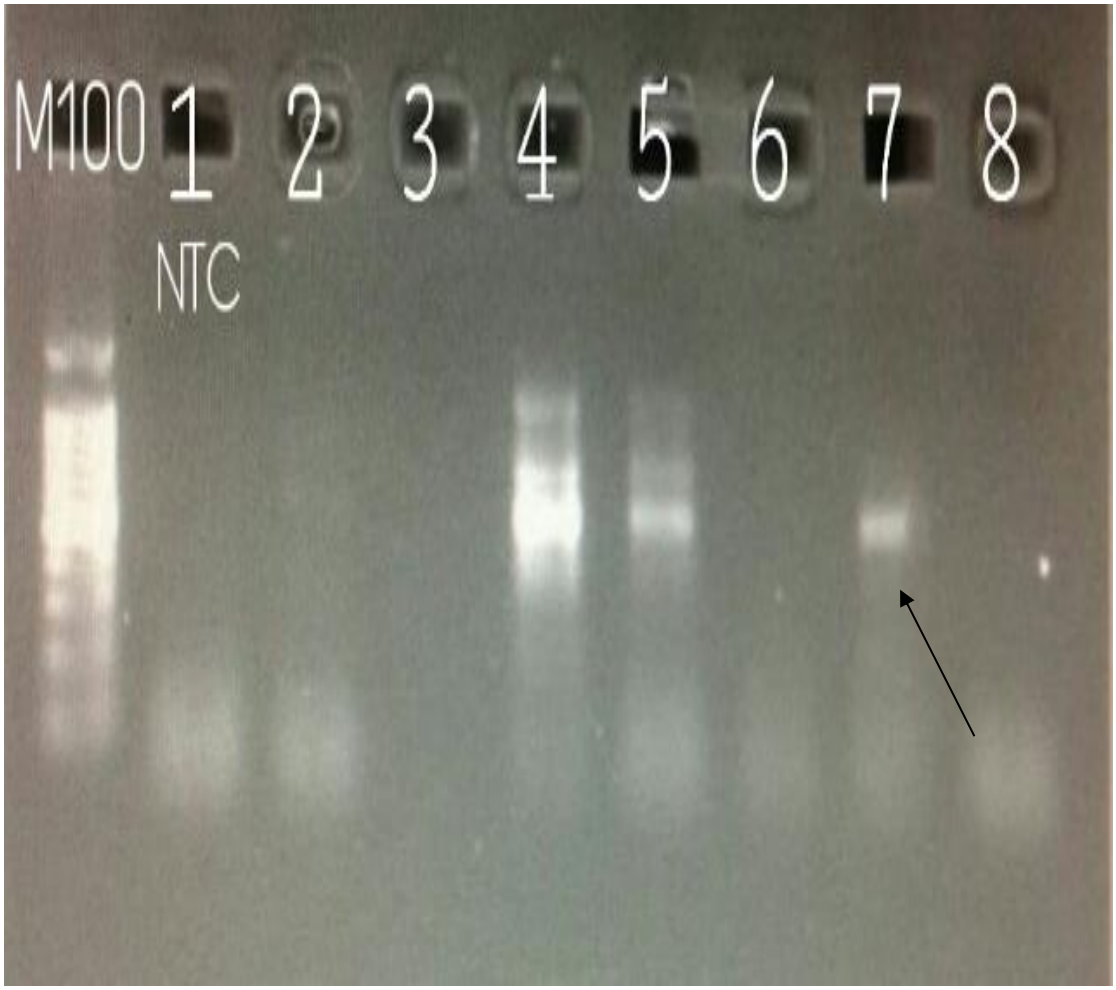

Figure 4.6: PCR product for E1 gene of rubella virus on 1.5% agarose gel.

M= DNA marker 100 bp lane 1=NTC (negative test control)

Lanes 2, 3, 6, and 8 are negative samples

Lanes 4, 5 and 7 are positive samples with band length 621 bp

PCR results gave positive result with two cases (1.2%) inhabited Omdurman, one case in Khartoum, one case in Khartoum north, no cases in East Nile and outside Khartoum State. There was significant association between PCR results and residence (table 4.10).

Table 4.10: The association between PCR result and residence among apparently healthy pregnant women

PCR result	Residence					Total
	Khartoum	Omdurman	Khartoum North	East Nile	outside Khartoum State	
Positive	1(.6%)	2(1.2%)	1(.6%)	0(.0%)	0 (.0%)	4(2.4%)
Negative	55(32.5%)	53(31.4%)	17(10.1%)	37(21.9%	3 (1.8%)	165(97.6%)
Total	56(33.1%)	55(32.5%)	18(10.7%)	37(21.9%	3 (1.8%)	169(100.0)%

P-value = ≤ 0.05

Significant differences= 0.694 (Non- significant)

PCR gave positive result with three pregnant women in third trimester (1.8%), one in second trimester and no cases in first trimester. There was no significant association between PCR results and trimester (table 4.11).

Table 4.11: The association between PCR result and trimester among apparently healthy pregnant women

PCR result	Trimester						Total		Significant differences
	First trimester	%	Second trimester	%	Third trimester	%	No.	%	
Positive	0	.0%	1	.6%	3	1.8%	4	2.4%	0.497 N.S
Negative	29	17.2%	57	33.7%	79	46.7%	165	97.6%	
Total	29	17.2%	58	34.3%	82	48.5%	169	100.0%	

P-value = ≤ 0.05

N.S: Non significant

PCR yield the same results in winter and summer (two cases in each). There was significant association between PCR result and season of year (table 4.12).

Table 4.12: The association between PCR result and season of year among apparently healthy pregnant women

PCR result	Season				Total		Significant differences
	Summer	%	Winter	%	No.	%	
Positive	2	1.2%	2	1.2%	4	2.4%	0.224 N.S
Negative	39	23.1%	126	74.6%	165	97.6%	
Total	41	24.3%	128	75.7%	169	100.0%	

P-value = ≤ 0.05

N.S: Non significant

PCR showed high positive in pregnant women with moderate socioeconomic compare with low one and no significant differences between PCR result and socioeconomic situation. Also socioeconomic situation had no risk to get rubella infection (table 4.13).

Table 4.13: The association between PCR result and socioeconomic situation among apparently healthy pregnant women

PCR result	Socioeconomic situation				Total		Significant differences
	Moderate	%	Low	%	No.	%	
Positive	3	1.8%	1	.6%	4	2.6%	0.129 N.S
Negative	155	91.7%	10	5.9%	165	97.6%	
Total	158	93.5%	11	6.5%	169	100.0%	

P-value = ≤ 0.05

N.S: Non significant

O.R= 0.193 (Normal range= 0.018 to 2.034)

PCR results was found the same in primagravida and multigravida (two cases in each) and no significant differences between PCR result and gravidity. Correspondingly gravidity (mainly primagravida) considered as risk for rubella infection (Table 4.14).

Table 4.14: The association between PCR result and gravidity among apparently healthy pregnant women

PCR result	Gravidity				Total		Significant differences
	Primagravida	%	Multigravida	%	No.	%	
Positive	2	1.2%	2	1.2%	4	2.4%	0.332 N.S
Negative	46	27.2%	119	70.4%	165	97.6%	
Total	48	28.4%	121	71.6%	169	100.0%	

P-value = ≤ 0.05

N.S: Non significant

O.R= 2.587 (Normal range= 0.354 to 18.92) risk for primagravida

Families had members > 5 yielded high positive result compared with families had 2-5 members and no significant association between PCR result and family members (Table 4.15).

Table 4.15: The association between PCR result and family members among apparently healthy pregnant women

PCR result	Family members				Total		Significant differences
	2-5 members	%	> 5 members	%	No.	%	
Positive	0	0%	4	2.4%	4	2.4%	0.020 N.S
Negative	96	56.8%	69	40.8%	165	97.6%	
Total	96	56.8%	73	43.2%	100	100.0%	

P-value = ≤ 0.05

N.S: Non significant

O.R= 0.080 (Normal range= 0.004 to 1.512) no risk

CHAPTER FIVE
DISCUSSION

5.1. Discussion

Rubella infection is usually mild with non-specific (subclinical) symptoms. So it is frequently undiagnosed or misdiagnosed. However, the rubella virus remains an important public health problem due to the teratogenic effects and risk of miscarriage and stillbirth that may result from congenital infection, especially when the mother becomes infected during the first trimester of pregnancy (Martínez-Quintana *et al*., 2015).

In the present study, 2(0.56%) of pregnant women were IgM positive (recent infection), 356 (89.39%) were IgG positive (past infection) for rubella antibodies using ELISA technique (figure -1 and 2).

Concerning IgG results, this study was in agreement with the results of Gaber and Osman, 2017, Aljazeera State, Sudan (90%) and in countries that neighboring Sudan like results in Southern Ethiopia (86.3%) obtained by Tamirat *et al*., (2017), in Nigeria (89.4%) by Adewumi *et al*., (2013) and Alsibiani, 2014 in KSA (91.6%). Also in other countries such as India (88.2%) achieved by Gupta *et al*., (2014), and in Philippines (84.6%) by Lopez *et al*., (2016), in Togo (85%) by Mounerou *et al*., (2015**).**

The current study results was slightly lower than results obtained by Tahita *et al*., (2013) in Burkina Faso (95.0%), Adam *et al*., 2013 in Khartoum State (95.1%), Obijimi *et al*., 2013(96.6%) in southwestern Nigeria, Olajide *et al*.,(2015) in Nigeria (93.1%), Mamvura *et al.,* (2015) in Zimbabwe (92%), and, Abdul Jalel *et al*., (2017) (97.7%), Khartoum State.

Contrariwise, our results was higher than results obtained by El Feel *et al*., (2014) in Khartoum State (68.8%), Pennap and Egwa, 2016 in Nigeria (11.4%), Khan *et al*., (2017) in Pakistan (19.6%,), Gubio *et al*., (2017) in Nigeria (63.3%), Abdallah *et al*., (2015) in Khartoum State (47.8%), Zanga *et al*., (2017) in Democratic Republic of the Congo (58.97%).

The above high and low rate of rubella infection compared with our result could be attributed to several factors including; genetic susceptibility of participants, environmental factors, type of circulating genotype, and regional differences in endemicity of rubella.

Deviations of the current study results from the other previous studies conducted in Khartoum State may be due to different temporal pattern (in temperate areas, incidence is usually highest in late winter and early spring), perhaps study areas and personnel hygiene.

Noteworthy in Nigeria there are variation, this indicates that the distribution of rubella virus across Nigeria varies for reasons that could possibly be climatic (Olajide *et al*., 2015)

For IgM results, the presented study results were lower than results obtained by Abdallah *et al*., (2015) in Khartoum State (8.9%), Gaber and Osman, (2017), Aljazeera State, Sudan (20%), Obijimi *et al*., (2013) in southwestern Nigeria (6.7%), Olajide *et al*., (2015) in Nigeria (38.8%), Tamirat *et al.*, (2017) in Southern Ethiopia (2.1%), Gubio *et al.*, (2017) in Nigeria (33.3%), and Khan *et al.*, (2017) in Pakistan (3.6 %). In contrast our results near to results of Alsibiani, (2014) in KSA (0%) that could be due to vaccination introduced in KSA in 2002.

The high prevalence of the other previous studies conducted in Sudan compared with the present study possibly is due to the period between, since rubella occurs in a seasonal pattern with epidemics every 5–9 years (Adam *et al*., 2013).

The current study results illustrated that there was no relation between trimesters and gravidity with IgM and IgG results.

Concerning the gravidity, the presented results was showed no meaningful association with IgG result, which was in harmony with the result obtained by; Adam *et al*., (2013) in Khartoum state, Gaber and Osman , (2017)**,** Aljazeera State, Sudan**,** Mounerou *et al*., (2015), in Togo, Alsibiani , (2014) in KSA , Pennap and Egwa, (2016) in Nigeria , Gupta *et al*., (2015) in India and Lopez *et al*., (2016) in Philippines, Tamirat *et al.*, (2017) in Southern Ethiopia, Olajide *et al*., (2015) in Nigeria **,** and Zanga *et al*., (2017) in Democratic Republic of the Congo. But disagreed with current IgM results**.**

No difference was found between the trimesters of pregnancy and IgG result, this was agreed with the results done by Abdallah *et al*., (2015), Gaber and Osman, (2017)**,** Lopez *et al*., (2016), Tamirat *et al.*, (2017), Tahita *et al*., (2013), Olajide *et al*., (2015), and Zanga *et al*., (2017).

But both IgG and IgM results disagreed with result obtained by Pennap and Egwa, (2016) in Nigeria, Obijimi *et al*., (2013) in Nigeria, Mounerou *et al*., (2015), in Togo and Khan *et al*., (2017) in Pakistan. For IgM result it was similar to finding addressed by Gaber and Osman, (2017), Aljazeera State, Sudan.

Regarding the distribution of rubella exposure by members of family, season and socioeconomic situation, there was no association between and IgM results. For knowledge there were no studies on IgM result with season and family members as risk factors for acquisition rubella infection.

Concerning socioeconomic situation results, there was no association between IgM result and socioeconomic situation, which is contrary to the findings of Khan *et al*., (2017) in Pakistan, Olajide *et al*., (2015) in Nigeria.

Statistical analysis showed that there was no significant association between history of miscarriage, number of miscarriage, previous skin rash and birth child with congenital abnormalities with presence of rubella antibodies (IgG).

Alongside previous skin rash, our result incompatible with the result obtained by Khan *et al*., (2017) in Pakistan but compatible with El Feel *et al*., (2014) in Khartoum State, Olajide *et al*., (2015) results in Nigeria. That we expected this study was conducted on subclinical women and the findings depends on fluke.

There was no relation between previous miscarriage and IgG result that was matched with the results done by; Alsibiani, (2014), Tamirat *et al*., (2017), Tahita *et al*., (2013), Abdallah *et al*., (2015) Olajide *et al*., (2015) and Gaber and Osman, (2017). On the other hand it was harmonized with the Abdallah *et al*., (2015) results. We anticipate could be from other TORCH agents.

Also there was no association between IgG result and number of miscarriage that was matched with the result obtained by Alsibiani, (2014) in KSA.

The current study found there was no association between IgG and birth child with congenital abnormalities which concord with the result obtained by Olajide *et al*., (2015) in Nigeria**.** That could be from other teratogenic agents.

Conversely, the current study demonstrated there was no association between IgG result and the members of family and season. For knowledge, there was no previous study had studied the association between these variable and risk of infection.

Just as reported by Adam *et al*., (2013) in Khartoum State the prevalence of rubella antibodies IgG did not associated with the residence that was conformed to these results.

PCR results showed 4 (2.4%) were positive and 165(97.6) this is contrary to the findings of Zanga *et al*., (2017) in Democratic Republic of the Congo (60%), because his study was prospective cross-sectional; and they followed-up the participants from the first to third trimester, while the presented results done from apparently healthy women. The

statistical analysis of categorical data did not show any significant association between PCR results and residence, trimester, and season.

Found that; number of family members and socioeconomic situation weren't risk factor for acquisition rubella infection and in contrast, gravidity (primagravida) considered as risk factor.

For knowledge, there were no studies similar to our study to compare.

5.2. Conclusion

Rubella virus genome was detected by molecular techniques in few apparently healthy pregnant women and high exposure rate to rubella infection in the past. There was no association between infection and season, trimester. Gravidity (primagravida) found to be a risk factor for infection; season and number of family members had no risk.

5.3. Recommendations

Screening of pregnant women periodically for rubella virus should be done by RT-PCR or antigen detection based techniques or more sensitive techniques (real time PCR or LAMP-PCR or NASBA) rather than using of ELISA which is an important tool to identify active infection and to avoid the risk of congenital rubella syndrome and management.

Pregnant women should aware with risk of rubella and it is complications, and vaccination should be introduced.

Further studies which include genetic characterization of circulating wild-type viruses to support molecular epidemiologic studies and sequencing are recommended to be done.

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Appendix (A)

Sudan University of Science and Technology

Questionnaire

Molecular Detection of Rubella virus among Apparently Healthy Pregnant Women in Khartoum State

Code: …… Age: …… Telephone No.: ……

Residence: ……..……..

Socioeconomic situation: High ☐ Moderate ☐ Low ☐

Family size: 2-5 members ☐ 6-10 members ☐ >10 members ☐

Education: Illiterate ☐ Primary ☐ Secondary ☐ University ☐

Occupation: House wife ☐ Worker ☐ Student ☐

Obstetric profile

Trimester: First ☐ Second ☐ Third ☐

Gravidity: Primigravida ☐ Multigravida ☐

Medical History:

- History of **S**kin rash: Yes ☐ No ☐
- history of preterm death: : Yes ☐ No ☐
- Arthritis: Yes ☐ No ☐
- Miscarriage**?** Yes ☐ No ☐ If yes how many time? .....
- The last abortion. .....
- **B**irth child with congenital abnormalities: Yes ☐ No ☐

If yes specify: Cataracts ☐ Cardiac abnormalities ☐ deafness ☐

Growth retardation ☐ Rash ☐ Hepatosplenomegaly ☐

Jaundice ☐ Meningoencephalitis ☐ others ☐

- **V**accinated against rubella? Yes ☐ No ☐

Investigation results:

ELISA results: IgM ☐ IgG ☐

PCR result: +ve ☐ -ve ☐

Appendix (B)

Consent form

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

وثيقة الموافقة المستنيرة للمشاركة فى بحث علمي

عنوان البحث: الكشف الجزيئي لفيروس الحصبة الألمانية لدي النساء الحوامل السليمات ظاهرياً في ولاية الخرطوم

الباحث: وفاء محمد عبدالله عبدالرحيم.

مقدمة : الحصبة الألمانية عبارة عن مرض فيروسي يصيب كل الفئات العمرية وينتقل عن طريق الرزاز الملوث. وأعراض المرض تشبه الحصبة. قد يحدث المرض بدون أعراض ظاهرة بنسبة ٥٠%.

تتمثل خطورة المرض في أثره على الجنين في بطن المرأة الحامل خاصة الثلاث أشهر الأولى ومن ضمن هذه الآثار: تشوهات خلقية كالصمم والماء الأبيض بالعين و تشوهات بالقلب وقد تؤدي لموت الجنين. كما يمكن أن يحصل سقوط للحمل أو موت الجنين بالرحم.

الهدف من الدراسة: تهدف هذه الدراسة للكشف عن وجود فيروس الحصبة الألمانية لدي النساء الحوامل السليمات ظاهريا في ولاية الخرطوم. حيث يمكن أن تكون المرأة الحامل لديها المرض بدون أعراض ظاهرة ويمكن ان تنقل المرض للآخرين.

تتطلب مشاركتك في هذه الدراسة إجراء بعض الإختبارات لعينة الدم التي لا يزيد مقدارها عن ٥ مل. و ستفحص هذه العينات في معمل الأبحاث بالمعمل القومي للصحة العامة (استاك) وجامعة السودان للعلوم والتكنولوجيا- الجناح الغربي. وستحفظ العينات بالمعمل حتى إكتمال مشروع البحث. كما ستطرح بعض الأسئلة في الإستبيان (مرفق).

المخاطر : قد يتعرض المشارك في البحث لألم بسيط نتيجة لوخز الحقن وسوف تتخذ كل تحوطات السلامة المعملية لأخذ العينة.

البدائل : البديل للمشاركة في الدراسة هو عدم المشاركة ولك كل الحرية المطلقة لإختيار المشاركة أو عدم المشاركة في هذه الدراسة.

إنهاء المشاركة : سيتم إنهاء المشاركة فى الدراسة إذا قررت الإنسحاب من الدراسة او إذا قرر الباحث بأنك غير مستوفيه لشروط المشاركة فى البحث.

المشاركة التطوعية : المشاركة في هذه الدراسة طوعية وإذا قررت عدم المشاركة فإنك لن تتعرضى لأى مضايقات.

السرية : كمشاركة فى الدراسة ستكون هويتك ومحتويات الإختبارات المعملية سرية فى جميع المنشورات المتعلقة بنتائج الدراسة ويمكن الإطلاع عليها من قبل الباحثين ولجان الكلية فى حدود النظم والقوانين المطبقة بهذا الخصوص . هذه الدراسة بغرض الحصول على درجة الدكتوررراة في الأحياء الدقيقة بكلية المختبرات الطبية. كل المعلومات المتحصلة سرية للغاية و عند الإنتهاء من الفحص المعملي سوف يتم إبلاغك عن النتائج.

الأشخاص الذين يمكن الإتصال بهم للإستفسار عن نتائج البحث : يمكن الإتصال بالباحث على رقم الموبايل٠٩٢٥٢٥٤٥٢٤

الجزء الثاني

أنا ..... أوقع علي هذه الموافقة بعد ان شرح لي الباحث انني سأشارك في بحث علمي وأجاب علي كل تساؤلاتي بخصوص هذا البحث.

وبتوقيعي هذا أقر بأننى موافقة علي اخذ العينة (الدم) لغرض البحث.

المشاركة في البحث أو من يوقع عنها :

الإسم : .....

التوقيع أو البصمة : .....

التاريخ :.....

صلة القرابة :.....

(اذا كان الموقع غير المشاركة)

الباحث:

الإسم: وفاء محمد عبدالله.

التوقيع : .....

التاريخ : .....