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Immunological and Molecular Detection of Rubella Virus in Pregnant Women in Diyala Province

A Thesis

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Dedication

To:

My Wounded Country Iraq

The Dearest My Father and My Mother

My Brothers and Sisters

My Grandfathers and Grandmothers

My Beloved Wife *and*

*My Daughters "Narjis ,Zainab, Fatima
& Hurra*

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Summary

Rubella is caused by an RNA virus in the Rubivirus genus and the *Togaviridae* family. Popular clinical symptoms involve fever, rash, cough, lymphadenopathy, and often arthritis. While most infections with rubella cause mild, self-limiting measles-like disease, the real danger occurs when the fetus is infected with the rubella virus. As the virus may attack any portion of the growing fetus, any organ may be damaged, this number of congenital malformations known as congenital rubella syndrome, may involve the brain, heart, hearing, and vision

This study was intended to determine rubella virus seroprevalence and molecular detection of virus in pregnant women. The samples included 240 women who were attended during the period from October 2018 to May 2020 to Al-Batool Teaching Hospital for maternally and children in Baqubah-Diyala province.

Women's age ranged from 16-45 years; their age mean was 26.38 ± 5.11 years. Data collection included age, education, number of previous abortions, gestational age, and residence. Sera were collected from blood of each woman, and the antirubella IgG, IgM were detected by using Electrochemiluminescence , ELISA, On Site Rubella IgG/IgM Rapid tests and RT-rPCR assay were used to detect rubella viral RNA. The results revealed that anti-rubella IgG antibodies for the 240 pregnant women were ranged from (203+ve) 84.58% to (215+ve) 89.58% depending on the method employed. While the anti-rubella IgM antibodies for the 240 pregnant women ranges from (12+ve) 5% to (13+ve) 5.41% depending on the used method. Only 8 from 240 serum specimens of pregnant women were positive for rubella viral RNA with (3.33%) by RT-rPCR test.

This study revealed that the lowest rate of IgG seopositivity was at the age group of 15-20 years, while the highest rate was at an older age

group of >32 years. Also the study revealed that the rate of IgG seropositivity was higher (92.13%) among women who live in the urban areas while represent (82.30%) among women who live in the rural areas with significant relation ($P < 0.05$). In regard to the history of abortion, the IgG seropositivity were (61.42%) of pregnant women who had history of abortion with significant relation ($P < 0.05$), however the recurrent abortion among pregnant women have the past of one or more abortions with insignificant difference ($P > 0.05$).

There was no significant association between seropositivity of rubella and level of education and trimesters of gestation. Molecular detection of viral infection were done by using revers transcription real-time PCR (RT-rPCR). Rubella positive RT-rPCR samples were selected for genotyping of PCR products depending on the E1 gene amplified by RT-nPCR. Three samples of eight was positive by RT-rPCR test was produced bands sized 719bp by using RT-nPCR by the second pair of primers depending on primers amplification flanking region of genotype 1E, and two tested samples were produced bands sized 219-271 bp by using RT-nPCR by the third pair of primers indicated 2B genotype.

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List of Abbreviations

AALA	American Association for Laboratory Accreditation
AF	Amniotic fluid
B19	Parvovirus
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDC	Centers for Disease Control
CIAs	Commercial immunoassays
COI	country of origin information
CRS	Congenital rubella syndrome
CSO	Central statistical organization

DBS	Dried blood spots
EBV	Epstein–Barr virus
ECLIA	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FUS	Fuchs' uveitis syndrome
GVAP	Global Vaccine Action Plan
HPA	Health Protection Agency
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IU	International units
nm	Nanometer
MMR	Measles, Mumps, Rubella Vaccine
MOG	Myelin oligodendrocyte glycoprotein
NCBI	National Center For Biotechnology Information
OnSite	Rapid onsite diagnosis method
ORF	Open reading frames
PCR	Polymerase chain reaction
RCV	Rubella combined vaccine
R0	Reproductive rate
PHLN	Public Health Laboratory Network
RNA	Ribonucleic acid
RT-rPCR	Reverse Transcription Real time polymerase chain reaction
RT-nPCR	Conventional Reverse Transcription Nested PCR System
RV	Rubella virus
S.D.	Standard deviation
SP-ORF	Structural protein open reading frame
TORCH	toxoplasmosis, rubella, cytomegalovirus, and herpes test
UK-NSC	UK National Screening Committee
WG	Weeks of gestation
WHO	World Health Organization

1.1 Introduction

Rubella is caused by an RNA virus in the Rubivirus genus and the *Matonaviridae* family (belonged formerly to *Togaviridae*) (Walker *et al.*, 2019).

The actual host receptor is inaccessible to humans as the only normal host (Nguyen *et al.*, 2015). The rubella virus is spread from human to human by sick people coughing or sneezing (Lambert *et al.*, 2015), or transmitted by direct contact, as with air droplets, breastfeeding, and respiratory or pharyngeal secretions (Kumakura *et al.*, 2013). Popular clinical symptoms involve fever, rash, cough, lymphadenopathy, and often arthritis (CDC, 2015), while in up to 50 percent of cases remain asymptomatic or subclinical, clinical diagnosis of rubella during pregnancy is therefore difficult to obtain (Cozza *et al.*, 2015).

Though most infections with rubella cause mild, self-limiting measles-like disease, the real danger occurs when the fetus is infected with the rubella virus (Lambert *et al.*, 2015). After reach to 20 weeks of pregnancy, problems such as miscarriage or birth defects can occur if childbearing women were infected by virus (Lulandala *et al.*, 2017). As the virus may attack any portion of the growing fetus, any organ may be damaged (Dobson, 2014), this number of congenital malformations known as congenital rubella syndrome (CRS) may involve the brain, heart, hearing, and vision. (Murhekar *et al.*, 2018). While there are some indicators of CRS during the neonatal phase, the emergence of certain disorders has been appeared after the age of 2 years (De Jong *et al.*, 2013). The most prominent abnormalities related to rubella are hearing impairment, cardiac disorders, cataracts, and retinopathy with pigmentation. Microcephaly, developmental regression, hepatosplenomegaly, purpura, jaundice,

meningoencephalitis, and radiolucent bone disorder are the lesser-known symptoms (Dobson, 2014).

Congenital rubella syndrome diagnosis is restricted to healing because the antiviral medication is not accessible (Motaze *et al.*, 2019). Although no particular treatment is available for rubella or CRS, extremely safe live-attenuated rubella vaccinations are required to avoid rubella and CRS. The vaccine against rubella is safe and efficacious (Reef and Plotkin, 2013), both natural infection and vaccine administration thus have lifetime security (Caroca *et al.*, 2017). Laboratory diagnosis of CRS includes anti rubella immunoglobulin rubella M (IgM) in cord blood or baby serum, immunoglobulin G (IgG), in addition polymerase chain reaction (PCR). The maternal rubella infection sometimes goes unnoticed, as there is always no rash (Boshoff and Tooke, 2012). Reliable laboratory confirmation of confirmed cases of rubella is a prerequisite to successful control of this disease's removal process (Mulders *et al.*, 2016).

Rubella is planned for removal in five World Health Organization (WHO) regions by 2020 under the Global Vaccine Action Plan (WHO, 2019). The usage of rubella vaccinations in regular childhood immunization increase from 99 (51%) countries in 2000 to 168 (87%) countries in July 2019, and widespread rubella transmission has been disrupted in the Americas since 2009 (WHO, 2020). For many developed countries, though, rubella virus transmission is deemed a significant public health concern (Lulandala *et al.*, 2017) because vaccination was does not take place in many developing countries (Brooks *et al.*, 2013). The vaccination was used in other countries ' national immunization systems, (Macartney *et al.*, 2017), including Iraq.

Mumps ,Measles ,rubella vaccines (MMR) was incorporated into the 1988 Iraq National Immunization Plan, and the vaccine is regularly given in two doses: twelve to fifteenth months of age and a second dose about four and six years of age for females. (WHO-MOH Iraq, 2009).

1.2 Aims of the study

This study was planned to inspect a sample infection of the awareness of Iraqi pregnant women about the risk of rubella infection during pregnancy; assessing their attention to pre-conception prevention and knowledge of their serological status, correlated with their socio-demographic characteristics, to verify the likelihood of achieving the elimination of congenital rubella syndrome. Therefore, the aim of this study was to:

- 1- Determine seroprevalence of the rubella virus; precisely the seropositivity rate of rubella immunoglobulin G (IgG) and immunoglobulin M (IgM).
- 2- Compared by three laboratory techniques for rubella IgG and IgM detection. Additionally, molecular detection of viral RNA through revers transcription real-time polymerase chain reaction (RT-rPCR).
- 3- Rubella positive RT-rPCR samples are selected for genotyping depending on the E1 gene amplified by RT-nPCR.
- 4- Studies the relationship between some of these parameters in a sample of Iraqi pregnant women with and without prior history of abortion with different socio-demographic and reproductive factors.

2.1 History of Discovering Disease

Rubella was at first considered to be alternative of the measles or scarlet fever and was termed the 3rd disease. The term Rubella have its origin from the Latin word meaning “little red” (Atkinson *et al.*, 2007). In 1814, it was first discovered to be a distinct disease from the measles in German therapeutic literature thus delivery its nickname the “German measles” (Best *et al.*, 2005). In 1914, Alfred Fabian Hess theorized that rubella was produced by a virus, established on work with monkeys (WHO, 2007).

Rubella was first defined in the mid-eighteenth century. Friedrich Hoffmann finished the first clinical description of rubella in 1740, which was established by de Bergen in 1752 and Orlow in 1758 (Wesselhoeft, 1949).

In 1912, German measles come to be a nationally notifiable illness in the United States, needing United States healthcare suppliers and laboratories to record all detected cases. In the primary decade of recording, a median of 6,000 German measles- related loss of live was informed every year. In 1914, Hess suggested a viral etiology, and in 1938, Hiro and Tosaka established his etiology by passage the disease to children with nasal washings from an infected individual with a severe case (Atkinson *et al.*, 2007).

In 1941, Norman Gregg described congenital cataracts in 78 babies whose mothers had maternal rubella in early pregnancy. These were the main cases described of congenital rubella syndrome (CRS) (Zuckerman *et al.*, 2009). In 1962 rubella was first isolated by Parkman and Weller who then went on to discovery the common characteristics of the virus (Lee and Bowden, 2000).

Rubella virus is a causative agent of rubella disease or so-called “German measles.” Though most cases of contagion lead to a slight, self-limiting measles-similar illness (Brooks *et al.*, 2013). Rubella simply crosses the placenta of infected pregnant women; the real danger arises when rubella virus infects the fetus – particularly during the first trimester when infection can lead to abortion or congenital rubella syndrome (CRS) (Dontigny *et al.*, 2008). In 1941, an Australian ophthalmologist, Norman McAlister Gregg, recognized the relationship between congenital defects and rubella throughout pregnancy, thus establishing the teratogenic potential of the rubella virus (Hesketh, 2012). It has been noted a significant increase in the number of congenital cataract cases being seen in his clinical practice, suggesting to linkage of history of maternal “German measles” in 78 of these specimen (Gregg, 1991).

2.2 Rubella Virus

Rubella first isolated from cultural cells in 1962 (Weller and Neva, 1963). In shape it is oval or circular, 40- to 80-nm in diameter, and it is enveloped icosahedral virus (Mangala *et al.*, 2013). Rubella is created by an RNA virus belonged to Rubivirus genus and *Matonaviridae* family (formerly belonged to *Togaviridae*) (Walker *et al.*, 2019). And is an enveloped virus with a positive-sense, single stranded RNA genome (Plotkin *et al.*, 2011). The 9762-nucleotides genome has two nonoverlapping open reading frames (ORFs). One is 5’ proximal, encompassing two-thirds of the genome, and codes two non-structural proteins (NSP) (P150 and P90). The other is 3’ proximal, including the remaining third of the genome, and encodes three structural proteins (two glycoproteins, E1 and E2 and the capsid protein (Abernathy *et al.*, 2009),

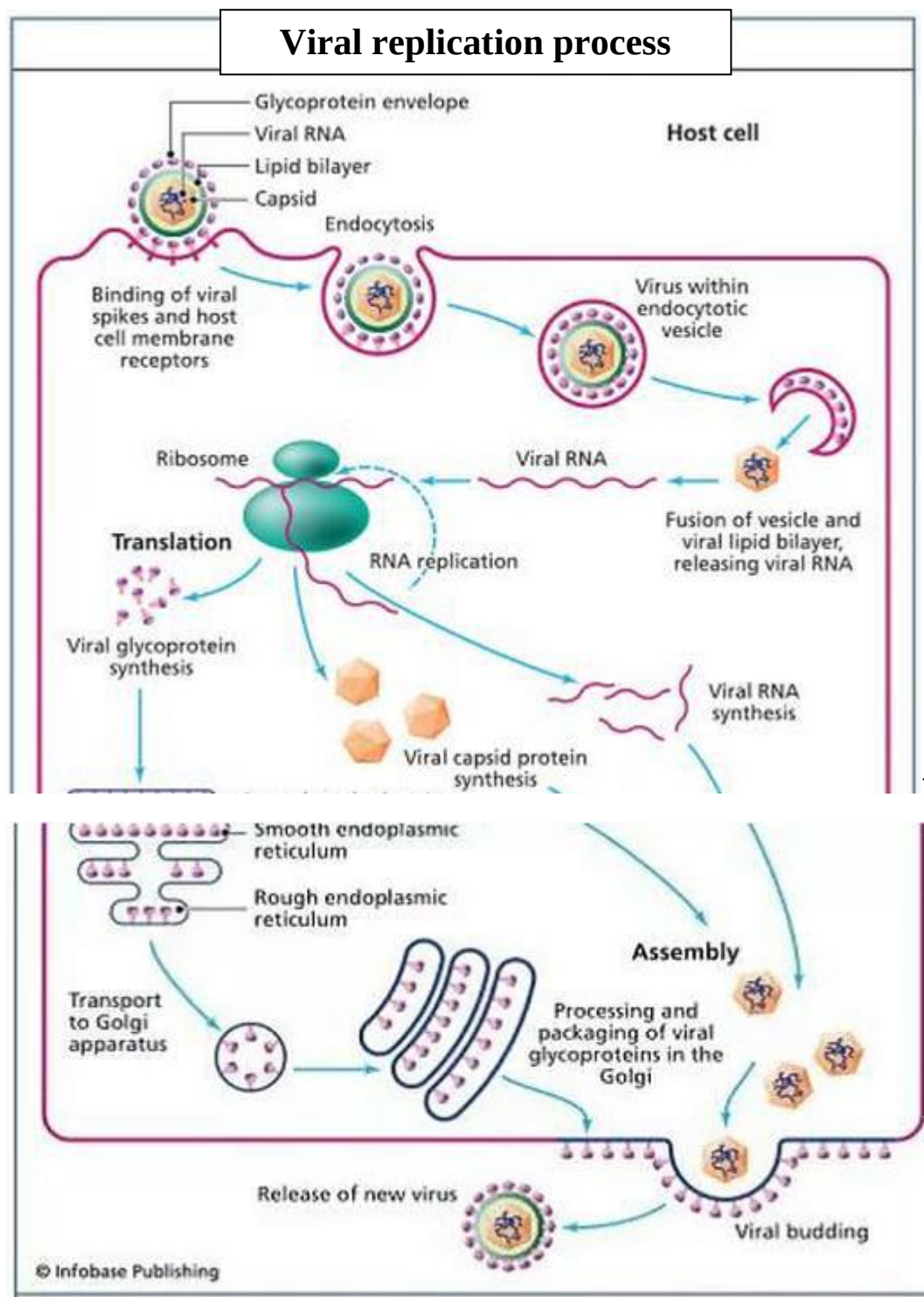


Figure 2.1 Viral replication process (Plotkin, 2006).

The E1 glycoprotein is considering immunodominant in the humoral response that inducing against the structural proteins and comprises neutralizing and hemagglutinating determinants (Chen and Icenogle, 2007 ;

Bellini and Icenogle, 2007).

Capsid protein interacted with genomic RNA and assembled into core fragments of icosahedral roots. Ultimately, the resultant nucleocapsid interacts in the cell membrane with the cytoplasmic domain of E2, culminating in the budding and development of mature virions. Phosphorylation controls RNA-binding behaviors, potentially slowing the assembly of viruses during the viral replication process as show in figure (2-2) (Prasad *et al.* , 2013). Capsid protein in the virion dimers, which is related to a disulfide molecules. Modulates genomic RNA Replication, modulates subgenomic RNA transcription through communication with C1QBP/Sfp32 humans. Induces both perinuclear clustering of mitochondria and the development of intramitochondrial plaques dense in electrons; the hallmarks of cells tainted with the rubella virus. Induces apoptosis, as expressed in cells transfected (Mukhopadhyay *et al.* , 2006).

The glycoprotein envelope E1 is a viral fusion protein of class II. Fusion activity is inactive, as long as E1 in mature virion is bound to E2. Attachment of cell- and clathrin-mediated endocytosis by viruses, Endosome acidification can induce heterodimer dissociation of E1 / E2 and concomitant trimerization of the E1 subunits. This homotrimer E1 requires fusion and promotes the release in the cytoplasm of viral nucleocapsid after endosome and viral membrane fusion (Claus *et al.*, 2006). E1 cytoplasmic tail modulates viral movement, and the residues of tyrosines are important for this function (DuBois *et al.* 2013). The 481 amino acid glycoprotein E1envelope Is a Category 1 transmembrane protein with many important functional domains, including antigens, hemagglutination reduction, and neutralization epitope (Frey, 1994).

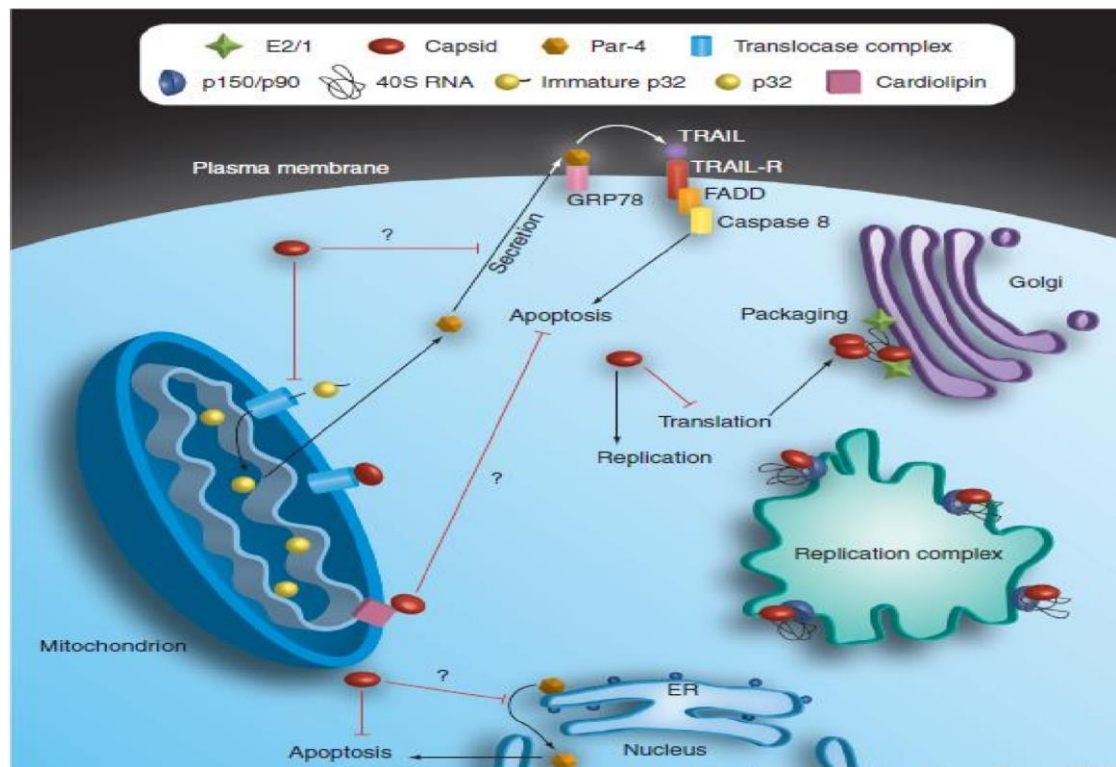


Figure 2.2 Different functions of rubella capsid protein in an infected cell (Ilkow *et al.*, 2010)

According to WHO guidelines, a 739-nucleotide window within the coding region E1 is needed for genotype identification (WHO, 2005). A total of 12 genotypes are currently accepted for RV(1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, 1J, 2A, 2B, and 2C) and 1 midgenotype (1a). Four genotypes, 1E, 1G, 1J and 2B, have been identified most frequently and two genotypes, 1E and 2B, have a common geographical distribution. "Temporary" for a range of factors, including the lack of reference viruses generally considered, and the remaining genotypes (1B, 1C, 1D, 1E, 1F, 1G, 2A, 2B, and 2C) are referred to as genotypes that have been recognized, and others are geographically confined, occurring regularly (WHO, 2013). Gen bank now contains complete genome sequences for 21 RVs (only sequences for other viruses, such as vaccine strain RA27/3, exist). Roughly half of the genomes (11/21) have come from nature and vaccine viruses since the 1960s (Reef and Plotkin, 2007). Gaining entire sequences of viral

genomes can lead to a better understanding of the virus. Each of the mentioned genotypes should contain at least 2 well-defined reference viruses that need an open reading frame (SP-ORF) of 3,192-nucleotide structural proteins was sequenced. A region of 739 nucleotides (nucleotides 8,731–9,469) within the SP-ORF was selected as the minimum sequence space needed for genotyping through comparability with reference virus sequences (Abernathy *et al.* 2011).

Proposed or approved All 13 genotypes can now be collected as guide Sequences of Viruses. Genotype 1a virus genomes, currently found in the 1960s vaccine and laboratory viruses, Cannot assemble as one group; and can be subdivided further. The bootstrap values are stable for all genotypes bar 1D, utilizing the sequences SP-ORF. For certain genotypes, the bootstrap values are less stable via the 739-nucleotide sequence range. A legitimate 739 nucleotide sequence review aims to correctly cluster reference viruses; not bootstrap guidelines (WHO, 2005). Knowledge in the field of molecular epidemiology may be used to generate fresh strain after an epidemic or to assist the entire new region (Abernathy *et al.* 2011). All identified RVs are a common serotype, but there is adequate genetic variability between wild RVs to enable virological observation and removal efforts to be acceptable (Icenogle *et al.* , 2006).

The molecular composition of the rubella virus was first identified in 1967 through the use of antigen-antibody complexes under electron microscopy. (Best *et al.* 1967; Kistler *et al.*, 1967). Additional training Characterized by electron microscopy accumulated rubella virus particles, as calculated in diameters between 50 and 85 nm (Oshiro *et al.* , 1969). Rubella virus covered by a Pleomorphic nucleocapsid connected by lipid membrane extracted from the host (Battisti *et al.*, 2012). The two protein The E1 and E2 pulses are inserted onto the virus' outer membrane sheet. E1

protein was responsible for endocytosis mediated by receptors; and it also reflects the immunodominant antigen (Petruzziello *et al.*, 1996). The sum of antibodies against E1's neutralizing domain may be useful as a defensive alliance against the rubella virus (Cordoba *et al.*, 2000; Wilson *et al.*, 2006). The glycoprotein E2 envelope is membrane-bound and is forming networks between E1 protein rows. Viral binding of E2-protein bind to the cell receptors to enter the host cell. Interaction between the proteins E2 and E1 leads to active transport to the plasma membrane as show in figure (2-3) (Elgenaid *et al.* 2018).

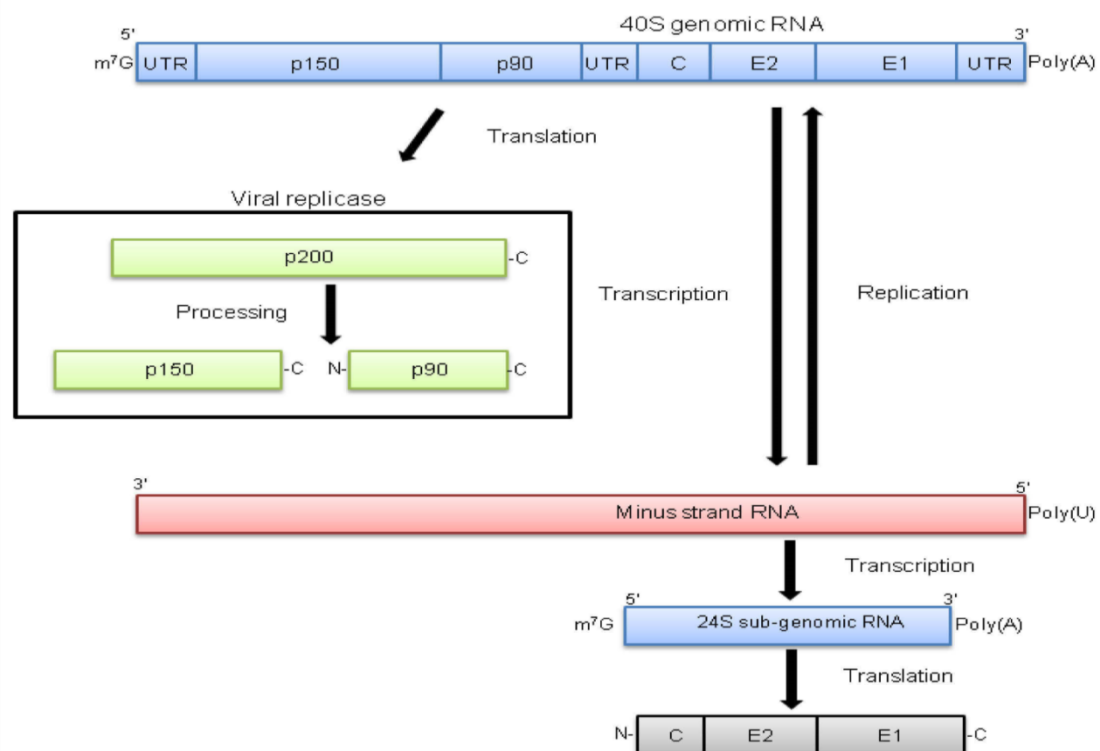


Figure 2.3: Rubella virus genomic structures (Lee and Bowden, 2000).

There have to date been no clearly identified cellular receptors for rubella virus. The binding on non-permissive cells of the rubella E1 receptor It makes *in vitro* infection of myelin glycoprotein oligodendrocyte

(MOG) and ectopic MOG production (Cong *et al.*, 2011). Nonetheless, MOG is a successful candidate for a cellular receptor in a biological sense; especially for infections of mothers that invade the fetus. There is a broad degree of homology between the proteins E2 rubella and MOG that may illuminate rubella antibodies' capacity to induce rat cell demyelination (Besson *et al.*, 2001). Tissue parts, gastrointestinal tract, human central nervous system CNS and placenta stain for MOG low to high, but all other natural tissues stain negative (Uhlen *et al.*, 2010). Rubella capacity to invade the placenta and CRS-related neurological pathologies, the theory that MOG is a potential rubella receptor, together with the presence of MOG on all types of tissue, is established. MOG involvement absence also, (i.e., vascular, lymphocyte, or skin) States that MOG is not the main collector of rubella collected. More work on finding the suspected the rubella virus-host receptor will have useful insight into virus pathogenesis, and aid direct new vaccine applicants (Cong *et al.*, 2011).

2.3 Pathogenicity

In many developing countries, infection with rubella virus is reflected a chief public health concern (Lulandala *et al.*, 2017). Clinical appearances and severity of disease differs with age. In adults and mainly in post-pubertal women, rubella infection represent an important causes of arthralgia/arthritis (White *et al.*, 2012). Adults typically have non-specific prodromal systemic indicators including arthralgia and low grade fever (Reef and Plokin, 2013). Children usually existent with a distinctive red maculopapular rash or may be without symptoms; though, fetus infection had the potential to be ultra-severe or fatal (Duszak, 2009).

Previously the vaccination date, and in countries that did not applied rubella vaccination such as many of unindustrialized countries, rubella has

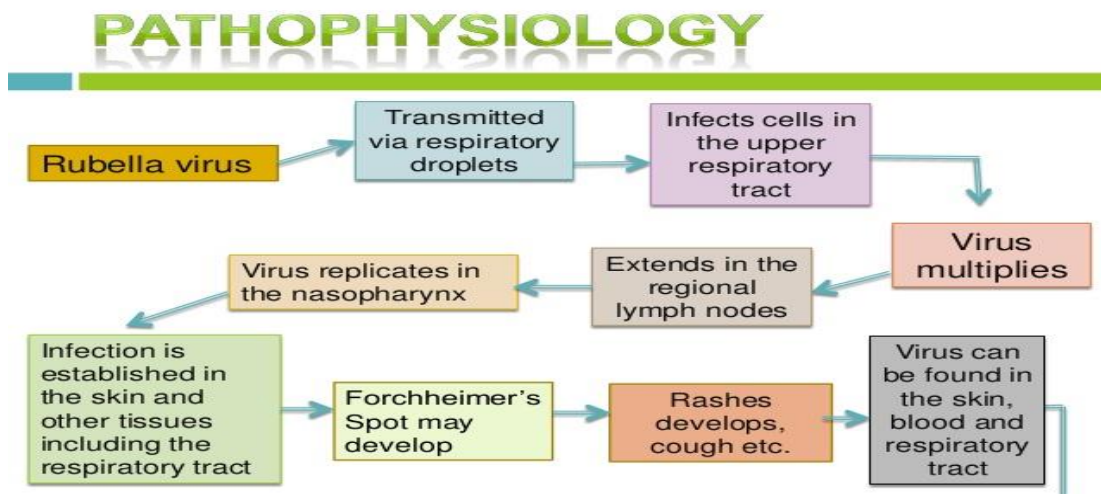


Figure 2.4 Proposed pathogenesis of postnatal rubella (Feigin and Cherry, 1992).

usually happened in the spring, with sporadic cases seeming year-round in moderate climates (Gahr *et al.*, 2014). Only known reservoir of rubella infection are the humans, and the rubella virus is transferred by direct interaction, its transmitted with air droplets, breast-feeding, and pharyngeal or respiratory secretions (Kumakura *et al.*, 2013). After inhaled the virus, it is replicates in the mucosa of respiratory tract and cervical lymph nodes, thereafter reaching the target organs by systemic circulation as show in figure (2-4) (Dimech *et al.*, 2016). Enlarged of lymph nodes are common symptoms and may past a few weeks. Joint pain in adults was common, Complications could be involve testicular swelling, bleeding problems, and nerves inflammation (Atkinson, 2011). Further symptoms may also occur such as fever, sore throat, and fatigue. The infective period ranges about 8 days before to 8 days after the onset of rash. Viremia is temporary and it can be detected throughout the week before the rash onset. Measles or influenza is more contagious than rubella; since numerous rubella cases were asymptomatic, the attack rate truly is indeterminate. Rubella had a worldwide distribution. The occurrence of rubella differs according to age and the geographic region. In developed countries, rubella epidemic had

happened every 5 to 9 years. Before the introduction of immunization programs in 1968, infection mainly affected the 5 to 9 year old groups, analogous to the primary school years (Huong *et al.*, 2013). Up to two years old of age, children with CRS are considered a source of infection and virus can be found in their throat, urine and stool and susceptible persons could be infected with rubella through interaction with these children (Bale, 2014).

2.4 Signs and Symptoms

Asymptomatic or subclinical was up to 50 percent of rubella events (Gilbert *et al.*, 2017). In clinically evident instances, a prodromal condition that involves fatigue, malaise, and adenopathy, mostly in the postauricular lymph nodes, after an incubation time of 13 to 20 days; occurs in viremia. Small pink papules describe a maculopapular rash which usually lasts 1 to 3 days. It is atypical, purpuric, or scarlatiniform on occasion. Clinical diagnosis of rubella is dramatically inaccurate as specific eruptions occurrence of many viral diseases includes parvovirus B19, herpes virus in animals (6 and 7), contact of enterovirus and toxoplasmosis, or allergic reaction (Bouthry *et al.*, 2014). Rubella had symptoms that are similar to the symptoms of influenza. But, The major sign of infection with the rubella virus is the presence of a rash (exanthem) on the face, which then spreads to the neck and limbs and generally disappears after 3 days (The three-day measles are sometimes referred to as) (Banatvala and Gray, 2004). Typically the facial rash clears off before moving through other parts of the body. Other signs include low-grade fever (CDC, 2015), enlarged glands (suboccipital lymphadenopathy and subsequent cervical); Knee pain, exhaustion, and conjunctivitis (Edlich *et al.*, 2005). The glands or lymph nodes that are swollen survive for up to a week and fever never increases beyond 38 ° C. German measles rash looks blue, or light gold.

The rash induces swelling which often occurs for almost three days. After a few days, the rash vanishes without a surface mark or peel. When the rash is clear the skin may shed into very tiny flakes where it was concealed by the rash. Forchheimer's sign exists in 20 percent of instances and is identified by tiny red papules in the soft palate region (Robert *et al.* , 2006).

Polyarthritis and polyarthralgia are the main conditions that typically arise in adult women that generally last 3 to 4 days; these effects seldom last beyond 1 month. While uncommon, other occurrences include postinfection encephalitis (About 1 in 6000 cases; more common outbreaks have higher rates). There have been sporadic cases of thrombocytopenia, hemorrhagic disorders, and Guillain Barre syndrome (Figueiredo *et al.*, 2008). Epidemics usually go unrecognized in tropical countries owing to the absence of clear symptoms of safety in sick babies (Brooks *et al.* , 2013).

2.5 Diagnosis of Rubella Infection

Rubella diagnosis is typically established by raising different types of rubella-specific antibody (rubella-specific IgG) in the blood, whether through virus production from respiratory secretions, urine whether skin or by virus identification (PHLN, 2010). A robust laboratory corroboration of questioned rubella cases is a strategy for successful surveillance in the elimination process of this disease (Mulders *et al.* , 2016).

2.5.1 Natural Immunity

Next a rubella virus infection, humoral and cell-mediated immunity were developed. Antibodies IgG and IgM are detected about 14-18 days after infection with rubella when the rash appears nearby as show in figure (2-5) (WHO, 2007). The right diagnosis of infection with rubella depends

on the knowledge of the antibody kinetics. RV-specific IgM assessment is the standard approach for validation of the rubella laboratory (Hubschen *et al.*, 2017). It was proposed that the best time to receive serum for the analysis of RV-specific IgM would be between 4 and 28 days following rash (Lanzieri *et al.*, 2014; McLean *et al.*, 2014). RV-ELISA detected IgG appears slightly longer (5–8 days before the emergence of rash) and permanent (Elise *et al.*, 2014). RV-IgG produces healthy volumes the maximum and remaining rates of RV-IgG are very inconsistent between a few days to a few weeks at any time; depending on the patient's condition and the use of the exam, this varies between commercial immunoassays. (Bouthry *et al.*, 2016).

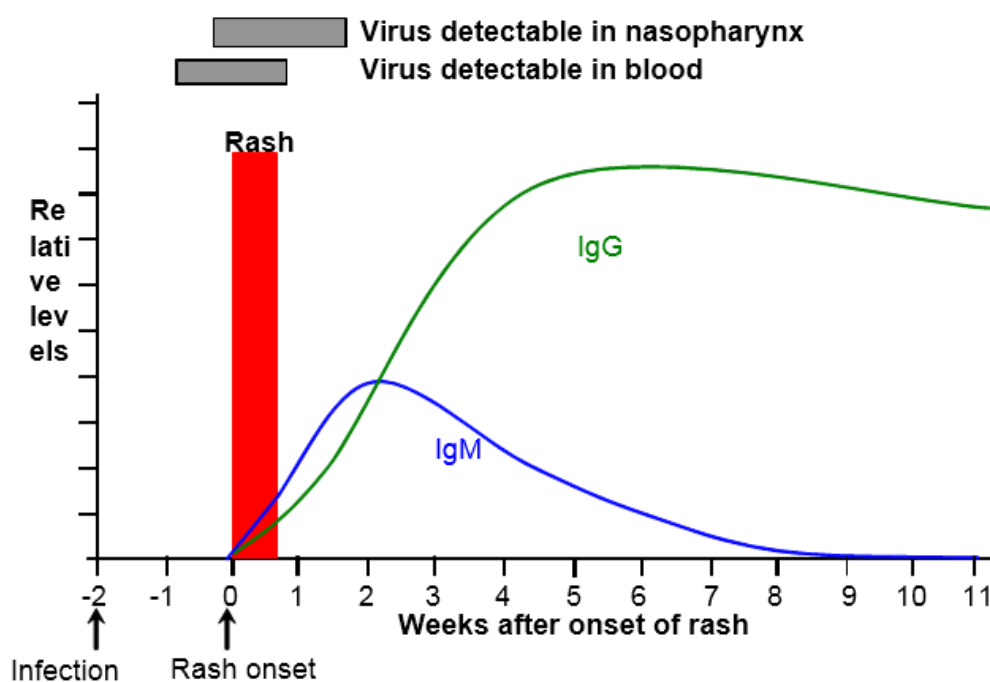


Figure 2-5 Immune response in typical rubella infection (WHO, 2007)

A strong titer of RV-IgG isn't a symptom of a new primary infection. Seroconversion or a substantial rise in rubella IgG titer in a mixture of acute and convalescent process serum checks in a related assay suggests an acute infection (Hubschen *et al.*, 2017). Keep swabs are the best sampling forms for RV genome recognition and RV isolation together; plasma/serum

samples can be sub-optimal, particularly for RV isolation (Uchinoa *et al.*, 2020). The positive culture of the rubella virus is usually not done (WHO, 2007). Reverse-transcriptase polymerase chain reaction (RT-PCR) cold can also be used to diagnose rubella viruses in the nose, teeth, scalp, urine, and cerebrospinal fluid samples (Abernathy *et al.*, 2013). False-positive or false-negative outcomes are common, depending on the nature of the assays and the worker's experience (Alphonsus *et al.* , 2015). False-negative IgM tests also happened while serum samples were collected within the first 3 days after the occurrence of rash (WHO, 2018), while the significant increase was measurable while collected 4 days after the onset of rash. In comparison, the optimum RV genome detection will be provided in RT-PCR assay in the first 7 days after rash onset (Kurata *et al.*, 2018). Cross-reaction with rheumatoid cold results in false-positive IgM (Wandinger *et al.*, 2011), including certain viruses such as Parvovirus B19, enterovirus and adenovirus (Yermalovich *et al.*, 2014), Epstein Barr virus (EBV), could cause false-positive rubella antibody tests (Lazar *et al.*, 2016). Those who have rubella, or rubella shot, in their blood, began having resistance against the infection. Such antibodies are protecting them from developing rubella because they once ever associate with it. While rubella antibodies can be estimated at age over 21, such antibodies can be extracted from the blood such individuals offer (Young *et al.*, 2015).

2.5.2 Determination of Rubella Immune Status

In some developing countries, pregnant women are usually screened to detect and immunize susceptible women for rubella antibodies (HPA, 2011).

Rubella is an exanthematous infectious virus (Cozza *et al.* , 2015), therefor Pathogenicity of the rubella virus is difficult despite the lack of

animal models to replicate the human sickness development (UK-NSC, 2010). Rubella pathways mediate teratogenesis, and the virus' associations. The host immune response isn't completely known (Bouthry *et al.* , 2014).

Because of the present epidemiological condition this is required that rubella serology be administered during there is proof of tolerance to two doses of rubella vaccination before the first prenatal visit. When an organism is initially seronegative, so at 20 weeks of development (WG) further serology is done; individuals remaining seronegative at 20WG can get the next delivery vaccine (Elise *et al.* , 2014).

Rubella immunity is systematically calculated by detecting the RV-IgG with immunoassays enzymes that have objective data per milliliter in foreign units (IU). The identification of rubella-specific IgG antibodies in many individuals, including pregnant women, is critical for evaluating seroprevalence (Shih *et al.* 2016). Due to the assay used, the RV-IgG tests and perception cold are discordant while the findings are definite In IU per milliliter, and in both immunoassays, the same universal level is compared (RUB-1-94) (UK-NSC,2012). Primary studies indicate that in cases where discrepancy results appear, women are usually immune. In these cases, the immunoblots are used as special assays. Latex agglutination experiments are no longer approved but are qualitative or semiquantitative assays (HPA, 2011).

2.5.3 Diagnosis of Maternal Rubella Infection

Thanks to its weak and unspecific clinical signs the diagnosis of rubella is challenging and ineffective (Best *et al.*, 2002). Within developing countries, clinical treatment of rubella is even less effective, because it has become rare (Cordier *et al.* , 2012). Laboratory diagnosis is important for confirming current infection with rubella and is developed by examining

seroconversion, The Kinetics of RV-IgG, RV-IgM, and RV-IgG avidity and rubella virus detection via the polymerase reverse transcription chain reaction in nasopharyngeal secretions (RT-PCR) (WHO, 2007).

In situations where the pregnant woman was in touch with potential contagious rubella, a serum sample of RV-IgG would be screened to monitor its immune status as early as possible (< 12 days). The patient might reassure when he received a good RV-IgG test. When the woman becomes breastfeeding, it is recommended That RV-IgG and RV-IgM titers are assessed 3weeks later to eliminate infectious asymptomatic central rubella doctors Be mindful the RV-IgG may become stable within a few days or weeks of diagnosis initiation, based on the individual being treated and the assays used following the initial diagnosis. (Kurtz and Anderson, 1985). Consequently a female seropositive could have been compromised a few weeks ago at the first screening examination For eg, In the case of ultrasonic More serological studies on the usable initial serum (in specific RV-IgM and RV-IgG-avidity tests) should be carried out on abnormalities (including intrauterine growth retardation), amniotic fluid (AF) rubella RT-PCR should also be treated In countries that prefer to avoid rubella, particularly if the woman was born in a country where the usual rubella vaccination system has not been implemented during childhood. (Vauloup and Grangeot, 2007).

For places where the rubella virus has completely disappeared, except clinical Rash, seroconversion and precise valuations of RV-IgM should be carried out closely and further investigations should be carried out. (RV-IgG Avidity and/or Immunoblot) can be tested in various laboratories. During the primary infection, RV-IgM is observed almost continuously (if experiments are conducted within 2 months of infection initiation) It can be tested in certain ways utilizing the most sensitive technique; (Grant *et al* . ,

1985). A positive outcome of RV-IgM is usually obtained through vaccine activation or non-specific immune system activation in countries that support the vaccine. Large levels after subsequent vaccine RV IgM may be observed for months or even years. RV-IgM that re-appear after reinfection, and there have been reports of false-positive reactivity. Positive RV-IgM is of crucial prognosis in endemic rubella countries (Thomas *et al.* , 1999).

It has been shown that Rubella virus-related immunoglobulin G avidity allows to date of the main infection of rubella (Mubareka *et al.* , 2007). Various techniques are used to calculate the quantity of the avidity index and generally the use of a denaturing agent to prevent a feeble avidity antibody from binding to the antigen (Vauloup *et al.* , 2007). In new primary infections, a weak RV IgG avidity index is recurrently calculated (< 1–3 months); a strong RV-IgG avidity index eliminates the latest primary infection (<3 months). The RV-IgG avidity can be treated with care in circumstances where the RV-IgG titer is extremely high. The next vaccine is steady growth and mostly stability of the RV-IgG avidity index at a temperate value (Vauloup and Grangeot, 2007).

2.5.3.1 Prenatal Diagnosis

When the maternal infection is detected, a prenatal examination of congenital rubella infection is advised based on the diagnosis of RV-IgM in the fetal blood or the detection of the virus genome in the fetal blood; Biopsies of ammonium fluid or chorionic villus (Best and Enders, 2006). Detection of rubella virus in biopsies of chorionic villus suggests a villi infection but not an infection of the fetus. A pregnancy evaluation with precision is around 100%, while the responsiveness is more than 90% if the following factors are found: I at least 6 weeks between the infection and the

specimen; (ii) after 21 WK the specimen is collected; and (iii) the RT-PCR Test is processed and shipped frozen (The RV-IgM fetal blood is obtained and transferred at 4 ° C). (Macé *et al.* , 2004).

2.5.3.2 Postnatal Diagnosis of Congenital Infection

Postnatal diagnosis of congenital rubella infection dependent on the presence of a particular RV-IgM by ELISA immunocapture, which is stable and successful up to 100% of infected neonates < 3 months old (Thomas *et al.* , 1993). A congenital infection could be identified in situations where The RV-IgM test is positive, either by isolating the rubella virus or by detecting RT-PCR in the nasopharyngeal swabs and urine (Bosma *et al.* , 1996). An option to distilled oil, (Manikkavasagan *et al.* , 2010), Bloodstains (DBS) or dissolved (Rota *et al.*, 2011). Producing a postnatal diagnosis of congenital infection is important to have a clear follow-up treatment plan when an infection is identified, regardless of whether a CRS clinical presence (including cognitive and visual surveillance) is detected. If a child is contaminated in utero it will excrete the virus for months or perhaps years in urine and saliva (Best and Enders, 2006).

2.6 Rubella Re- infection

Rubella reinfection is an infection of rubella in a person who previously has rubella antibodies induced by a recorded normal rubella infection or active immunization with rubella (Best *et al.* , 1989). Rubella reinfection is typically subclinical and sometimes challenging to diagnose; nevertheless, the titer of antibodies (IgG and/or IgM) may be substantially enhanced. Reinfection arising during pregnancy is unclear, so the danger of contamination to the fetus is hard to identify. Any potential experiments have shown that the chance of CRS is less than 5 percent most likely (HPA, 2011). In pregnant women, among viruses isolated from cases of rubella

reinfection, a replacement of amino acids was found in the E1 area, indicating antigenic shift is not responsible for reinfection of rubella (Bosma *et al.* ,1996; Frey *et al.* , 1998).

2.7 Congenital Rubella Syndrome

More than 100,000 children with congenital rubella syndrome (CRS) were estimated to be born worldwide (Vynnycky *et al.* , 2016). According to the WHO global estimates, there were more than 110,000 babies who born with CRS exceeded worldwide, making rubella a principal cause of congenital anomalies (CDC, 2013 b). The main risk of CRS is found in countries without rubella vaccination programs, these happening in some developing country (Grant *et al.* , 2017), for example, southeast Asian area and African region, High concentrations in southeast Asia (around 48%) (Vynnycky and Admas, 2008) and Africa (around 38%) (Goodson *et al.* , 2011), and these regions had a substantial portion of the 121 344 universal of rubella cases reported during 2009 (Chang *et al.* , 2017). It was estimated that 105,000 children with CRS each year were born globally from 1996–2010, 38 percent of them were from India (Murhekar *et al.* , 2020). CRS was estimated to occur in France in 2011 at 1.01 per 100 000 live births (Bouthry *et al.* , 2014). Rubella virus pathogenesis is responsible for recurrent non-lytic fetal infections which may impact any tissue. CRS pathogenesis is multifactorial (Lee and Bowden, 2000). For several organs, the RV antigen has been recognized by fetuses with congenital rubella infection, including the head, liver, rein, back, spleen, and ciliary body. (Nguyen *et al.* , 2015).

Non-inflammatory necrosis is found in the chorion epithelial tissue and throughout such endothelial cells. Such cells are passed to the fetus and fetal organs like the liver, neck, brain, and mouth, causing ischemic lesions

and thrombosis. In the case of rubella infection, actin assemblage is blocked actively or implicitly, contributing to cell mitosis suppression and the production of organ originator Bones. The immune system may play a part, as interferon and cytokines tend to be upregulated in human fetal cells contaminated with rubella, which may interfere with cell growth and differentiation and therefore lead to congenital defects (Adamo *et al.* , 2008).

The rubella virus persists in newborns with CRS and is noticed for many months in saliva, urine, and cerebrospinal fluid; this may be described by a cell-mediated immune deficiency. Anomalies of T cells of young adults with CRS have been identified, possibly leading to organ-specific autoimmune disease (Best and Enders, 2006).

In 2014, 141 CRS cases of 114 nations, the bulk have come from South-East Asia (n=86/141), but the writers themselves could be inaccurate in coverage (Grant *et al.*, 2015). This figure has already been calculated in developed countries to be as large as 100,000 cases per year (WHO, 2013). In CRS, the rubella virus is capable of infecting the placenta, fetal level, and modifying the role of many fetal systems by interacting with the formation of organs and inducing systemic inflammation (Lazar *et al.* , 2016). CRS includes anomalies in the auditory, cardiac, sensory, and ocular systems (Yazigi *et al.* , 2017). Could be a prenatal diagnosis of fetal infection recommended in situations where the main rubella infection develops within the first 4 months of pregnancy. While technology has been planned, it is not permanently possible to diagnose rubella prenatally (Cimino *et al.* , 2013). There is also recurrent infection consistent with CRS, and recurrent infection with rubella virus intraocular is found in patients with Fuchs' uveitis syndrome (FUS) diagnosis (Suzuki *et al.* 2010). Detection of RNA rubella virus in the eye-watering humor of a patient aged

28 and 73 diagnosed with CRS and FUS suggests the infection may continue for decades (Winchester *et al.* , 2013; Abernathy *et al.* , 2015).

Infection with rubella that occurs just before birth and during early pregnancy may contribute to fetal death, miscarriage, or congenital defects (Murhekar *et al.* 2018). The degree of exposure depends on the time of pregnancy during which infection happens. The greatest prevalence of CRS is observed in women of childbearing age in states with high rubella susceptibility (Reef *et al.*, 2013). The child mortality rate for CRS may be as high as 33 percent and lately, pulmonary arterial hypertension has been recognized as a significant cause of death in CRS cases (Toizumi *et al.* , 2014).

If childbearing women that's infected before 20 weeks of pregnancy, complications like this miscarriage or CRS with delivery defects affecting the heart, brain, hearing, and vision may happen (Lulandala *et al.* , 2017). When rubella infection develops immediately before or within the first 8-10 weeks of pregnancy, it may cause multiple fetal abnormalities in 90 % of cases, including stillbirth or fetal waste. (Miller *et al.* , 1982). The likelihood of child defects decreases during conception with infection, and fetal abnormalities are rarely linked with maternal rubella after the 16th week of pregnancy as organogenesis is total (Winchester *et al.* , 2013), Although sensorineural hearing deficiency may occur as late as week 20 of gestation with infection (Grillner *et al.*, 1983). Congenital rubella syndrome disorders more often involve the eyes (e.g., cataracts, pigment retinopathy, microphthalmia, chorioretinitis, glaucoma), vision (e.g., sensorineural deafness); (Nguyen *et al.* , 2015), Congenital hearing impairment is the most severe sequela resulting in around 60 percent of instances, especially where illness happens during the 4th month of pregnancy (Caroca *et al.*, 2017), heart abnormalities (e.g., peripheral pulmonary artery stenosis,

ventricular septal abnormalities or patent ductus arteriosus) (Deb and Ubil, 2014), and brain disorders (e.g., microcephalus) are identified. (Simons *et al.* , 2017; Chauhan *et al.* , 2016), Granuloma development (Neven *et al.*, 2017), induces disease in immunologically stable people if inflammation arises in a favorable immune role (Perelygina *et al.* , 2016). Many who survive the historical infant may experience significant physical difficulties (e.g., vision and auditory impairments) and may pose an elevated risk of developmental disability, including autism. In reality, rubella can be considered a cause for autism preventable by the vaccine (Berger *et al.*, 2011). Congenital rubella infection was often linked with an elevated incidence of long-term symptoms correlated with endocrinopathies such as thyroiditis and insulin-dependent diabetes mellitus (Ginsberg *et al.*, 1984). The child may also get born with microcephalus, hemolytic anemia, thyroiditis, Thrombocytopenia, micrognathia and congenital adrenal hyperplasia (CAH), limb abnormalities, lip/palate clefts, large bone metaphysis outbreaks, and neural tube defects. Intellectual disability and somatic developmental delays in infants born with CRS can also be obvious (Meissner *et al.* 2016).

Advanced encephalopathy has also been shown in patients with CRS, such as subacute sclerosing panencephalitis (Weil *et al.*, 1975). Rare experiments explain the pathway where congenital rubella infection during pregnancy may trigger a malformed condition in the fetus. (Garcia *et al.* , 2016). The prevention of congenital rubella syndrome is not only based on intensive child immunization, but also on detecting and vaccinating people vulnerable to rubella (Vilajeliu *et al.*, 2015).

2.7.1 Fetal Infection Incidence

The hazards associated with the duration of conception at the diagnosis

of disease depend on congenital diagnosis and complication. Miller *et al.* illustrated this before WG 11, the rate of congenital infection reached 90%, dropped to 30% between 24 and 26 WG, and increased to close to 100% after 36 WG of pregnancy. The chance of chief fetal defects in the first 12 WG exceeds 85 percent, with about 20 percent of cases contributing to the first 8 WG's spontaneous abortion. The chance decreases quickly and ranges from 11 to 18 WG, reaching 0 percent next 18 WG of pregnancy (Miller *et al.* , 1982). In situations where pregnancy occurs after the rash, the probability of prenatal infection is most likely quite low, and the rash is associated with anticorps activity and viremia ending. In fetuses whose mothers had a rash before or within 11 days of their last menstrual cycle were determined to be among the most intrauterine infection. Teratogenic infections may occur in pregnancies where rubella rash develops after the last period of 3 to 6 weeks (Enders *et al.* , 1988).

2.7.2 Fetal manifestations

During the development of the fetus, viral rubella infection contributes to common heart defects cataract triads and sensorineural deafness, as well as several other defects. Such malfunctions may be categorized as acute, permanent, or late (Cooper and Alford, 2006). Hemolytic anemia, Temporary abnormalities in infant Small birth weight triggers hepatosplenomegaly, purpura thrombocytopenia, and meningoencephalitis. Ophthalmic defects (microphthalmia, cataracts, and retinopathy) induce irreversible abnormalities; cardiac abnormalities (patent ductus arteriosus and pulmonary artery hypoplasia) (Oster *et al.* , 2010), Manifestations inside the central nervous system (psychomotor or mental retardation and developmental delays) and craniofacial

(microcephalus) malformations. Deafness is the most severe condition which may be the only one shown (Hutton, 2016), especially where Infection happens from 12-18WG. Psychiatric, endocrine, and behavioral conditions are examples of late-onset. Research in the US has reported 125 individuals with congenital rubella Ophthalmic damage as the most prevalent condition (78 percent), in 88% of instances, sensorineural deafness (66%), psychomotor retardation (62%) and heart abnormalities (58%), and other organ disorders resulted. (Givens *et al.* , 1993). CRS is linked with autoimmune disorders, and several reports have indicated an elevated incidence of diabetes and thyroid disorders (Forrest *et al.* , 2002; Takasu *et al.* , 2005).

2.8 Vaccination

The vaccine was produced in the national immunization programs of several countries (Macartney *et al.* , 2017), Then rubella vaccines were introduced in 1969, three rubella vaccines (HPV-77, Cendehill, and RA27/3) were permitted (Papania *et al.* , 2014). In many countries, vaccination strategies are aimed at eliminating rubella and CRS (Grant *et al.*, 2015). The most frequently used rubella vaccines are focused on the active, attenuated RA 27/3 strain developed in human diploid cells; China and Japan use the BRD-2 and To-336 strains respectively (Kremer *et al.* , 2006). The rubella vaccine is secure and effective (Reef and Plotkin, 2013). Rubella vaccinations initiated as monovalent formulations or that may be diagnosed with measles, vaccinations for moles, mumps, and varicella (Davidkin *et al.*, 2008). The degree of response of antibodies to a single dose exceeds 95 percent. The degree of reaction after two doses is 100 percent, and immunity is observed at age over 21 amid diminishing immunoglobulin G (RV-IgG)-specific rubella virus titer (LeBaron *et al.* , 2009). However, vaccine or natural infection can vaccinate people in the

past (Vilajeliu *et al.* , 2015). In certain nations, the rubella vaccine program is similar to the measles vaccination regimen with two doses up to 24 months (McLean *et al.* , 2013), Throughout 2012, the first dosage of MMR was adjourned to 12 months for the first dosage and 3-4 years for the second dose (Ministerio *et al.* ,2018).

The efficiency of the vaccine is almost 95%, without important side effects (WHO, 2015). Rubella vaccines are usually well accepted, and the majority of side effects are moderate. There may be rashes (5 percent), fever (15 percent), parotiditis, or intermittent lymphadenopathy. Temperate and serious (Demicheli *et al.*, 2012). In postpubertal female subjects following immunization, as well as arthralgia and arthritis, there was a significantly higher occurrence of acute joint appearances (Tingle *et al.*, 1997). Preferably, anti-rubella IgG titer requirements should be assessed before marriage or abortion, and in cases of negative titer, immunization should be performed (Ghafourian *et al.* , 2015).

Rubella immunization during breastfeeding or probably during the next month, vaccination is contraindicated as a live attenuated vaccine and as a potential teratogenic threat of the rubella vaccine; Therefore, unvaccinated pregnant women are warned to avoid contact with infected persons; they should be vaccinated directly next delivery (Ghanaat and Soleiman, 2019). Studies performed the Centers for Disease Control (CDC) did not reveal some problems linked to rubella in people immunized during pregnancy or 3 months before conception, while some babies reporting subclinical rubella-specific immunoglobulin M (RV-IgM) infections were positive. (CDC, 1989). A retrospective quantitative analysis of 94 specimens found that in the immunized population there were no more malformations or contaminated neonates than in the control community (Bar-Oz *et al.*, 2004). Numerous publications after this research have

recorded a loss of CRS after inoculation during pregnancy (Castillo *et al.* , 2011; Minussi *et al.* , 2008). Maternal antibody (IgG) is transferred to the fetus through the placenta during pregnancy, giving infants protection in the first few months of life (Van den Berg *et al.* , 2014).

The rubella vaccine is primarily intended to prevent congenital rubella infection. The period needed to extract rubella and CRS is entirely based on the software used and may vary from under 10 to 30 years (WHO, 2014). One alternative may be by vaccinating young girls or women of childbearing age to raise CRS. Another method, seen in most developing nations, vaccinates primary boys and girls in children and adults vulnerable to rubella to prevent transmission of the rubella virus. Many vaccine campaigns have been implemented in multiple states around the world. In 2010, in conjunction with measles vaccination, 67 percent of The Member States used rubella vaccinations in their regional immunization programs (Orenstein *et al.* , 2018). If childhood RCV coverage breakdown below a critical threshold, CRS occurrence can increase beyond rubella endemic CRS levels by increasing the typical age of infection without adequately decreasing rubella incidence (Winter *et al.* , 2018). The latest immunization initiative, the 2012–2020 Action Strategy for Global Measles and Rubella aims for measles and rubella in at least 5 WHO regions by the end of 2020 (WHO, 2014). However, where measles and rubella remain prevalent, problems in the provision of immunization facilities and sickness control need to be resolved by committed professional assistance from WHO and development collaborators (O'Connor *et al.* , 2017). To accomplish this aim, vaccination coverage of two dosages of rubella and measles or rubella, measles, and mumps vaccinations will perform at least 95 percent. Along with the WHO national measles and laboratory network on Rubella, epidemiological control of measles and rubella was organized.

Emphasizing the laboratory (RV-IgM) and description of the viral genotypes should provide a suitable approach to outbreaks (WHOa, 2012).

Globally, as a result of the WHO vaccination program Rubella cases have fallen from 250 102 in 2006 to 22 427 in 2015 to eliminate measles and rubella. However, CRS whiffles reported at an average of 180 cases per year during the same period (minimum 64 in 2006 and maximum 302 in 2012) (WHO, 2017). There has also been a considerable decrease in the number of reported rubella cases from 29 617 in 2012 to 2437 in 2015 in Europe (WHO, 2016). In a transition phase, these findings are to be anticipated before women of childbearing age have been vaccinated in most countries. There were 282 confirmed cases of CRS in 2015, although this classification is a difficulty and several orders of magnitude might have underestimated the actual number. (Giambi *et al.* , 2017).

Compared with measles-rubella had a lower basic reproductive rate (R_0), to indicate that eradication of rubella could be easier to reach than the eradication of measles. This principle is backed up in reality in the UK, Where measles outbreaks continue to occur among adolescents born during this period a time of diminished collective trust in MMR vaccine owing to the still unconfirmed correlation of MMR with childhood autism (Wise, 2013). Though rubella vaccine coverage is about the same as measles vaccination coverage, no significant rubella outbreaks have been recorded in the United Kingdom, indicating elimination may have been accomplished and maintained (Manikkavasagan *et al.* , 2010). According to measles, where significant outbreaks of one dose of measles vaccine have been recorded in people of broad coverage, vaccine deficiency this does not appear to play a part in ongoing rubella transmission following one single dose of rubella vaccine. Whilst usually two doses are required, a 1998 article by Andrew Jeremy Wakefield was high coverage with a wave, a

single dose seems to have been appropriate for terminating the transmission (Demicheli *et al.* , 2013; Strebel *et al.* ,2013). A leading advocate for retired British physician and scientific expert anti-vaccination, lancet, written in one of the world's largest and well-established science papers (Aylesworth, 2016). The report suggested a connection between the vaccines for measles, mumps, and rubella and bowel disorder, and autism (Godlee *et al.*, 2011). Although several research indicated a link between the MMR and the autism spectrum disorder, there was no substantial support between the research of Uno *et al.* (Uno *et al.* , 2015).

2.9 Epidemiology

Rubella is the leading infectious cause of congenital delivery defects. Observable progress has been made in introducing the rubella vaccine in developing countries, decreasing global injustice in its use and falling the numbers of reported cases of rubella and CRS, although almost 105 000 infants are born each year with avoidable CRS (Vynnycky *et al.*, 2016). Rubella vaccine use in routine childhood immunization enlarged from 99 (51%) countries in 2000 to 168 (87%) countries in July 2019, and the region of the Americas has disturbed endemic rubella transmission from 2009 (WHO, 2020). At the height of the pre-vaccine period, rubella became a deadly viral disorder concerning children and young adults worldwide. The number of cases of rubella has declined in many countries due to the introduction of the rubella vaccine programs and, since 2009, its sporadic spread has been disrupted in the WHO condition of the Americas (Andrus *et al.*, 2011).

Immunization with live attenuated rubella virus vaccine has demonstrated the potential to prevent infection, and one of the most dangerous complications as CRS. While there has been much progress,

rubella remains a significant pathogen and a global public health concern. Of starters, Japan's latest rubella epidemic, with more than 11,000 rubella cases reported during the first 6 months of 2013 and at least 13 CRS reports, illustrates the possibility that a limited immunization program results in large outbreaks. (Minakami *et al.*, 2014).

Countries that have High rubella sensitivity levels in women of childbearing age present the greatest potential danger to CRS. This occurrence varies due to epidemiological and social disparities within and within countries. Before the development Rubella vaccination prevalence ranged from 0.1–0.2 per 1,000 live births to 0.8–4.0 per 1,000 live births during contagious cycles during rubella epidemics. (Cutts *et al.*, 1997). Seventy percent of the cases of rubella in the Japanese outbreak resulted in males aged 20 to 39, indicating the weakness of an original program that provided the rubella vaccine only to young females. In 2012, Poland and Romania have conducted rubella outbreaks, which primarily impacted males as a result of a vaccine policy initially dedicated to the female vaccine (Paradowska *et al.*, 2013). Of this cause, there must be a global commitment to the management of rubella then elimination and finally eradication (Janta *et al.*, 2012).

A study of the pre-vaccine epidemiology of rubella in Africa (2002–2009) showed that the total Positive rubella IgM events were 7.3 years old (4.2–9.0 years interquartile) (Goodson *et al.*, 2011). The recorded seasonal pattern in West Africa and Southern Africa was more apparent, with a bimodal variation in Central and Eastern Africa with transmission throughout the year. In an African region, the threshold for herd immunity was estimated at between 85 percent -91 percent. Accordingly, When vaccine campaigns will reach and sustain immunization coverage beyond this point that results in increased community immunity, rubella may be

removed (Cutts *et al.*, 2000). Broad epidemics of rubella may contribute to high rates of illness, as witnessed during 1964-65 in the U.S. An estimated 12.5 million rubella cases were associated with this disorder, including >2,000 cases of encephalitis, >11,250 cases of fetal waste and >20,000 cases of CRS (There were: >8,000 cases of deafness; 3,580 cases of deaf/blind babies; and 1,800 of those 20,000 mentally impaired babies); (Plotkin, 2008).

The specific reproductive rate (R_0) for rubella was measured in European countries at about 3-8 and as large as 12 in crowded developed countries (Cutts *et al.*, 2000). The herd immunity threshold for breaking rubella transmission in the United Kingdom and the United States was estimated to be between 85% -88% and 67% -87% in European countries based on different mixing patterns based on the mean infection age at the pre-vaccine date. (Edmunds *et al.*, 2000).

Rubella virus is a candidate for global elimination because human beings are the only established host, safe and highly effective vaccines (995 percent after a single dose), reliable diagnostic and molecular assays are available, and since 2009 there has been evidence of continuing disruption of viral transmission throughout the Americas. (Andrus *et al.*, 2011).

Three of the six WHO regions set goals for rubella regulation and/or elimination (Perry *et al.*, 2015). By 2010 the Americas planned and acknowledged the eradication of rubella and CRS in 2009 (Alya *et al.*, 2013). The European area is expected to remove rubella by 2015 although Residents or foreign visitors entering the European regions from rubella endemic regions can introduce viruses (MMWR, 2013). The Western Pacific area is planning to dramatically increase the regulation of rubella and prevention of CRS By 2015 (< 1 CRS event per 100,000 live births).

The European, Eastern Mediterranean and South-East Asian areas are yet to begin to monitor or remove rubella goals (Martínez-Quintana *et al.*, 2015).

At the May 2012 World Health Assembly (WHA), the aim for the elimination of rubella in five of the six WHO regions by 2020 was certified by all 194 Member States As part of the Regional Vaccine Action Plan (GVAP) Decade for Vaccinations (WHO, 2013). Main supporters of the Measles and Rubella Program (US Red Cross, United States Centers for Disease Control and Prevention, United Nations, UNICEF, and WHO) fired the Regional Measles and Rubella Policy Plan 2012–2020 (WHO, 2012). The program designs a planet without measles, rubella, and congenital rubella syndrome with the existing syndrome as markers to this end central monitoring and national targets of removal. Plan according to plan (WHO, 2017) includes a five-pronged approach to 1) reach and preserve elevated rates of community protection through providing two-dose vaccine coverage of measles and rubella; 2) tracking illness by efficient surveillance; and forecasting programmatic activities. (Orenstein *et al.* 2018), 3) Success in planning for outbreaks and reacting promptly to outbreaks, 4) engaging and contributing in increasing public trust and desire for vaccination, and 5) having the requisite work And production of cost-effective programs and upgrade of vaccine and diagnostic services (Lambert *et al.*, 2015).

In 2011, the WHO revised its guidelines on the usage of rubella vaccines with a strong suggestion Countries who have not yet taken rubella vaccines should seize the chance to use swifter measures to track and remove RCV in their immunization programs (WHO, 2011). As both countries have goals for elimination, their strategies for vaccine delivery include A forum for the production of rubella and CRS with the usage of

combination vaccinations (e.g., measles, rubella vaccine, MR vaccine, or measles, mumps, MMR rubella vaccine). For countries that have newly introduced the rubella vaccine, the preferred solution is to continue with a wide age range MR campaign immediately followed by the introduction of either one or two doses of MR or MMR vaccine in the standard protocol depending on the country's measles vaccination program. The future promotions will have the shot MR or MMR. Countries will also strive to target children and vaccinate teenage Women and/or women of childbearing age by regular jobs or public promotions (CDC a, 2013).

A (paradoxical effect) fear that continued poor rubella vaccination coverage in babies and young children could decrease rubella sensitivity during childhood was one of the key factors restricting widespread usage of the rubella vaccine; It leads to a greater risk of women of childbearing age before the date of pre-vaccination as the infection is not protected or exposed to these individuals. It has the statistical ability to result in an elevated probability of CRS beyond the period of pre-vaccine. Country experience and methodological simulations recommend that countries reach and sustain 80% or more of the vaccine coverage with at least one dose of RCV given by standardized programs and/or regular complimentary immunization activities to avoid an increased risk of CRS (Lambert *et al.*, 2015).

Rubella vaccines are particularly cost-effective, owing to their good efficacy and comparatively low expense. For a spread of > 80% of Latin America's industrialized and less advanced countries and the Caribbean Rubella vaccine cost-benefit analyzes found that the benefits exceed the costs and are thus economically vindicated by the rubella vaccine; particularly when paired with the measles vaccine (Hinman *et al.*, 2002).

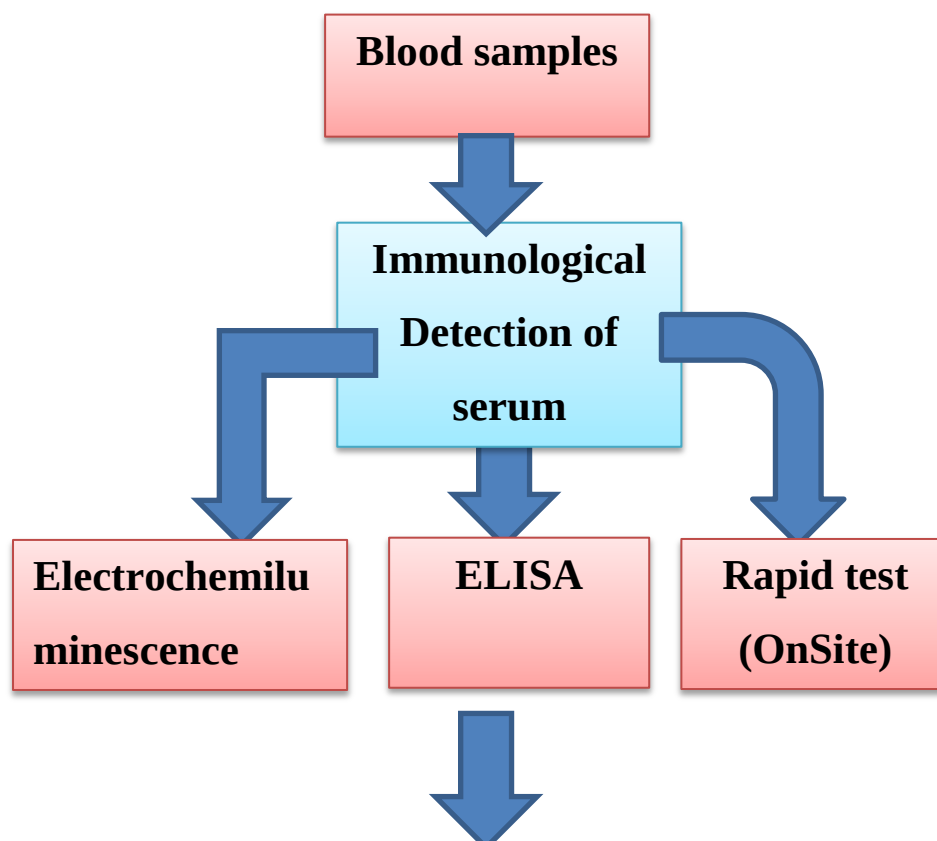
These experiments have however not been carried out in low-income African and Asian countries (Babigumira *et al.*, 2013).

3. Materials and Methods

This study was conducted during the period from October 2018 to May 2020 at Al-Batool Teaching Hospital for maternally and children in Baqubah-Diyala province and the Al-Nahrain University –Forensic DNA research and training center.

3.1 Subjects

The study consisted of 240 pregnant women who suffered from gestational Disruption or had a history of previous abortions. The age of pregnant women was 16-45 years, their mean age was 26.38 ± 5.11 years. A consent form explaining the dimensions of the study was obtained from each woman either as a written form or an oral form. Data include age, education, number of previous abortions, gestational age, via a straightforward interview, and a well-prepared questionnaire, the residence was received. Blood samples from these women were collected only if they had no records of serious or extreme diseases.



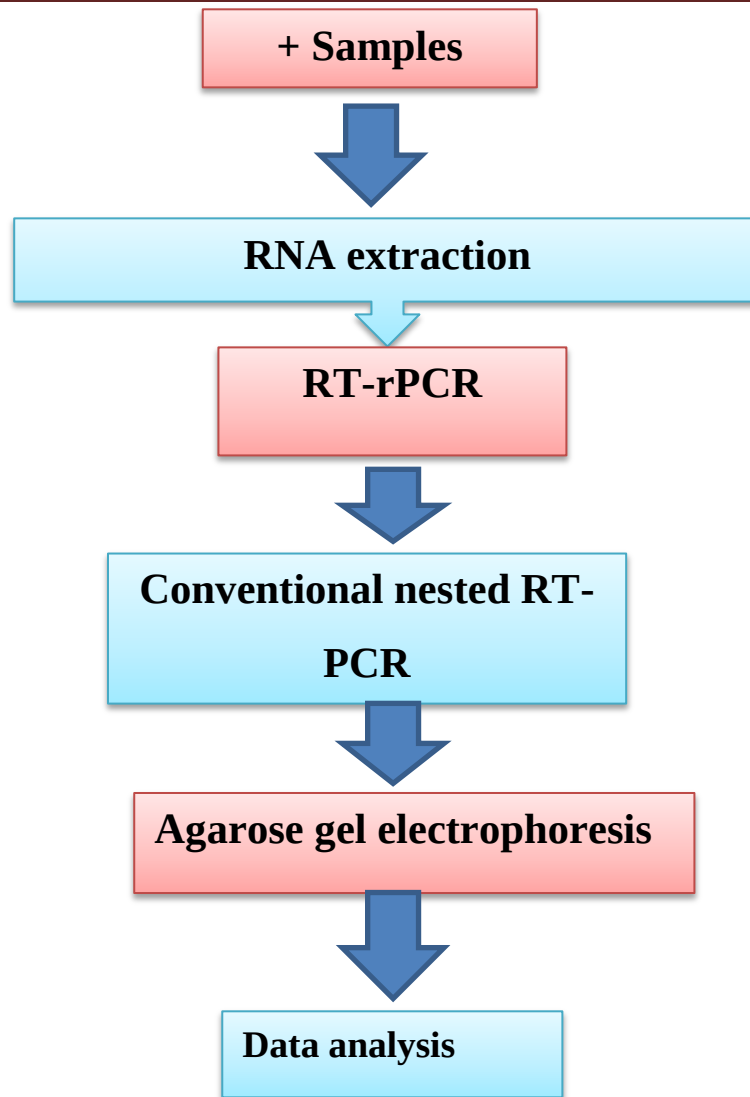


Figure 3-1: Study plan

3.2 Instruments

The instruments used in this study are listed in table 3-1.

Table 3-1: Instruments used with their manufacturing company and country of origin.

Instruments	Manufactured Company	Country
Autoclave	Hirayama	Japan

Beaker	Hirschman	Germany
Printer at Bioelisa	Le. M. R.	Germany
Reader Bioelisa	Bechman Coulter	England
Bioelisa washer	Biotek	Spain
Butte for gel preparation	Hirschman	Germany
Centrifuge	Hettich	Germany
Cooling Centrifuge	Hettich	Germany
Cylinder (pig, small)	Hirschman	Germany
Deep freeze	Biomedical	Germany
Disposable syringes	Unlock	UK
Electronic balance	vGenuis	UK
Eppendorf tube	Eapif	Germany
Eppendorf racks	Lot	China
Face mask	Lot	China
fast Real-time PCR	Applied Biosystems	USA
Gloves	Broche	Malaysia
Gradient PCR virility 96 well	Hirschman	Germany
Hot plate	Stuart	UK
Ice machine	Vision	Korea
Incubator	Memmert	Germany
Laboratory coats	Broche	Malaysia
Laminar airflow	Thermo lab	China
Micropipette disposable tips	Lot	China

Micropipette (0.5-10ul) (10-100ul) (100-1000ul)	Slammed	Germany
Multimate pipette	Slammed	Germany
Microwave	Hirschman	Germany
Nanodrop 2000	Thermo scientific	USA
PCR Microplate 96 well	Hirschman	Germany
pH meter	Bp3001 /Trans	Germany
Pipettes classic	Hirschman	Germany
Disposable pipette tips	Lot	China
The plastic tube with plain gel	Lot	China
Plate centrifuge	Hirschman	Germany
Power supply for electrophoresis + tank	CSL Midi	UK
Reader lens	S. P. I.	Japan
Refrigerator	Arcelik	Turkey
Sterile pipette tips with filters	Lot	China
Thermo mixer	Eppendorf	Germany
Water bath	Instrument grant LTD	England
Vortex mixer	Stuart	UK
Tube racks	Lot	China
UV Transit spectrum	Hirschman	Germany

3.3 Chemicals and Kits

The chemicals and kits used in this study are listed in table (3-2).

Table 3-2: Chemicals as well as kits used by company and producer country in the study.

<i>Chemicals and Kits</i>	<i>Manufactured Company</i>	<i>Country</i>
Rubella IgG Cobas E 411	Roche	Germany
Rubella IgM Cobas E 411	Roche	Germany
Rubella virus IgG ELISA Kit	Abnova	Spain
IgM ELISA virus rubella kit	Abnova	Spain
Rubella IgG / IgM Rapid on-site kit	Biotech	Germany
Real-time PCR search for qualitative identification of Infectious rubella	Cacace	Italy
RNA extraction kit	Sacace	Italy
GoTaq® 1-Step RT-qPCR System	Promega	USA
Absolute ethanol Alcohol	Scharlau	
Agarose	Oxygen	Spain
Nuclease-free water	Promega	USA
Tris base	Applichem	Germany

3.4 Blood Collection and Sera Separation

Blood collection was done using disposable syringes and needles. Each individual had obtained 5 ml of venous blood in the appropriate vein.

The collected blood was passed directly into sterile plain gel activator tube then allowed to stand at room temperature for 10 minutes for clotting activator included in the tube time to react, Then the tube was centrifuged at room temperature to separate the serum at 5000 rpm for 10 minutes; primary rubella diagnosis makes up a gel tube directly inside the COBAS INTEGRA 411 system, which provides a good location for serum. The rest of the serum was dispensed through two closely packed, sterile, and polished Eppendorf tubes (-20° C) until analysis time to estimate immunological and molecular parameters.

3.5. Electrochemiluminescence method, immunological detection of anti-rubella virus antibodies

3.5.1. Immunological detection of anti-rubella virus IgM antibodies:

3.5.1.1. Contents of Rubella IgM Cobas E 411 kit

The IgM antibodies to rubella virus in pregnant women's serum were detected by the Electrochemiluminescence immunoassay method using Rubella IgM Cobas E 411 kit (Roche/ Germany). The contents of this immunological detection kit were mentioned in Table [3-3].

Table 3-3: The contents of the Rubella IgM Cobas E 411 kit

Content	Description	Quantity
M Streptavidin-coated microparticles (transparent cap)	Streptavidin-coated microparticles 0.72 mg/ml; preservative	1x6.5 ml
R1 Anti-h IgM-Ab~biotin; Rubella-specific recombinant antigen (gray cap)	Biotinylated monoclonal anti-h IgM antibody (mouse) > 500 ng/mL, Rubella-like particles (RLP) approximately 0.1 U/mL; sodium	1x10 ml

	phosphate buffer pH 7.7; preservative	
R2 Anti-Rubella (black cap)	Anti-Rubella antibodies labeled with ruthenium complex > 400 ng/mL; sodium phosphate buffer pH 7.7; preservative	1x10 ml
Calibrator1 Negative calibrator 1 (white cap),	Human serum, non-reactive for anti-Rubella IgM; preservative	2x1 ml
Calibrator2 Positive calibrator 2 (black cap)	Anti-Rubella IgM approximately 700 U/mL (Roche units) in buffer; preservative	2x1 ml

3.5.1.2. Principle of Rubella IgM Cobas E 411 kit

Total time of assay: 18 minutes 1st incubation: 10 µl of sample was automatically pre-diluted 1:20 with diluent universal. Biotinylated monoclonal antihuman IgM specific antibodies and Rubella specific re combine antigen were add and react with anti rubella IgM antibodies that found in the specimen to produce a complex. Next then incubation: Next adding of ruthenium labeled) Rubella specific antibodies and streptavidin coated micro-particles the complex becomes bound to the solid part through interaction of biotin with streptavidin. Then reaction mixtures were aspirated into the measuring cell where the micro-particles are magnetically captured on the electrode surface. Unbound materials are then removing by ProCell/ ProCell M. Voltage application to the electrode were induces chemiluminescent emission which is measured via photomultiplier. Results were determines by a calibration of curve which is instrument exactly created by 2 point calibration and a principal curve providing by the reagent barcode. The results were determines automatically by the software via relating the signal of electrochemiluminescence that obtained from the

reaction product of the sample which compared with the signal of the cutoff value which previously obtained by calibration.

3.5.2. Immunological detection of anti-rubella virus IgG antibodies:

3.5.2.1. Contents of Rubella IgG Cobas E 411 kit

The pregnant women's serum anti-rubella virus IgG antibodies were detected by the chemiluminescence immunoassay method using Rubella IgG Cobas E 411 kit (Roche/ Germany), the contents of this immunological detection kit were mentioned in table (3-4).

Table 3-4: The contents of the Rubella IgG Cobas E 411 kit

Content	Description	Quantity
M Streptavidin-coated microparticles (transparent cap)	Streptavidin-coated microparticles 0.72 mg/ml; preservative	1x6.5 ml
R1 Anti-h IgG-Ab~biotin; Rubella-specific recombinant antigen (gray cap)	Biotinylated monoclonal anti-h IgG antibody (mouse) > 500 ng/mL, Rubella-like particles (RLP) approximately 0.1 U/mL; sodium phosphate buffer pH 7.7; preservative	1x10 ml
R2 Anti-Rubella (black cap)	Anti-Rubella antibodies labeled with ruthenium complex > 400 ng/mL; sodium phosphate buffer pH 7.7; preservative	1x10 ml
Negative calibrator 1 (white cap),	Human serum, non-reactive for anti-Rubella IgG; preservative	2x1 ml

Content	Description	Quantity
Positive calibrator 2 (black cap)	Anti-Rubella IgG approximately 700 U/mL (Roche units) in buffer; preservative	2x1 ml

3.5.2.2. Principle of the kit anti-rubella virus IgG antibodies

Total period of assay: 18 minutes 1st incubation: 10 μ L of samples were incubated with biotinylated monoclonal anti human IgG antibody, (Rubella like particles) and a ruthenylated monoclonal anti Rubella antibody particle. In addition a biotinylated Rubella virus specific recombinant antigen E1 (*E. coli*) and E1 labeled with ruthenium complex) react with anti-Rubella IgG from the specimen to form a sandwich complex, then incubation addition of streptavidin-coated micro-particles. The reaction mixture is aspirated into the measuring cell where the micro-particles are magnetically captured onto the electrode surface. Then removed of unbound substances by ProCell/ ProCell M. Voltage application to the electrode are induces chemiluminescent emission which is measured via a photomultiplier. Results are determined by a calibration curve which is instrument specifically generated by two point calibration and a master curve provided by the reagent barcode.

3.6. Immunological identification of antibodies to the rubella virus by ELISA.

3.6.1. Rubella virus IgG ELISA Kit

3.6.1.1. Principle of the Assay

Rubella antigens purified were coated on the surface of micro-wells. Diluted sample serum was added to the wells, and if the specific Rubella IgG antibodies present, binds to the antigen during the period incubation. After wash the wells to eliminate unbound sample, human IgG antibody conjugated with horseradish peroxidase (HRP) was added and incubated at 37°C for 30 minutes. Conjugate that unbounded was removed via a following washing step. Then added the solution of TMB reagent to the micro-wells. The reaction of enzyme conjugate catalytic was stopped at a specific time. The color generated intensity was proportional to the quantity of IgG specific antibody in the sample. The results are read via a micro-well reader compared in an equivalent manner with calibrators and controls.

3.6.1.2. Component of the kit

Table (3.5): Component of the Rubella virus IgG ELISA Kit

	Component	Amount
1	Microtiter Wells: purified Rubella antigen coated wells (12 x 8 wells)	96 wells
2	Enzyme Conjugate Reagent (red color): Red cap. 1 vial	12 ml
3	Sample Diluent (blue color): Natural cap. 1 bottle	22 ml
4	Negative Control: Range stated on label. Natural cap	100 µL/vial
5	Cut-off Calibrator: Yellow cap. Rubella IgM Index = 1	100 µL/vial
6	Positive Control: Range stated on label. Red cap.	100 µL/vial
7	Wash Buffer Concentrate (20 x): Natural cap. 1 bottle	50 ml
8	TMB Reagent (One-Step): 1 vial	11 ml
9	Stop Solution: 1N HCl, Natural cap. 1 vial	11 ml

3.6.1.3. Preparing the reagent

- ❖ All reagents permitted to reach room temperature (20-25°C) before use.
- ❖ One volume (1x) of Wash Buffer (20x) should be diluted with 19 volumes of distilled water. For example, dilute 50 ml of Wash Buffer (20x) into 950 ml distilled water to make 1000 ml of wash buffer (1x). Wash Buffer was stable for 1 month at 2-8°C. Before use try to mix well.

3.6.1.4. Procedure for Assay

1. The required number of coated wells was hooked on the holder.
2. The test samples, positive control, negative control, and calibrator was diluted 1:40 by adding 5 µl of the specimen to 200 µl of Sample Diluent, Mixed well.
3. Distributed one hundred microliter of diluted sera, calibrator, and controls into the proper wells. For the reagent blank, dispense 100 µl. Sample should be diluent in 1A well position. Tap the holder to eliminate air bubbles from the fluid and mix well.
4. The plate was incubated at 37°C for 30 minutes.
5. At the end of incubation period, removed liquid from all wells. Rinse and flick the micro-titer wells 5 times with diluted wash buffer (1x) and then one time with distilled water.
6. One hundred (100 µl) of enzyme conjugate were added to each well. Mix gently for 10 seconds.
7. The plate was incubated at 37°C for 30 minutes.
8. Enzyme conjugate was removed from all wells. micro-titer wells then rinsed and flicked 5 times with diluted wash buffer (1x) and then one time with distilled water.
9. One hundred (100 µl) of TMB reagent were dispensed into each well. It had mixed gently for 10 seconds.

10. The plate was incubated at 37°C for 15 minutes.
11. One hundred (100 µl) of stop Solution (1N HCl) were added to stopped the reaction.
12. Mixed gently for 30 seconds. It was important to assured that the positive control and positive sample turned from the blue color changes to yellow color completely while the negative sample were colorless. Note: Make sure that were no air bubbles in each well before reading.
13. The O.D. was read at 450 nm within 15 minutes with a micro-well reader.

3.6.2. Rubella virus IgM ELISA Kit

3.6.2.1. Principle of the Assay

Rubella antigens purified were coated on the surface of micro-wells. Diluted sample serum was added to the wells, and if the specific rubella IgM antibody present, binds to the antigen during the period incubation. After washes the wells to removable unbound sample, antibody to human IgM conjugated with horseradish peroxidase (HRP) was added and incubated at 37°C for 30 minutes. Unbound conjugate was removed via a following washing step. Then add the solution of TMB Reagent to the micro-wells. The enzyme conjugate catalytic reaction was stopped at a specific time.. The color generated intensity was proportional to the quantity of IgM specific antibody in the sample. The results were read via a micro-well reader compared in an equivalent manner with calibrators and controls.

3.6.2.2. Component of the kit

Table (3.6): Component of the Rubella virus IgM ELISA

	Component	Amount
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1	Microtiter Wells: purified Rubella antigen coated wells (12 x 8 wells)	96 wells
2	Enzyme conjugate reagent (red color): red cap. 1 vial	12 ml
3	Sample diluent (blue color): natural cap. 1 bottle	22 ml
4	Negative control: range stated on label. natural cap	100 µL/vial
5	Cut-off calibrator: yellow cap. rubella IgM index = 1	100 µL/vial
6	Positive control: range stated on label. red cap.	100 µL/vial
7	Wash buffer concentrate (20 x): natural cap. 1 bottle	50 ml
8	TMB reagent (One-Step): 1 vial	11 ml
9	Stop solution: 1N HCl, natural cap. 1 vial	11 ml

3.6.2.3. Sample Preparation

- ❖ Blood samples were collect and the sera were separated.
- ❖ Samples were refrigerated at 2-8°C for up to 7 days or freezing for up to 6 months. Repeated freezing was Avoided and melting of serum sample.

3.6.2.4. Assay Procedure

1. The number of required wells were placed and hooked on the holder.
2. The test samples, positive control, negative control, and calibrator was diluted 1:40 by adding 5 µl of the specimen to 200 µl of Sample Diluent, Mixed well.
3. Distributed one hundred microliter of diluted sera, calibrator, and controls into the proper wells. For the reagent blank, dispense 100 µl. Sample should be diluent in 1A well position. Tap the holder to eliminate air bubbles from the fluid and mix well.
4. The plate was incubated at 37°C for 30 minutes.

5. At the end of incubation period, removed liquid from all wells. Rinse and flick the micro-titer wells 5 times with diluted wash buffer (1x) and then one time with distilled water.
6. One hundred (100 μ l) of enzyme conjugate were added to each well. Mix gently for 10 seconds.
7. The plate was incubated at 37°C for 30 minutes.
8. Enzyme conjugate was removed from all wells. micro-titer wells then rinsed and flicked 5 times with diluted wash buffer (1x) and then one time with distilled water.
9. One hundred (100 μ l) of TMB reagent were dispensed into each well. It had mixed gently for 10 seconds.
10. The plate was incubated at 37°C for 15 minutes.
11. One hundred (100 μ l) of stop Solution (1N HCl) were added to stopped the reaction.
12. Mixed gently for 30 seconds. It was important to assured that the positive control and positive sample turned from the blue color changes to yellow color completely while the negative sample were colorless. Note: Make sure that were no air bubbles in each well before reading.
13. The O.D. was read at 450 nm within 15 minutes with a micro-well reader.

3.7. Immunological detection of anti-rubella virus antibodies by OnSite Rubella IgG/IgM Rapid Test

The OnSite Rubella IgM/IgG Rapid Test is a sideways flowed immunoassay for the semi-quantitative detection and differentiation of IgM and IgG antibodies to rubella virus in human plasma, serum or whole blood. It was intentional to be used by professionals as a screening test and

provides a primary test result to help in the diagnosis of infection with rubella virus.

3.7.1. Principle of the Assay

The OnSite rubella IgG/IgM rapid test was a lateral flowed chromatographic immunoassay. The assessment cassette consisting of:

- 1) A burgundy colored conjugate pad containing rubella virus antigens conjugated with colloidal gold (rubella conjugates) and a control antibody conjugated with colloidal gold.
- 2) A nitrocellulose membrane strip containing three test lines (M, G1, G2 lines) and a control line (C line). The M line is pre-coated with mouse anti-human IgM for detection of anti-rubella virus IgM. The G1 and G2 lines are pre-coated with mouse anti-human IgG for detection of different levels of anti-rubella virus IgG. The C line is pre-coated with a control line antibody.

When an adequate how much volume of test sample and specimen diluent were dispensed into the sample well, the specimen migrates by capillary action across the cassette. Anti-rubella virus IgM, if present in the specimen, was bound to the rubella conjugates. The immune-complex was then captured on the membrane by the pre-coated mouse anti-human IgM forming a burgundy colored M line, signifying an anti-rubella virus IgM positive test result.

If anti-rubella virus IgG present in the specimen it was bounds to the rubella conjugates. The immune-complex was then captured on the membrane by the pre-coated mouse anti-human IgG forming burgundy colored G1 and/or G2 test lines, signifying an anti-rubella virus IgG positive test result. An anti-rubella virus IgG titer ≥ 15 IU/mL produces a

burgundy colored G1 test line. An anti-rubella virus IgG titer ≥ 250 IU/mL produces burgundy colored G1 and G2 test lines. Absence of any test lines (M, G1 or G2) suggests a negative result.

The test contains an internal control (C line) which should exhibit a burgundy colored line of the immune-complex of the control antibodies, regardless of color development on the test lines (G1, G2 and M). If the C line does not develop, the test result is invalid, and the specimen must be retested with another device.

3.7.2 Component of the kit

Reagents and Materials Provided

1. Individually sealed foil pouches containing:
 - a. One cassette device.
 - b. One desiccant.
2. Capillary tubes (10 μ L).
3. Sample diluent (REF SB-R0243, 5 mL/bottle).
4. One package insert (instruction for use).
5. Clock or timer
2. Lancing device for whole blood test.

3.7.3 Assay Procedure

Step 1: The samples and check pieces tacked to room temperature. Mixed well before performing the test, after the specimen is thawed.

Step 2: The pocket opened at the notch and remove the device. Place the test device on a clean, flat surface.

Step 3: The device labeled with the specimen's ID number.

Step 4: The capillary tube filled with a specimen that does not reach the length of the specimen. The test volume approximately 10 μL . For improved precision, switch specimen using a pipette which can produce a volume of 10 μL .

Hold the capillary tube vertically, dispense the entire specimen into the center of the sample well making sure that there are no air bubbles.

Immediately added 2 drops (about 60-80 μL) of sample diluent to the sample well with bottle positioned vertically.

Step 5: The timer was set up.

Step 6: Results were reader at 10 minutes. Positive results may be visible in as short as 1 minute.

Negative results could be confirmed at the end of the 15 minutes only. Any results interpreted outside of the 10-15 minute window should be considered invalid and were repeated. Discard used devices after interpreting the result following local requirements governing the disposal of devices.

3.8. RNA extraction kit

3.8.1. Content of the kit

“Ribo-Sorb-50”: isolation of RNA from clinical specimens

- Lysis Solution, 22.5 ml.
- Washing Solution, 20 ml.
- Sorbent, 1.25 ml.
- RNA-buffer, 5 x 0.5ml.

3.8.2. Specimen and reagent preparation

During extraction, the following controls were used:

- Positive control rubella virus-rec (Positive control of extraction, PCE): Ninety(90 μ l) of negative control (C–) was added and 10 μ l of rubella RNA C+ Rec to the tube labeled PCE;
 - Negative control (C–): (100 μ l) of negative control (C–) was added to labeled C–
 - Internal Control STI-87-rec (IC): Ten (10 μ l) of Internal Control RNA were added during the RNA isolation procedure directly to the sample/ lysis mixture.
1. Washing solution and lysis solution were warmed up to 60–65oC until disappearance of ice crystals.
 2. Prepare required quantity of (1.5 ml) polypropylene tubes including one tube for negative control of extraction and one tube for RNA positive control of extraction.
 3. To each tube (10 μ l) of internal control and (450 μ l) lysis solution was added.
 4. One hundred (100 μ l) of samples were added to the appropriate tube.
 5. Controls were prepared as follows:
 - One hundred (100 μ l) of negative control were added to each of the two control tubes.
 - Ten (10 μ l) rubella RNA C+ Rec was added to the tube labeled Cpos.
 6. The tubes were vortexes and centrifuged for 3-5 second.
 7. Sorbent vortexes vigorously and added (25 μ l) of it to each tube.
 8. Vortex for 5 to 7 second and incubated all tubes for 10 min at room temperature. Vortexes periodically.

9. All tubes were centrifuged for 45 second at 10000g and used micropipette with a plugged aerosol barrier tip, remove carefully and reject supernatant from all tubes without disturbing the pellet and changed tips between tubes.
10. Four hundred (400 μ l) of washing solution were added to each tube. Vortexes vigorously and centrifuge for 45 second at 10000g and used micropipette with a plugged aerosol barrier tip, remove carefully and discard supernatant from each tubes without disturbing the pellet.
11. Five hundred (500 μ l) of 70% Ethanol was added to each tube. Vortex strongly and centrifuge for 45 sec at 10000g and used micropipette with a plugged aerosol barrier tip, remove carefully and discard supernatant from each tubes without disturbing the pellet.
12. Repeated step 11.
13. Four hundred (400 μ l) of acetone was added to each tube. Vortexes robustly and centrifuged for 45 second at 10000g and used micropipette with a plugged aerosol barrier tip, remove carefully and discard supernatant from each tubes without disturbing the pellet.
14. All tubes were incubated with open cap for 10 min at 60°C.
15. The pellet was air dried, after that resuspend the pellet in (50 μ l) of RNA buffer. Incubate for 5 min at 60°C and vortexes periodically.
16. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g).

The supernatant contains RNA ready for using. The amplification can be performed on the same day of extraction. If this is not possible, the RNA preparations can be stored at -80°C for up to one month.

3.9. Reverse Transcription Real-Time PCR (RT-rPCR) test for Qualitative Detection of Rubella Virus

Rubella Real-TM Qual is a Real-Time test for the qualitative detection of Rubella virus RNA in the serum. Rubella RNA is extracted from specimens, amplified using RT amplification and detected by using fluorescent reporter dye probes specific for Rubella.

3.9.1. Principle of the assay

The detection of rubella virus by the polymerase chain reaction (PCR) was based on the amplification of the pathogen genome specific region by using specific primers. In real time PCR, the amplified product is detected by using the fluorescent dyes. These dyes linked to oligonucleotide probes that bind specifically to the amplification product. The real time monitoring of the fluorescence intensities during the real time PCR permits the detection of accumulating product without reopening of the reaction tubes after the PCR run. Rubella Real TM Qual PCR kit is a qualitative technique that contains the internal control (IC), which was used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. Rubella Real-TM Qual PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which activated by heating at 95 °C for 15 minute.

3.9.2. Content of the kit

- RT-G-mix-2, 0.015ml.
- RT-PCR-mix-1-FRT Rubella, 0.6 ml.

- RT-PCR-mix-2-FEP/FRT, 0.3 ml.
- Polymerase (TaqF), 0.03 ml.
- TM Revertase (M-MLV), 0.015 ml.
- Positive Control cDNA Rubella / STI (C+), 0,1 ml.
- RNA-buffer, 0.6 ml.
- Negative control (C-)*, 0.5 ml x 2.
- Positive control Rubella -rec**, 0.1 ml x 2.
- Internal control STI-87-rec (IC)***, 0.5 ml.

* * It was used as a negative extraction control in an insulation process.

** Using as positive extraction monitor use (10 µl) with C+ rink RNA and (90 µl) of the PCE negative power level insulation pipeline.

*** Ten (10 µl) of internal control were added to each sample during the RNA purification procedure directly to the sample/lysis mixture.

3.9.3. Reagents preparation (reaction volume 25 microliters)

1. The required numbers of reaction tubes were prepared.
2. Prepared a new sterile tubing reaction mix for each application: RT-PCR-mix-1, (5 µl) RT-PCR-mix-2, and (10 µl) (0.25 µl) RT-G-mix-2, (0.50 µl) TaqF polymerase and (0.25 µl) reverse M-MLV. Exhaustive centrifuges for tornado and 5 second. This mix will be used as soon as possible.
3. Each tube was filled with a response balance of fifteen (15 µl).
4. Ten (10 µl) extracted RNA samples were added to the appropriate reaction mix tubes, and pipetting was used to mixed well. (Recentrifuge all RNA-extracted tubes at average velocity 12000-16000 g for 2 minutes, then be cautiously supernatant. Note: Do not disturb the pellet as pellet may inhibit reaction).

Table 3-7: Prepare required quantity of reaction tubes for RT-PCR

The volume of reagents is x 1Reaction (μl):		10,0	5,00	0,25	0,50	0,25
Samples N RNA *	Reactions N **	mix-1 (RT-PCR)	mix-2 (RT-PCR)	mix-2 (RT-G)	Polyme rase TaqF	Reverses to (M-MLV)
4	6	60	30	1.5	3	1.5
6	8	80	40	2	4	2
8	10	100	50	2.5	5	2.5
10	12	120	60	3	6	3
12	14	140	70	3.5	7	3.5
....58	60	600	300	15.00	30	15.00

* specimens plus 2 extraction controls (N+2).

** specimens plus extraction and amplification controls (N+2+2).

5. For each panel 2 controls were prepared:

- Ten (10 μl) of RNA-buffer were added to the tube labeled negative control;
- Ten (10 μl) of rubella cDNA Pos and IC Pos was added to the tube labeled positive control;

The results were interpreted through the presence of crossing of fluorescence curve with the threshold line.

Rubella cDNA is detected on the JOE(Yellow)/HEX/Cy3 channel, IC DNA on the FAM (Green) channel.

Rubella cDNA exists in the DNA networks JOE (yellow)/HEX / Cy3 FAM (Green) and IC.

Table (3-8): Amplification program for plate used in Real-time RT-PCR

Stage	Temperature, °C	Time	Fluorescence detection	Repeats
1. Reverstranscription	50 °C	15 min	--	1
2. Denaturation	95 °C	15 min	--	1
3. Annealing	95 °C	5 s	--	5
	60 °C	20 s	--	
	72 °C	15 s	--	
4. Extention	95 °C	5 s	--	40
	60 °C	30 s	FAM,HEX/Cy 3/ Joe	
	72 °C	15 s		

Fluorescence is detected at stage 4 (60 °C) in FAM and HEX fluorescence channels.

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

Data Analysis:

Accumulation of rubella virus cDNA amplification product is detected in the JOE/Yellow/HEX channel, internal Control amplification product is detected in the FAM/Green channel.

The results were interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The results of analysis considered reliable only if the results obtained for positive and negative controls of amplification as well as for the negative control of extraction were correct.

3.10. Conventional Reverse Transcription Nested PCR System (RT-nPCR)

GoTaq® 1-Step RT-qPCR System (a,b,c,d) was a reagent system for quantitative analysis of RNA using a one step reverse transcription quantitative PCR (RT-qPCR) protocol. The GoTaq® 1-Step RT-qPCR System combines the benefits of GoScript™ Reverse Transcriptase and GoTaq® qPCR Master Mix for efficient, sensitive and linear one step RT-qPCR quantification over a wide range of RNA template inputs.

3.10.1. Content of the kit

1. GoTaq® qPCR Master Mix, 2X.
2. GoScript™ RT (Reverse Transcriptase) Mix for 1-Step RT-qPCR, 50X or Nuclease-Free Water for Minus-RT control.
3. Optional: Supplemental MgCl₂, 25mM.
4. Optional: Supplemental CXR Reference Dye, 30Mm.
5. Nuclease Free Water.

3.10.2. Protocol

1. GoTaq ® 1-Step RT-qPCR components and RNA models and priming pairs were thawed on ice at room temperature or 37 ° C.

2. Inspect externally to ensure thawing has been achieved. Then directly thoroughly combine a single thawed part solution. Turn using a low-velocity vortex mixer Minimizing aeration. Hold the reagents on ice for thawing.
3. All reagents that include GoTaq® qPCR Master Mix were protected from light.
4. Combined the component volumes in a nonstick sterile tube, on ice (Table 3-9). After each addition, mix the combinations gently and thoroughly, by drawing up and down in the pipette tip. The vortex mixer was used low speed to minimize aeration.
5. The reaction volumes were carefully dispensed to the wells of the reaction plate on ice, using attention to avoid cross contamination.
6. The wells were covered with an optical plate seal.
7. The plates were centrifuged for up to 1 minute at room temperature to collect the reaction volumes and eliminated residual air bubbles from the well contents.
8. The reactions were kept chilled on ice or in a cold block whenever possible. Protect the reactions from light. The plate was kept on ice during transfer to the instrument.

The reaction plates were placed into the prepared instrument (prewarmed and programmed). The run was started immediately.

Table 3-9: GoTaq® 1-Step PCR Reaction Mix.

Component	Volume per 20µl Reaction	Volume per 50µl Reaction	Final Concentration in Reaction
GoTaq® qPCR Master Mix, 2X	10µl	25µl	1X
Forward Primer, 10X	2µl	5µl	50–300nM

Reverse Primer, 10X	2 μ l	5 μ l	50–300nM
GoScript™ RT Mix for 1-Step RT-qPCR, 50X or Nuclease-Free Water for Minus-RT control	0.4 μ l	1.0 μ l	1X
RNA Template (500fg–100ng) (or Nuclease-free Water for No-Template Control)	4 μ l	10 μ l	variable
Optional: Supplemental MgCl ₂ , 25mM*	__ μ l	__ μ l	$\geq$ 2mM
Optional: Supplemental CXR Reference Dye, 30 μ M**	__ μ l	__ μ l	$\geq$ 33nM**
Nuclease-Free Water	to 20 μ l	to 50 μ l	—

*Add to supplement the MgCl₂ provided in Master Mix

**Guidelines for addition of CXR Reference Dye (30 μ M) to the reaction mix to achieve a final concentration of 0.5 micromol:

For 20 μ l reactions batch 31 μ l per 100-reaction or 78 μ l per 100-reaction batch for 50 μ l reactions.

First cycle nested RT-PCR was performed as mention above and the run program detailed Table 3.10, the second cycle followed the same program with eliminated the reverse transcription step, and the DNA template was 2microliter from the PCR product of first cycle.

Table 3.10: program the thermal cycling conditions.

Initial incubation step	Inactivation	Denature	Anneal	Extension	Final extension	Final hold
Reverse transcription	Hold	Cycle (40)			Hold	Hold
55 C° 30 min	95 C° 10 min	94 C° 10 sec	55 C° 15 sec	72 C° 1 minute	72° 5 min	4C ∞

Table 3.11: Primer sequences for three nested reverse transcription-polymerase chain reaction genotyping assays

Nested assay set number	Primer name	Primer sequence (5'–3')	PCR product size	Nucleotides targeted
1	RV8633F	AGCGACGCGGCCTGCTGGGG	945	8,731–9,469
	RV9577R	CGCCCAGGTCTGCCGGGTCTC	873	
	RV8669F	GTGATGAGCGTGTTCCGCCCTT		
	RV9541R	GTGTGTGCCATACACCACGCC		
2	RV8812F	CAACACGCCGCACGGACAAC	766	8,869–9,469
	RV9577R	CGCCCAGGTCTGCCGGGTCTC	719	
	RV8823F	ACGGACAACCTCGAGGTCC		
	RV9541R	GTGTGTGCCATACACCACGCC		
3	RV8669F-2B	GTGATGAGCGTGTTCCGCCCT	328	8,731–8,869
	RV8996R	CCACGAGCCGCGAACAGTCG	271	
	RV8691F-2B	CTAGCTACGTCCAGCACCC		
	RV8961R	CAAACCGGGGAGGCCCA		

Three pairs of published primers designed in the highly concerned regions 1E of rubella virus for the detection and amplification of the virus by (Lazar M, Abernathy E, Chen M, Icenogle J, Janta D, Stanescu A, Pistol A, Santibanez S, Mankertz A, Hübschen JM, Mihaescu G, Necula G, Lupulescu E.).

Primers were obtained commercially from Advanced Scientific Bureau (ASCO IQ) |Head of Commercial Support Department Baghdad, Al Harthiya, Dist. 213, ST. 44, Building 44.

All primers were supplied in lyophilized forms dissolved in nuclease free water to give final concentration of 100 picomol/ μ l as recommended by provider and store in deep freezer as stock solution until use.

Work solution was prepared by adding 90 ml of nuclease free water to 10 ml of primer.

3.10.3. Experimental optimization:

For each stage program temperature, duration and number of repeats for optimal results. As a guide, Table (3-10) outlines general incubation parameters that are appropriate for most assays. The program modified as necessary for optimal results, based upon assay time requirements, the required level of sensitivity, and the specificity of the target/primer sequences. Experimental optimization might include variation of the following:

- A. Reverse transcription temperature ($>37^{\circ}\text{C}$).
- B. Reverse transcription time (greater or less than 15 minutes).
- C. Reverse transcriptase inactivation/hot-start activation time and temperature.
- D. Denaturation, annealing and extension times and temperatures of each qPCR cycle.
- E. Number of steps in each qPCR cycle (e.g., two-step or other)

Increased or reduced number of cycles.

3.11. Agarose gel electrophoresis

The following solutions were prepared as described by Sambrook and Rusell (2001).

A- Preparation of 1X TBE buffer:

The 1X TBE buffer was prepared from 10X TBE buffer (as a stock solution) by adding (100 ml) from a stock solution to (900 ml) D.W (1:9 dilution of TBE: D.W).

B- preparation agarose gel:

The steps of agarose gel preparation are as follows:

1. The amount of 1X TBE (80 ml) were taken in a beaker.
2. Agarose powder (0.8 gm^s) were added to the buffer
3. The solution was heated to the boiling point used micro wave.
4. Ethidium promide (0.3 ul) of (0.5 mg/ml) was added to the Agarose solution.
5. The solution was left to cool down at 50-60 C.
6. The casting of the horizontal agarose gel, The Agarose solution was poured into the gel tray. When agarose solidifies after 30 minutes at room temperature, carefully remove the comb. After that, the gel tray was placed in the gel tank. The tank was filled with 1X TBE buffer until it reached 1-2mm over the surface of the gel.
7. DNA sample was mixed with Bromophenol blue 3:1 (loading dye) then was loaded into the wells in Agarose gel.

3.12 Statistical Analysis

Data Analysis was computer aided. The statistical analysis was done using SPSS (Statistical Package of Social Science) version 20 computer software. Frequency distribution and percentage for selected variable were done. The independent t-test was used and P-Value (equal or less than 0.05, 0.001) was considered as the level of significance (Nici, 2004).

Note: The comparison of significant when the probability (P-value) in any test were:

* = Significant difference ($P < 0.05$). ** = Highly Significant difference ($P < 0.001$).
NS= Non significant difference ($P > 0.05$).

Results and Discussion

In this study two hundred and forty pregnant women were recruited. Their mean age was 26.36 ± 5.11 years (range 16-45 years). Almost more than half of the women were 26 year old or younger.

4.1. Rubella virus seroprevalence rate

Using three methods Electrochemiluminescence (ECLIA), the enzyme-linked immunosorbent assay (ELISA), and the on-site rapid diagnostic test (OnSite) to diagnose Rubella-specific IgG and IgM antibodies in the sera of pregnant women.

The results in Table 4.1 showed that 210 (87.5%), 203 (84.5%), 215 (89.58%) of specimens was IgG antibodies positive for rubella by ECLIA, ELISA, and OnSite methods respectively and 30 (12.5%), 37 (15.4%), 25 (10.41%) were negative for rubella IgG antibodies respectively.

Table 4.1: ECLIA, ELISA, OnSite results of anti-rubella IgG antibodies in pregnant women.

Method	IgG Positive no.	Percentage %	IgG Negative no.	Percentage %	Total	p-value
ECLIA	210	87.50 %	30	12.50 %	240	0.25 NS F=1.358
ELISA	203	84.58 %	37	15.41 %	240	
OnSite	215	89.58 %	25	10.41 %	240	

Results in Table 4.2 showed that 12 (5%), 12 (5%), 13 (5.41%) of sera were positive for rubella IgM antibodies by ECLIA, ELISA, OnSite method respectively, and 228 (95%), 228 (95%), 227 (94.58%) were negative for rubella IgM antibodies by methods respectively.

Table 4.2: ECLIA, ELISA, OnSite results of anti-rubella IgM in pregnant women.

Method	IgM Positive	Percen. %	IgM Negative	Percen. %	Total	P-value
ECLIA	12	5 %	228	95 %	240	0.89 NS F=0.115
ELISA	12	5 %	228	95 %	240	
OnSite	13	5.41%	227	94.58 %	240	

Results in Table 4.3 showed that only 7 (2.91%), 6 (2.50%), 8 (3.33%) of sera were IgG and IgM antibodies positive for rubella by ECLIA, ELISA and OnSite methods respectively and 25 (10.41%), 31 (12.91 %), 20 (8.33%) were negative for rubella IgG and IgM antibodies by methods respectively.

Table 4.3: ECLIA, ELISA, OnSite results of anti-rubella IgG and IgM in pregnant women.

Method	IgG&IgM Positive	Percentage %	IgG&IgM Negative	Percentage %	P-value
ECLIA	7/240	2.91 %	25/240	10.41%	0.49 NS F=0.706
ELISA	6/240	2.50 %	31/240	12.91%	
OnSite	8/240	3.33 %	20/240	8.33%	

Anti-rubella IgG complete seroprevalence in pregnant women that were obtained in the present study ranged from 84.58% to 89.58% depending on the laboratory used. This result is almost similar to a previous study conducted in the target population, Diyala province, Iraq by Hasan *et al.* (2011) which obtained higher rate (91%) of rubella IgG. While the synchronized study conducted in Zakho city, north of Iraq has obtained a slightly lower rate (83%) (Naqid *et al.*, 2020). Another study also synchronized with this study conducted in Baghdad has obtained a higher

rate (95%) for IgG and (9.8%) percentage for IgM antibodies (Mohammed and Kokaz, 2019).

Several studies conducted in Iraq were obtained a variant percentage of IgG and IgM rubella antibodies. Mentioned some for example, not a full survey : (81%) for IgG and (0.0%) for IgM, (85.90%) for IgG and (4.55%) for IgM, (28%) for IgG and (8.7%) for IgM, (42%) for IgG, (80.7%) for IgG and (21.1%) for IgM antibodies, these studies were conducted by Abid and Fayad (2017), Saadoon (2016), Salman (2016), Khadair *et al.* (2019) and Mahmood *et al.* (2015) respectively.

Studies in the neighboring countries to Iraq had reported variable results. The seropositivity of rubella IgG and IgM antibodies was observed in women with pregnancy (75.3%) and (1.2%) respectively in Iran (Rasti *et al.*, 2016), however, the seropositivity rate of rubella virus in pregnant women in Turkey (the northern neighbor of Iraq) were recorded (86.5%), (0.5%) for IgG and IgM respectively (Parlak *et al.*, 2015). Another study displayed in Turkey, showed that the seroprevalence of rubella is 88% (Gundem and Kalem, 2017). In Jeddah (Western region of Saudi Arabia) where screening for rubella serostatus was accomplished among pregnant women attending the prenatal clinic, most of the women 9410 out of 10,276 (91.6%) were immune or IgG positive, whereas the remaining 866 (8.4%) were susceptible or IgG negative (Alsibiani, 2014).

Similarity worldwide studies had reported variable results. In Sweden, rubella seropositivity was (89%) of all female and (93.4%) of the male donors tested (Seruyange *et al.*, 2018). In Canada (87.6%) of pregnant women are rubella seropositive (Gilbert *et al.*, 2017). In Italy the number of women protected to rubella was (82.2%) (Lo *et al.*, 2014). Protection rates of anti-rubella IgG in citizens in sub-Saharan Africa was (64.1%) (Caroça *et al.*, 2017). The rubella seroprevalence among pregnant women was

(93.1%) while non-pregnant women (90%) in Nigeria (Olajide *et al.*, 2015). In Mexico the rubella seropositivity was turned to be (97.1%) (Alvarado-Esquivel *et al.*, 2016).

Seroprevalence studies offer information on the susceptibility to infection of certain populations, including women of childbearing age (Seruyange *et al.*, 2018). Nonetheless, all of these studies refer to the same message: that some women of childbearing age may be susceptible to rubella infection that may lead to CRS which is a serious concern for rubella infection during pregnancy since immunity to rubella is of particular importance in young adults women. Consequently, it advises (1) taking any opportunity to evaluate rubella serostatus in women of childbearing age (e.g., consultation with preconceptions), (2) all pregnant women should be tested for their rubella serostatus, and (3) susceptible adult women should be vaccinated either preconception or postpartum before discharging from the hospital (Dontigny *et al.*, 2008).

In this study only 12 (5%) of pregnant women were positive for IgM antibodies. This is due to that rubella IgM typically declines the next 2-3 months post-infection (Vauloup and Grangeot, 2007), this result affirms that women positive for IgM had an active infection by the virus. The women serum tested positive for anti-rubella IgM and IgG negative reflects the occurrence of the new seroconversion (a new infection with rubella virus) or reinfection (Best *et al.*, 2005). While those who exhibited IgG positive sera with negative IgM reflects those immunized or previously infected exposed, which constituted the majority of the studied women.

These differences can be attributed to some chief factors. The first one is the implementation of the rubella immunization program within the national schedule. Almost all countries that do not use the rubella vaccine, like most African countries, had a relatively low rate of IgG positivity,

while countries using rubella vaccination like Iraq usually have a high rate of IgG positivity. The second factor is regarding study methodology and including of different sample size, population mass, differences in the studied population, chances of virus intake, herd immunity at the time of virus intake and population ethnicity (WHO, 2011). These variances may be attributed to the differences in the reference range of the used tests and the laboratory techniques in employment in the studies, as used different laboratory methods and the variance in the cutoff point of the assay, and the changes in the sensitivity and specificity of laboratory procedure (Singh *et al.* , 2016). Besides that attributed to the differences in the endemicity of the rubella virus. These differences may further be ascribed to the variations most importantly the immunization status of the participants.

Furthermore, These variations can be illuminated by outbreaks of rubella infections in certain zones than others that offer immunity among the infected people, particularly in the urban areas; where the primary schools are crowded by youngsters that enable the rapid and mass spread of the disease between the population (Huong *et al.* , 2013), the interrupted vaccination, reduced vaccination coverage such in some rural areas, or poor adherence of the individuals with the immunization program, and sometimes by the absence of the vaccine because of the circumstance of Iraq. Notice that this study does not discern the origins of anti-rubella IgG in participants. Whether from natural infection or previous childhood vaccination, as in targeted region, premarital or prenatal vaccinations are not regularly done. Furthermore, even the MMR immunization the system was often disrupted during infancy, at least over the last 16 years, owing to prohibition and unsuitable security circumstances. Unfortunately, this study ignored the vaccination status of the participants in this study as there were no personal family records of vaccination.

These differences in results among laboratory techniques that are used in this study (ECLIA, ELISA, The rapid onsite) diagnosis method can be attributed to many chief factors such as its variance in the gray zone, cutoff, and the antigens that using in complete virus assays (or recombinant antigens). In Iraq, many test procedures are used to identify persons whether or not they are contaminated or immunized with the rubella virus. The rapid onsite diagnosis method is used in private laboratories on a large scale and the Electrochemiluminescence method is used in the laboratories of government health centers and the ELISA method is used on a narrow scale.

Some laboratories select to report a “gray zone” to designate insecurity in the degree of defense conferred by low anti-rubella virus IgG levels (Dimech *et al.*, 2013), In Iraq, the gray zone scale used most frequently is 10 to 15 IU / ml. For clinical trials, Samples within the grey region should be retested. Nonetheless, considering that certain commercial IgG immunoassays anti-rubella virus used 10 IU/ml as safety level, the thresholds used by manufacturers to have the greatest practicable distinction between negative and positive outcomes vary (Enders *et al.*, 2013).

The development of rapid and perfect laboratory competencies for the identification of pathogens is essential in the application of effective prevention and control strategies against infectious diseases (Coker *et al.* , 2011). Although much progress has been made in diagnostic testing such as that for the care of individual patients or the quick detection of pathogens during disease outbreaks, several of these testing modalities need a skillful practices and complex equipment, which has restricted their use for onsite testing (Caliendo *et al.* , 2013).

To encourage the usage and employment of onsite diagnostic testing, particularly for pathogen identification in the field during contagious disease outbreaks, the tests must be simple to use, rapid (i.e., short time to complete), cost-effective (e.g., requires less reagent and sample volumes), field-portable (e.g., does not require complex instrumentation or power sources), and have the ability to detect many pathogens simultaneously (Drain *et al.* 2014).

More importantly, the advance of laboratory competencies that support the surveillance of infectious diseases in distant locations and firm to reach populations, where specimens collection and testing can be achieved away from central testing services, is crucial to permit medical and other public health measures to be readily implemented (Singh *et al.* , 2016).

Immunity to RV is usually normally evaluated by detecting IgG-specific rubella virus, typically through commercial immunoassays (CIAs) that utilize enzymatic detection or chemiluminescent processes. These CIAs necessities were often calibrated to the universal level of the WHO and registered data per milliliter in universal units. In general, the cut-off levels for immunity are usually set at 10 IU / ml (less frequently 15 or 20 IU / ml) (Dimech *et al.* 2008). Consequently, the usage of global methods means that serological assays are strongly consistent, so the findings obtained from various assays are entirely equivalent. However, the antigens used in the tests (total virus or recombinant antigens), conjugate, and assay configuration (indirect, competition, sandwich, or capture) are diverse from assay to assay. In these circumstances, experimental tests of RV IgG and their definitions for the same serum, based on the CIA used, can be varied and even discordant. Many studies had reported differences between different assays (Enders *et al.*, 2013) which could do have a diagnostic

effect. Nonetheless, this disparity may contribute to (i) addle clinical management of pregnant women, (ii) excessive revaccination of persons previously immune, and (iii) reports of bogus seroconversion among those with small RV IgG titers (Bouthry *et al.*, 2016).

The latter could illustrate why Texas report found a high risk of infection with rubella during pregnancy (6.8%) (Hutton *et al.*, 2014). Variances in assay cutoffs and other variables (e.g. antigen used) may also contribute to discrepancies in the comparability of determinations of seroprevalence, which are used gradually to track success in rubella management activities (Bouthry *et al.*, 2016).

Several reports had shown that the quantitative results of the IgG anti-rubella virus recorded across separate assays are not reliably related (Guillet *et al.*, 2012). Consequently, specific clinical definitions may be provided to persons submitting to laboratories utilizing different assays, especially if their IgG anti-rubella virus rates are low (Dimech *et al.*, 2013).

Many novel commercial IgG assays against rubella viruses had recently become available. Assays need testing before regular use is implemented (Clsi, 2008). A common method of validation used by laboratories is the comparison of the results that obtained from the assay with those obtained from the assay routinely used by the laboratory (Diepersloot *et al.*, 2001). However, when there is a difference in the quantitative or qualitative data obtained from the two assays, it is difficult to explain the right outcome of the test (Guillet *et al.*, 2012). Studies demonstrate that the specificities and sensitivities of many commercial immunoassays are comparable (Greenwood *et al.*, 2010; Medici *et al.*, 2008).

However, since most commercial anti-rubella IgG immunoassays use 10 IU / ml as the threshold titer for immunity. Numerous assays report

various methodological results with the same sample. Correspondingly the findings produced from a sample of an individual being evaluated in one assay cannot be compared with results obtained in other assays. Accordingly, when results are obtained from acute and convalescent samples and each sample is tested in different assays, the results may look like a seroconversion to anti-rubella virus IgG, with the early sample testing negative in one assay and later samples testing positive in another. Potentially, these results may be interpreted as evidence of a recent rubella virus infection. This condition may lead to concern for the patient or even a recommendation for termination of pregnancy. Hence, consecutive samples from the same individual should be tested together with the same assay (Guillet *et al.*, 2012).

As new (CIAs) are presented to the market, researchers compared the latest assay findings to those collected from their daily assay testing problems in clarifying discordant check outcomes. It is not advised to check samples of discordant test outcomes on a third examination (Miller, 1998). This is recommended to equate qualitative findings with those reported from a reference norm gold check (Clsi, 2008).

Of the 12 samples assigned a positive status when testing by rapid onsite diagnosis method but having negative clinical anti-rubella IgG immunoassays are the product of ELISA testing, 7 of these samples had positive results in both ECLIA and rapid on-site diagnostic checking however, the ELISA check was null. The adverse outcome may have been attributed to a lack of sensitivity of the assays. Generally, commercial anti rubella virus IgG immunoassays have sensitivity greater than 98% and specificity of greater than 85% (Medici *et al.*, 2008).

The use of a strict cutoff of 10 IU/ml invariably results in the reporting of false positive and negative test results. Many Iraqi laboratories

testing for anti-rubella virus IgG select to report results between 10 and 15 IU/ml as positive nonetheless adding a clinical clarification stating that the clinical significance of the result is doubtful. The sources for this doubt are the imprecision of the assay and biological false reactivity of some samples in the assay. Establishing and reporting a gray zone allow laboratories to follow the industrialist's instructions while acknowledging the uncertainty of the clinical interpretation at low levels of antibodies (Dimech *et al.*, 2013).

The approach taken did not consider factors such as biological difference and the complexity of the proteins being detected, the reactivity of these antigens with antibodies of differing levels of avidity and affinity, the features of the assay being used, and the phase of disease of the patient being tested (AALA, 2012).

While an international reference standard for calibrating assays for the diagnosis and measurement of IgG anti-rubella virus has been available since the 1980s, it has not led to greater levels of standardization between commercial assays and issues reported in 1992 remain. It may be time for the scientific community to question the relevance of quantification of anti-rubella virus IgG. It may be possible for manufacturers of commercial assays to assign a cutoff for their assays by maximizing sensitivity and specificity using well validated panels of samples with a known status, disregarding the WHO standard, and no longer reporting the anti-rubella virus IgG results in IU/ml but as a qualitative result (Dimech *et al.*, 2013).

Diagnosis of acute rubella virus infection or reinfection can be made by detecting rubella virus-specific IgM and recognizing a clinically related increase in the specific IgG concentration of the rubella virus (WHO, 2008; Best and Enders 2007). Quantitative IgG tests are also required to better

diagnose and treat pregnant women who might have been exposed to the rubella virus (Enders *et al.*, 2013).

The assay that does not include a gray zone, and is capable of minimizing the number of experiments assisted or repeated serum specimens used in daily testing. In certain instances, a pregnant woman's rubella immune state may be identified from a qualitative examination; but there are requests where a quantitative test outcome for rubella IgG is needed (diagnosis of reinfection with rubella virus, for example). One principle for rubella virus reinfection in persons with previous immunity (i.e., documented immunization or rubella antibodies) is a clinically related rise in antibody concentration, or both (Best *et al.*, 1989).

Viral infections in pregnancy lead to maternal and fetal morbidity and mortality (Silasi *et al.*, 2015). To a large extent, the detection of rubella depends on antibody positivity. While anti-Rubella IgG seropositivity indicates a prior exposure or immunization, IgM seropositivity displays acute or recent exposure to rubella infection (or reinfection) during pregnancy (Best *et al.*, 2005). Rubella infections have been reported to cause serious complications during pregnancy, including miscarriage, stillbirth, and reproductive failure (Dobson, 2014). There is very limited data on the incidence of infections in the province of Diyala, Iraq. Rubella seropositivity in the target population, province of Diyala, Iraq ranged between 28 % (Salman, 2016) reach up to 91 % conducted by (Hasan, 2011). To test rubella seroprevalence in pregnant women, rubella-specific IgM and IgG antibodies are critical.

Although the incidence of CRS has been decreased worldwide attributable to expanded rubella immunization coverage (WHO, 2012b), it remains a deadly and exorbitant disease in regions where pregnant women are not vaccinated from the rubella virus and have no protective IgG levels.

According to WHO Rules, the primary aim of the rubella vaccine is to avoid infection with congenital rubella and CRS. Any of two strategies for the usage of rubella containing vaccinations is focused entirely on reducing CRS by vaccinating young girls or women of childbearing age; or both. As rubella control progresses towards elimination, the sensitivity and specificity of surveillance systems should increase. If resources license, periodic seroprevalence surveys could be used to monitor the effect of an immunization program; these surveys could include the collection of samples from women attendance antenatal clinics. In all steps of rubella control, rubella surveillance should be combined with the measles surveillance system (WHO, 2011). Integrated measles mumps rubella (MMR) vaccine by lot country as this is a cost-effective strategy, since 1) rubella and measles clinical symptoms are similar and 2) rubella and measles affect the same age groups (Martínez-Quintana *et al.*, 2015).

A primary problem for Iraq is the detection and elimination of measles and rubella as a member of the WHO.MMR schedule was incorporated into the Iraq national immunization program in 1988, and the vaccine is usually given in two doses: at twelve to fifteenth 12-15 months of age and, for females, a second dose between four and six years of age (WHO-MOH Iraq, 2009). The percentage of MMR vaccine programs has coverage from 67 % up to 97% of the population goal along the period from 1988 to 2008 years. The rise and fall in the proportion of immunizations are caused by the conditions that Iraq is going through, such as wars, economic blockades, and sectarian fighting. Women whose birth is less than 1988 were not immunized with the rubella vaccine, which means that all women over the age of 32 did not undergo the immunization program.

To date, no rubella or measles monitoring system has been implemented in Iraq for women of childbearing age, and there was no accurate estimation of the percentage of pregnant women at risk of acquiring CRS. So time after the vaccine drive in 1988, current information about the immunity status of pregnant women against these viruses is lacking. On the other hand, after the fall of the regime in Iraq or after the occupation, and for a decade, the lack of a routine screening system to detect women of childbearing age at risk for rubella before marriage or conception has led to a gap in our knowledge. It is unknown whether the level of immunity against rubella in women who participated in the 1988 campaign has changed significantly.

The high percentage of women that exhibit a negative serological status for this infection and the very low rate of pre-conception tests, suggested that these women were giving very little care to the prevention of congenital rubella. Few women under this study know of rubella as a disease, nor of them know that rubella infection may have potential destruction to the foetus. Fewer of them were conscious the dangerous of TORCH agents and rubella and, lastly, none of them had the rubella test before pregnancy. All these findings testify about the decreasing awareness in the importance of infectious diseases and their prevention that has spread over the last few decades. As a result, there were witnessed a decrease in both the awareness about these diseases and the related prevention, as shown in particular by the remarkable decrease of immunization coverage. This study established that the perception of the rubella risk in pregnancy by women is very poor. Today there were witnessing a significant “cultural gap” concerning prevention that needs, on one hand, a cultural growth of the population and, on the other hand, more awareness on the part of all healthcare workers about immunizations and the rubella issue (Squeri *et al.*, 2017).

Note: the socio-demographic and reproductive factors comparisons were made based on the results of the Electrochemiluminescence (ECLIA) method.

4.2. Rubella-specific IgG and IgM Abs in the different age groups of pregnant women

Results in Table (4.4) regarding the age groups, These findings showed that the highest seropositivity rate for rubella IgG was among those 21-26 years of age group, while the lowest rate was among women more than 32 years old, therefore, there was a significant difference between age groups ($P=0.047$). Moreover, the results showed that the highest anti-rubella IgM seropositivity rate was among those with 21-26 years of age, while the lowest rate was among women more than 32 years old. However, there was an insignificant difference between the age groups ($P=0.13$).

Table (4.4): Detection of Rubella- specific IgG and IgM antibodies among different age groups of pregnant women

Age groups (years)	Result of IgG		Result of IgM	
	No. positive (%)	IgG titer mean $\pm$ SD IU/ml	No. positive (%)	IgM titer mean $\pm$ SD COI
15-20	46 (21.9%)	160 $\pm$ 54	3 (25%)	2.07 $\pm$ 0.37
21-26	67 (31.9%)	152 $\pm$ 41	4 (33.3%)	2.12 $\pm$ 34
27-32	60 (28.5%)	149 $\pm$ 44	3 (25%)	2.04 $\pm$ 0.30
> 32	37 (17.6%)	139 $\pm$ 38	2 (16.6%)	1.99 $\pm$ 0.28
Total	210/240		12/240	
P-value	0.047 S*		0.13 NS	

S*: significant NS: no significant

Statistical analysis showed that pregnant women's age was directly associated with the anti-rubella IgG, a finding consistent with the results of most other studies that increased the rate of positive anti-rubella IgG by age such as (Hasan, 2011; Facciola *et al.*, 2019 ; Zahraei *et al.*, 2020 ; Yalcin *et al.*, 2008). Mwambe and his co-workers observed that with each year's increase in age, the risk of contracting rubella increases by 1.6% (Mwambe *et al.*, 2014). This association can be clarified by a rise in the risk of sensitivity to wild rubella infection as the age increases. Studies documented negative association between IgG positivity rate and age groups are conducted by Honarva *et al.* (2013), Byrne *et al.* (2012) and Mora-Garcia *et al.* (2011).

According to results in Table (4.4), it turns out that the positive rubella IgM was present in all age groups and almost the same proportions. This indicates that the virus can infect women at any age; this result suggests that women positive for IgM may have an active infection with the virus.

In the current study, there was no statistically significant association between the age group and degree of anti-rubella IgM positive rate, a result consistent with several other studies (Salman, 2016 ; Abid and Fayad, 2017). In other studies conducted in Iraq, there was a lack of association between both IgG and IgM with age such as Naqid *et al.* (2020), Saadoon (2016), Mohammed (2015). While Mohammed and Kokaz (2019) showed a lack of association between IgG and age while inversely associated between age and anti-rubella IgM positivity rate.

The IgG Ab positivity level to this virus in the > 32 age group were lower than other groups, this can be explained whenever progress is in an age whenever decline is in rubella IgG titer after infection or vaccination by the rubella virus. This syndrome may be attributed to repeated exposure to the rubella virus by the younger age groups, with reduced seropositivity

with age (Malakmadze *et al.*, 2004). Waning immunity is not significant after natural rubella infection but it has been reported that the levels of the antibody declines over time in the vaccinated population (Davidkin *et al.*, 2000). Although this work is limited to pregnant women, it is clear that in pregnant women in target population, rubella immunity is widespread, yet a substantial percentage rate of pregnant women remains unprotected from this infection, notably in older age groups. This reinforces the need for a secure, tailored rubella immunization with a second dose in adolescence to further boost the weakening immunity seen in the < 32-year-old age group (Gupta *et al.*, 2006).

The age group of > 32 years old women has antibodies against rubella. Since the rubella vaccine is rarely given to adults in Iraq before 1988, this results suggest that a great number of women become infected during childbearing age or previously. Elimination of measles and rubella is a primary issue for Iraq as a World Health Organization member. MMR training was integrated into the 1988 Iraqi National Immunization Programme, and the vaccine is routinely given in two doses: at 12-15 month of age and, for females, a second dose between 4-6 years of age (WHO-MOH Iraq, 2009). The of MMR vaccine programs has rate from 67 % up to 97% of the population goal along the period from 1988 to 2008 years. The rise and fall in the rate of vaccinations-course are caused by the conditions that Iraq is going through, such as wars, economic blockades, and sectarian fighting. Women whose birth is less than 1988 were not vaccinated with the rubella vaccine, which means that all women over the age of 32 did not undergo the vaccination program.

To date, no rubella or measles monitoring system has been implemented in Iraq for women of childbearing age. There was no accurate estimation of the number of pregnant women who are at risk of acquiring

CRS. Accordingly, a decades since the vaccine program in 1988 there is a shortage of reliable knowledge on the immune status of pregnant women against such viruses.

According to the statistics of the Iraqi Ministry of Planning, central statistical organization, the population of Iraq is approximately thirty-nine 39 million in 2019, and by performing a calculation, 48% of the Iraqi population is female, this means nearly 18.7 million women in the Iraqi population, and 49% of these women are of childbearing age, which approximately 9.16 million women at childbearing age (CSO, 2018). According to this study, 12.5% of sera were negative for rubella IgG antibodies, that is, approximately 1.145,000 of Iraqi women are of childbearing age and at risk of contracting rubella virus and developing CRS, taking into consideration that Iraqi women who become 32 years old or older were not immunized against rubella, as MMR schedule was incorporated into the Iraq National Immunization Program in 1988.

4.3. Association of Rubella IgG Ab in Pregnant women with History of Abortion:

A data in Table (4.5) indicates the association between anti-rubella IgG antibodies and abortion and the number of recurrent abortion. The total rate of abortion was 61.42%. The rate of recurrent abortion was 32.85%, 15.71%, and 12.85 % for one, two, and three abortions or more abortion respectively. Furthermore, pregnant women had a history of abortion was significant associated IgG ($P < 0.05$), However, the abortion frequency insignificant difference among pregnant women with a history of one abortion or more with non-significant relation ($P > 0.05$).

Table 4.5: Association of seropositive rubella IgG antibodies in Pregnant women with history of abortion.

No. of pregnant women with anti-rubella IgG antibodies	History of abortion							
	Nonabortion		1 abortion		2 abortions		3 abortions or more	
	No. %	IgG titer mean $\pm$ SD IU/ml	No. %	IgG titer mean $\pm$ SD IU/ml	No. %	IgG titer mean $\pm$ SD IU/ml	No. %	IgG titer mean $\pm$ SD IU/ml
210	81 38.57%	142 $\pm$ 42	69 32.85%	154 $\pm$ 44	33 15.71%	161 $\pm$ 50	27 12.85%	157 $\pm$ 46
			129 (61.43 %) (156 $\pm$ 48)					
Abortion	P = 0.034	P < 0.05	Significant					
Abortion Frequenc.	P = 0.382	P > 0.05	Non Significant					

The current study showed a significant relationship between the history of miscarriage and the presence of rubella IgG antibodies (as shown in Table (4.5), 61.58% of the total seropositive pregnant women had a history of abortion. The miscarriage may be due to the effect of rubella on systemic and local (placenta) immunological response. There was non-significant relation ($P > 0.05$) between the presence of rubella IgG antibodies and the number of abortions. This finding may reveal the ability of the rubella virus to cause miscarriage only one time. This finding is consistent with the findings of most other studies that found a history of miscarriage and is significantly associated with rubella seropositivity, such as Saadoon (2016). Another study showed that there was a strong positive association between anti-rubella antibodies IgM, IgG, and abortion numbers (Mohammed, 2015). Rubella virus infection was linked as an etiological factor with spontaneous abortion in Bandar Abbas/South of Iran (Jahromi *et al.*, 2011). The relationship between abortion of pregnancy and rubella specific IgG and IgM antibodies was also reported by Kareem (2014). This study disagreed with Mohammed and Kokaz (2019) who were revealed a significant inverse association between previous abortion and IgG positivity. A study by Khadair *et al.* (2019) revealed a history of

miscarriage which is insignificantly associated with seropositivity of rubella virus.

Several factors induced miscarriage (Hwang, 2019). Rubella virus transmitted through the placenta to the fetus during the maternal viraemic process, the fetal damage tends to include all germ layers and is the product of the sudden death of some cells and chronic viral infection in others (Fokunang *et al.*, 2010). The rubella virus plays a significant role in the incidence of miscarriage in the study population (Ommieya *et al.*, 2019). Previous reports of etiological causes for abortion, such as infectious agent *Toxoplasma gondii* (Mohammed and Al-Janabi, 2019), Cytomegalovirus (Sheini *et al.*, 2018), Dengue virus (Sondo *et al.*, 2019), as well as immunological causes such as anti-nuclear antibodies (Cavalcante *et al.*, 2019) and *Chlamydia Trachomatis* have been proposed as significant causative agents for spontaneous abortion (Haghdoost *et al.*, 2019). (Adeleke and Adepoju, 2010).

4.4. Distribution of Rubella- specific IgG and IgM antibodies results according to a levels of education

The effect of the level of education in rubella seroprevalence is shown in Table 4.6. It is obvious that the highest detection rate about the virus was among those with secondary school education, followed by those higher educations and the lowest rate was among those with literacy educations for IgG results. Also the highest detection rate of antirubella IgM was among those with secondary school education and the lowest rate was among those with literacy educations for IgM result. The difference among these groups statistically insignificant with IgG ($P = 0.280$) and IgM ($P = 0.541$).

Table 4.6: Anti-rubella IgG and IgM antibodies according to education level:

Level of education	Result of IgG (RV)		Result of IgM (RV)	
	No. positive (%)	IgG titer mean $\pm$ SD IU/ml	No. positive (%)	IgM titer mean $\pm$ SD COI
Literacy	12 (5.7%)	144 $\pm$ 53	2 (16.6%)	2.09 $\pm$ 0.34
Primary school	52 (24.7%)	146 $\pm$ 46	3 (25%)	2.13 $\pm$ 0.28
secondary school	84 (40%)	154 $\pm$ 42	4 (33.3%)	2.05 $\pm$ 0.38
Higher school	62 (29.5%)	151 $\pm$ 50	3 (25%)	2.01 $\pm$ 0.31
Total	210/240		12/240	
P-value	0.280 NS		0.541 NS	

In the current study, there was no statistically significant association between the education level and rubella seroprevalence. This study is consistent with the results of other studies such as Olajide *et al.* (2015), whereas similar result has been reported by Hasan (2011), who found an insignificant effect of educational levels on the IgG positivity rate among pregnant women. While this study disagreed with Salman (2016) who revealed a significant association between the education level and rubella seroprevalence. Studies by Facciola *et al.* (2019) and Khadair *et al.* (2019) linked between these two variables and found a statistically significant association between them, as the women with a lower educational levels were more negative than those with a higher education level.

The motivation of health education regarding the risks of rubella infection seems beneficial for women to minimize the burden of the disease. The cultural levels was the more risk exclusively in the oldest

women, with a low cultural level, probably due to a reduced knowledge about the potential risk represented by rubella infection in pregnancy.

4.5. Association of anti-rubella antibodies seropositive with pregnancy gestational age

Table 4.7 demonstrates anti-rubella seropositive antibodies associated with gestational time of pregnancy. The highest prevalence of rubella IgG (35.7%) is observed in pregnant women in the first trimester of gestation while the lowest seropositive levels for rubella IgG are observed in women in the third trimester 30.0%. While the highest prevalence of rubella IgM (50.0%) is observed in pregnant women in the third trimester of gestation. There were no significant differences between the anti-rubella antibodies seroprevalence for pregnant women and the trimester of pregnancy.

Table 4.7: Seropositively rate of anti-rubella IgG, IgM according to gestational age:

Gestational age of a pregnancy	Result of IgG (RV)		Result of IgM (RV)	
	No. positive (%)	IgG titer mean $\pm$ SD IU/ml	No. positive (%)	IgM titer mean $\pm$ SD COI
1st trimester	75 (35.7)	146 $\pm$ 52	3 (25%)	2.03 $\pm$ 0.35
2nd trimester	72 (34.2)	155 $\pm$ 47	3 (25%)	2.10 $\pm$ 0.31
3rd trimester	63 (30.0)	151 $\pm$ 43	6 (50%)	2.07 $\pm$ 0.39
Total	210/240		12/240	
p-value	0.091 NS		0.133 NS	

In the current study, there was no statistically significant association between the seropositively of IgG and IgM according to gestational age

analysis consistent to the findings of several other similar studies (Honarvar *et al.*, 2013; Olajide *et al.*, 2015; Mohammed and Kokaz, 2019), they reported that there was insignificant association between the anti-rubella Abs and gestational time among pregnant women, while inconsistent with the study conducted by Saadoon (2016) who reported statistical significant association between the distribution of rubella antibodies and gestation age. Another study conducted by Mwambe *et al.* (2014) who revealed significantly high risk of contracting rubella virus infection was increased with gestational age.

Pregnancy is fairly split into three-trimester cycles in many cultures as a means to facilitate comparison to the multiple stages of fetal growth (Ifeanyi *et al.*, 2014). The first trimester begins at the beginning of the 13th week and is the three months of infancy that have been finished. Normally, the second trimester begins in the late 27th week. The third trimester will finish somewhere between week 38 and week 42 (Akinloye *et al.*, 2013). The first quarter period contained the largest abortion risk. In the second row, the development should be monitored and evaluated. The third trimester of birth represents the beginning of viability (Patricia *et al.*, 2013), and the fetal organs reach maturation during this trimester (Oke *et al.*, 2011).

In the present study rubella antibodies were found in pregnant women within all stages of pregnancy trimester but with different rates of the distribution rubella antibodies type and pregnancy trimester. The highest rate of seropositive for rubella IgG and IgM was within the third trimester of pregnancy, while the lowest rate of IgG and IgM were found in women within the first trimester of pregnancy as shown in table 4.7. These findings may be due to the ability of the virus to cause infection at any time during pregnancy. Concerning the high rate of primary and re-infection in the third

trimester, this may be due to that these groups are more in contact with infected individuals than pregnant women in the second or first trimester.

When a non-immune pregnant woman is infected during the first trimester, especially the first month, significant congenital malformations can occur as a result of maternal viremia and fetal infection. The increased rate of abnormalities during the primary weeks of pregnancy is attributed to the very sensitive organ development that happens at that time (Levinson *et al.*, 2014).

The proportion of pregnant participants in this sample during the first trimester was greater than those of pregnant women in their second trimester of their sixth. This mirrored the reality that women in their first trimester are often sent to obstetrics and maternity clinics, even more so than later pregnant women.

The complications and abnormalities of congenital infection rely upon the gestational age at diagnosis. Miller *et al.* have been reported that the congenital infection prevalence reaches 90 % before 11 WG, falls to 30% between 24 and 26 WG and rises to approximately 100 percent over 36 WG. In the first 12 WG, the incidence of significant fetal abnormalities is equivalent to 85%, with around 20 % of cases in the first 8 WG culminating in involuntary abortions. The risk falls rapidly and fluctuates between 11 and 18 WG, approximately 0 percent after 18 WG (Miller *et al.*, 1982).

The danger of contracting rubella infection during the different phases of pregnancy, is outlined: first trimester (weeks 0 to 13): if get rubella in the first trimester, get very good rubella. Large chance that baby may be infected (up to 51 %). The earlier that catch rubella in pregnancy is related to the offspring with the greater risk. By week 10, the damage to the offspring is decreased however they may experience problems in their sight

or hearing that might not become evident until they are older. Second trimester (14 to 26 weeks): the infant is already at risk (33 %) between weeks 14 and 15. Through their eyes or ears, they may experience symptoms that do not arise before the older men. The third trimester (stage 27 at birth): 29% of the baby's vulnerability is small after week 16 (Kareem, 2014).

4.6. Anti-rubella IgG and IgM results according to residence

Table 4.8 showed that the detection rate of RV IgG positively was highest among women who reside in urban areas (55.7%) compared to those who reside in rural areas (44.2%). The detection rate of RV IgM positively was highest among women who reside in rural areas (58.3%) compared to those who reside in urban areas (41.6 %). The statistical difference between the two groups was significant ($P = 0.018$) with IgG and insignificant ($P = 0.307$) with IgM.

Table 4.8: Distribution of rubella IgM and IgG according to different Residence:

Residence	Result of IgG (RV)		Result of IgM (RV)	
	No. positive (%)	IgG titer mean $\pm$ SD IU/ml	No. positive (%)	IgM titer mean $\pm$ SD COI
Urban	117 (55.7)	158 $\pm$ 53	5 (41.6)	2.09 $\pm$ 0.33
Rural	93 (44.3)	141 $\pm$ 45	7 (58.4)	2.05 $\pm$ 0.38
Total	210/240		12/240	
p-value	0.018 S		0.307 NS	

The significantly higher anti-rubella IgG positivity rate among urban compared to rural women in the present study was also reported by previous studies (Khadair *et al.* (2019) ; Saadoon (2016) ; Tahita *et al.* (2013) ;

Jahromi *et al.* (2011). Another study reached the truth, the women residing in the rural district has 1.09-time greater opportunity to have a protective IgG than women residing in an urban district (Mohammed and Kokaz, 2019). Result in this study is inconsistent with other studies as they showed the insignificant difference in the rubella specific IgG antibodies rate among urban and rural residence women (Salman (2016) ; Olajide *et al.* (2015) ; Mwambe *et al.* (2014) ; Honarvar *et al.* (2013).

Although the anti-rubella IgM seropositivity rate was higher in rural women compared to that of urban women, However, the difference was failed to reach the level of statistical significance. The insignificant difference in the rubella specific IgM antibodies rate among urban and rural residence women in the present study was also reported by other studies (Mohammed and Kokaz (2019) ; Salman (2016) ; Olajide *et al.* (2015). Other studies showed a significant difference in the rubella specific IgM antibodies rate among urban and rural residence women (Saadoon, 2016).

The significantly higher anti-rubella IgG positivity rate among urban compared to rural women may be due to the higher population growth in metropolitan environments and the spread of rubella virus through respiratory droplets are vulnerable to a higher chance of rubella infection in such regions. Furthermore, probably poor immunization coverage in some rural areas compared with urban areas, probably the rate of immunization for rubella is higher in an urban area, and even experience of rubella as a preventable disease is more common in metropolitan regions, with more interaction with facts about it than in rural areas.

Rubella is almost always diagnosed in central clinical laboratories. In resource-limited settings, transportation from rural communities to remote laboratories is often cost-prohibitive (Kiyaga *et al.*, 2013), meaning that

rubella infections (and attendant cases of CRS) routinely sweep through rural populations without testing or treatment (Reef *et al.*, 2011).

In this study, a large number of the seronegative women are from rural areas and have poor knowledge about the risk and threats of rubella infection to the fetus during the first trimester period, and this should aware the health service providers to take any action by universal immunization of the girls at the secondary schools and provide educational material and to improve knowledge about the health problems and health risks that might affect the pregnancy outcome, especially in non-educated population.

4.7. Molecular Results

4.7.1 Viral RNA genome extraction

The viral RNA genome was extracted from the serum samples. Concentration of extracted RNA was ranged between 20.4-39.0 ng/μl and the purity was ranged between 1.67-2.68. There were low concentrations

Table (4.9): Concentration and Purity of RNA Viral Measured by Nanodrop 2000 System

Samples	Concentration ng/μl	Purity 260/280
1	32.5	2.68
2	33.1	2.38
3	28.1	2.38
4	27.1	2.53
5	20.4	2.13
6	20.8	2.25
7	39.0	2.39
8	20.7	1.67

4.7.2 Reverse Transcription Real-time PCR test (RT-rPCR) for Qualitative detection of Rubella Virus

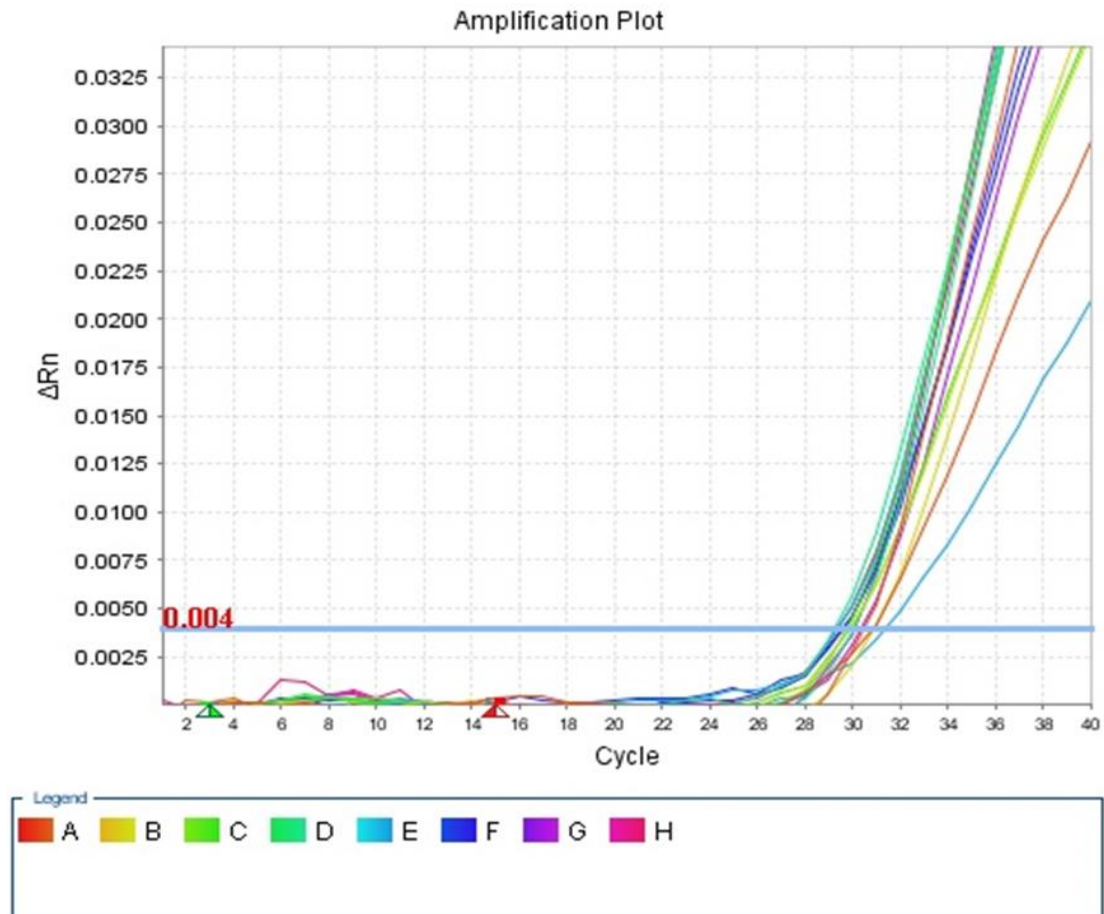
The results in Table 4.9 showed that only 8 from 240 specimens of pregnant women are rubella viral RNA positive with (3.33%) percentage, while 232 (96.66%) specimens of pregnant women are rubella viral RNA negative.

Table 4.10: Qualitative detection of Rubella Virus RNA by using RT-rPCR test

Method	viral RNA Positive	Percentage %	viral RNA Negative	Percentage %	Total
RT-rPCR	8	3.33 %	232	96.66 %	240

Lower Ct values (typically below 29 cycles) indicate high amounts of target sequence. Higher Ct values (above 38 cycles) mean lower amounts of your target nucleic acid.

It was probable that the blood specimens from the three rubella cases were negative due to the inhibition of the RT-PCR inhibitors. The inhibitors of the RT-PCR negatively affect PCR amplification efficiency during template preparation. Common inhibitors include various materials such as components of body fluids, proteins, organic and phenolic compounds, DNA fragmentation (**Burns et al., 2005**) The results in the present study were also consistent with the results of another study, which have been reported by **Mahmood et al. (2015)** showed that only 6 (10.5%) were positive for Rubella virus by using Real-Time RT-PCR test from 46 (80.7%) are positive by ELISA test.



Figur (4.1). The amplification plot of rubella virus by RT-Rpcr.

This negative result is very low concerning the positive result when measuring the samples depending on the presence or absence of rubella IgG and IgM antibodies. Where from 12 positive IgM results, which represent an acute infection, only 8 of them appeared positive in a way of RT-PCR. This result can be explained on the basis that the positive result with the RT-PCR technique appears only when the RNA of the virus is present in the sample. The duration of inflammation varies between about 8 days before the rash begins and about 8 day later. It takes two weeks from began to the end of disease, after which the virus fades from the body and screening for it by approved molecular methods is not possible. Therefore, test is negative while the antibodies remain for a longer period, and this explains why only 8 samples were found positive by RT-PCR. RT-PCR is a mixture of three steps: (1) reverse transcriptase (RT)-dependent RNA

translation into cDNA; (2) cDNA amplification using PCR; and (3) real-time identification and quantification of amplification products (Gibson *et al.*, 1996). Although it has become the method of choice to quantify RNA (Ginzinger *et al.*, 2002), several concerns directly affect the assay's reliability. These are mainly because (1) the results depend on the quantity of the template, the quality and the optimal design of the assay (Bustin and Nolan, 2004), (2) the reverse transcription reaction is not systematic and may thus be very complex (Stahlberg *et al.*, 2004) and (3) the data interpretation is extremely arbitrary and, if incorrectly carried out, obscures the real effects of the study (Bustin, 2002).

The recommended RT-PCRs for verifying rubella infection in the laboratory are tools that include assaying TaqMan in real-time. Conventional endpoint assays are also used for sequencing amplification products, especially nested formats. Though such clustered RT-PCRs are extremely reactive and need amplification in the case of low viral loads as well as in the case of suboptimal specimens such as serum (Hernandez-Rodríguez *et al.*, 2012). The same clinical procedures will be used to diagnose measles and rubella viruses, enabling concurrent or parallel studies. Quick and sufficient the collection of samples and proper transport and storage are necessary to avoid false-negative tests. Rubella virus viremia is small, so often there is no other material to be tested except for serum. The RT-PCR diagnostic assay would also be very open to the specific diagnosis of rubella virus (Claus *et al.*, 2016), especially when the patient is pregnant and at risk of developing congenital infections. Much as measles, 739 viral E1 gene nucleotides (WHO, 2013) based on rubella genotype findings is crucial to identifying the virus' epidemiology and verifying elimination (Mulders *et al.*, 2016).

Responsive, accurate, High-quality, and verified tests are expected to support the rapid removal of rubella from public health programs. Regional monitoring networks have a part to play in ensuring that suitable Clinical specimens shall be obtained in the appropriate manner and at the period needed during infection. Specimens planned for RT-PCR or in particular for virus isolation need to be properly transported and stored to prevent deterioration of the specimens (Abernathy *et al.*, 2009).

It is difficult to obtain appropriate Specimens for molecular virological information, including prompt planning by professional workers of the throat swab and oral fluid specimens and a tight reverse cold chain. These requirements can also not be achieved by routine measles and rubella testing (Zheng *et al.*, 2013). Alternate tests providing biological virological confirmation retrospectively, such as serum; they are a possible source of pre-vaccine-era reference data on rubella virus genetic diversity. Rubella virus RNA can be observed in an acute serum process (0-3 days after rash) specimens this is IgM-based rubella with the polymerase chain reaction and healthy samples can be genotyped (Puuta *et al.*, 2016).

4.7.3. Conventional RT-nested PCR Results.

Rubella positive samples by RT-PCR will be selected for genotyping depending on E1 gene amplified by PCR, and sequencing the PCR products. The GoTaq® 1-Step RT-qPCR System, is a reagent system for quantitative analysis of RNA using a one-step reverse transcription-quantitative PCR (RT-qPCR) protocol. Six (6) pairs of primers tested and analysis before used at the National Center for Biotechnology Information (NCBI) site to obtain the special segment in order to obtain the nitrogenous base sequences and determine the genotype of the rubella virus. many repeated attempts to obtain the gene fragment to determine the genotype of the rubella virus, applying special protocol with the kits, changed the

concentration of the polymerase enzyme (GoTaq® 1-Step RT-qPCR), changed and manipulating the program of the thermal cycling conditions such as the Annealing temperature, The nested assay 1 was used initially, all 8 samples that tested were found negative in this assay, then subsequently tested using the assays 2 and 3.

Three samples was gave bands sized 719bp by using set 2 depending on primers amplification flanking region of 1E genotype as shown in figure 4.2, and two tested samples were found positive to set 3 indicated 2B genotype as shown in figure 4.3.

From eight blood samples were real-time PCR positive, only five samples were gave bands, this result can be explained on the basis that sera are not optimal specimens for viral detection due to the low amounts of viral RNA present, another study also synchronized with this study conducted in Rumania, whereas RuV RNA was detected in 21.5% of IgM positive serum samples collected close to symptom onset, while genotypes were obtained from only 7.5% (Lazar *et al.*, 2016), also these numbers are in good agreement with a another study of RuV RNA in sera in which 26% of sera samples were real-time PCR positive and 12% yielded genotypes (Zheng *et al.*, 2013).

There are no previous studies on rubella virus genotyping in Iraq or in neighboring countries. Genotype 1E viruses were first identified in 1997 in the United States, Canada, the Caribbean, and Italy (Zheng *et al.*, 2003).

Viruses of the genotypes 1E and 2B had a wide geographic distribution and were frequently found, Genotype 1E viruses have been identified in 1 Middle Eastern country, 7 European countries, 3 Southeast Asian countries, 3 African countries, and 4 Western Pacific countries, and are the dominant genotype in China (Zhu *et al.*, 2010), while Viruses of genotype 2B were reported from 2 Middle Eastern countries, 5 European

countries, 4 Southeast Asian countries, 4 South and Central American countries, 3 African countries, and 2 Western Pacific countries (Abernathy *et al.*, 2011). Genotype 1E viruses were clearly predominant in the 17 of 31 provinces surveyed in China, it has been identified as the predominant viral genotype, circulating continuously throughout China since it was first identified in 2001 (Zhu *et al.*, 2012). In contrast, genotype 2B viruses have been sporadically detected in 2000 and 2008 (WHO, 2013). Genotype 1E and 2B was considered to be endemic in the Americas in part or all of the period from 1997 through 2007 (Icenogle *et al.*, 2011). Viruses of genotype 1E and 2B has been found in Arabic countries such as Tunisia, Sudan, Yemen, Egypt and Dubai (Abernathy *et al.*, 2011).

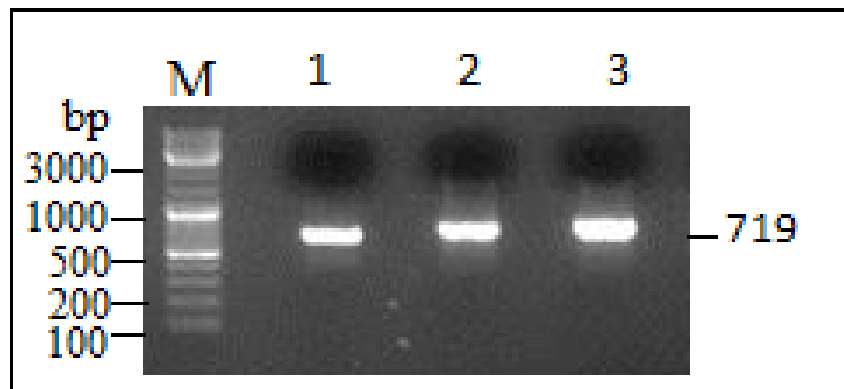


Figure (4.2). Gel electrophoresis analysis of PCR product of E1 flanking region of nested assay 2, sized 719bp. Lane M:100-10000bp ladder, lanes1-3 represent E1 gene in 1% agarose gel stained with red safe DNA dye and electrophoresed by 5vol/cm in TBA buffer then visualized under UV light.

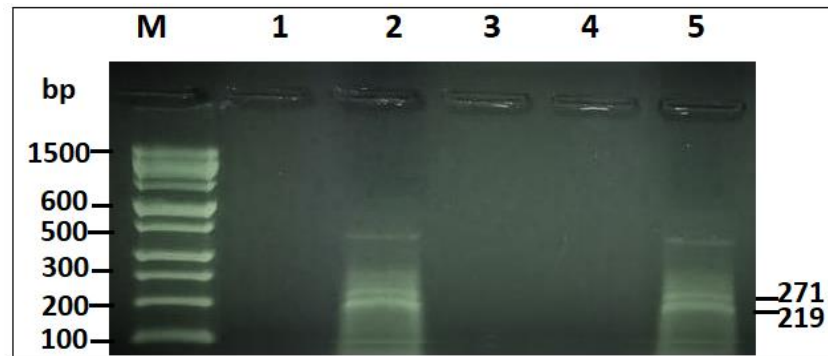


Figure (4.3): Gel electrophoresis analysis of PCR product of E1 flanking region of nested assay 3, sized 271, and 219 bp. Lane M:100-1500bp ladder, lanes1,5 represent E1 gene in 1% agarose gel stained with red safe DNA dye and electrophoresed by 5vol/cm in TBA buffer then visualized under UV light.

Nested PCR second round product of the E1 partial gene were sent for sequencing and failed to revealed good sequence because of samples degraded. Hernández-Rodríguez *et al.* (2012) refers to that a critical issue for rubella detection when using endpoint conventional PCR or real-time RT-PCR for diagnosis is the quality and the timing of collection of the clinical specimen. If it has been transported under suboptimal conditions, or collected late after onset of rash, or the patient has a low level of Viremia, a negative RT-PCR result may actually be diagnostically incorrect. Therefore, WHO (2013) recommends using IgM serology as reference standard and using RT-PCR only in combination with serology. However, during the first few days after rash onset, RT-PCR may be diagnostically more useful than serology. A positive RT-PCR result is diagnostic; a negative outcome must be interpreted with caution. Nested formats are mostly used for amplifying products for sequencing. While these nested RT-PCRs are very sensitive and allow amplification also in cases of low viral load and from suboptimal specimens such as serum, they require strict contamination control measures. Early and appropriate sample collection as well as adequate transportation and storage are essential to

avoid false-negative results. The recent study concludes are consistent with Claus *et al.* (2016) that Rubella virus Viremia is low and often no other specimen besides serum is available for investigation. The diagnostic RT-PCR assay should therefore be very sensitive to reliably detect rubella virus particularly when the patient is pregnant and at risk for congenital infection. As with measles, rubella genotype data, based on 739 nucleotides of the viral E1 coding region.

Conclusions and Recommendations

Conclusions

1. Since the vast majority of pregnant women were rubella IgG positive and few rubella IgM positive, this indicates that most of them result from vaccination or old infections, and a few are due to recent infections.
2. Only 8 of 12 IgM positive samples were found positive by RT-rPCR test.
3. (RT-nPCR) on E1 flanking region indicated genotypes 1E and 2B.
4. The detection of rubella infection depending on Ab-Ag complex is more suitable than RT-rPCR detection.
5. There was a significant association between seropositivity of rubella and age. The highest seropositivity rate for rubella IgG was among those over 32 years of age group, while the lowest rate was among women 15-20 years old.
6. There was a significant association between history of abortion and presence of rubella IgG.
7. There was a significant association between seropositivity of rubella and residence, the higher anti-rubella IgG positivity rate was among urban compared to rural pregnant women.

Recommendations

1. Further studies are required to screen all Iraqi/Diyala women with TORCH (toxoplasmosis, rubella, cytomegalovirus, and herpes infections).
2. The health care authorities need to set up an additional program of vaccination for unvaccinated women post-delivery or post-abortion.

3. Research is recommended to conduct other studies with large sample size to assess the need for comprehensive supplemental immunization programs.
4. Strength the premarriage girl's vaccination.
5. Efficiency of rubella vaccination.

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Appendix (1): The patient questionnaire list.**Name:****Age:****Trimester:**

1st

2nd

3rd

Education:

Literacy

Primary

Secondary

High school:

Abortion:

Abortion frequency

Residence:

Urban

Rural

Date:**Mobile No.:**

الخلاصة

الحصبة الألمانية هي مرض فيروسي يسببه فايروس الروبلا. الاعراض السريرية الشائعة هي الحمى والطفح الجلدي والسعال وتضخم العقد اللمفاوية وغالبا ما يسبب التهاب المفاصل. عادة ما تكون إمرضياته خفيفة الى متوسطة مشابهة لمرض الحصبة ، خطورة المرض الحقيقية تحدث عندما يصاب الجنين بفايروس الروبلا اثناء الحمل ، لأن الفايروس قد يهاجم أي جزء وأي عضو من الجنين كالدماع والقلب والسمع والبصر ويسبب له الأذى والتشوه و مجموع هذه التشوهات تدعى متلازمة الحصبة الألمانية الشائعة.

صممت الدراسة الحالية لمعرفة انتشار فايروس الحصبة الألمانية في النساء الحوامل. شملت العينات مائتي وأربعون(240) امرأة ممن راجعن مستشفى البتول التعليمي للنسائية والأطفال في مدينة بعقوبة للفترة من شهر تشرين الاول سنة 2018 الى شهر مايس 2020. تتراوح اعمارهن بين 16-45 سنة وبمتوسط عمري 26.38 ± 5.11 سنة. تتضمن البيانات التي جمعت العمر والتعليم وعدد حالات الاسقاط السابقة وفصل الحمل والسكن.

جُمع المصل من كل عينة وتم قياس الضدات النوعية لفايروس الحصبة الألمانية (IgG,) باستخدام ثلاثة طرق هي الطريقة الكهربية الكيميائية الومضية الذاتية (Electrochemiluminescence) وطرية الأليزا والطريقة السريعة (OnSite Rubella IgG/IgM Rapid tests). كذلك تم استخدام طريقة الزمن الحقيقي لتفاعل البلمرة التسلسلي (RT-rPCR) لاكتشاف وجود الحامض النووي الرايبوي للفايروس (RNA).

أظهرت الدراسة أن النسبة المئوية لوجود الضدات النوعية IgG تتراوح بين 84.58 % إلى 89.58 % بالاعتماد على الطريقة المستخدمة، بينما كانت النسبة المئوية لوجود الضدات النوعية IgM تتراوح بين 5% الى 5.41 % اعتمادا على الطرية المستخدمة. من مجموع 240 عينة فقط ثمانية 8عينات كانت موجبة باستخدام طريقة الزمن الحقيقي لتفاعل البلمرة التسلسلي. RT-rPCR. كانت أقل نسبة لوجود الضدات النوعية IgG في الفئة العمرية 15-20 وأعلى نسبة لها في الفئة العمرية الأكبر من 32 سنة. بينت الدراسة ان النسبة المئوية لوجود الضدات النوعية IgG في النساء التي تسكن المدن تشكل 92.13 % وهي اعلى من النسبة التي ظهرت في النساء التي تسكن في الريف 82.30 % وبفارق ذو دلالة معنوية $0.05 >$. اظهرت الدراسة ان 61.42 % من الحوامل الموجبة للضدات النوعية IgG قد عانت من حالات اسقاط سابقة وبفارق ذو دلالة احصائية $0.05 >$ ولا يوجد فرق ذو دلالة معنوية بين عدد حالات الاسقاط المتكرر.

التشخيص الجزيئي للاصابة بفايروس الحصبة الالمانية تم باستخدام النسخ العكسي للزمن الحقيقي لتفاعل البلمرة التسلسلي . تم اختيار عينات RT-rPCR ايجابية للحصبة الألمانية للتنميط الجيني وللكشف عن تسلسل القواعد النتروجينية لمنتجات تفاعل البلمرة التسلسلي وذلك اعتماداً على الجين E1 الذي تم تضخيمه بواسطة RT-nPCR. ثلاث من مجموع ثماني عينات ايجابية باختبار RT-rPCR انتجت قطع بحجم 719 باستخدام الزوج الثاني من البادئات المتخصصة بتضخيم النمط الجيني E1 ، وعينتين انتجت قطع بحجم 219- 271 أزواج من القواعد النتروجينية باستخدام الزوج الثالث من البادئات المتخصصة بتضخيم النمط الجيني B2.



وزارة التعليم العالي
والبحث العلمي
جامعة ديالى
كلية التربية للعلوم الصرفة

الكشف المناعي الجزيئي لفيروس الحصبة الألمانية في النساء الحوامل في محافظة ديالى

أطروحة مقدمة إلى

مجلس كلية التربية للعلوم الصرفة / جامعة ديالى

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم الحياة
من قبل

محمد حسين علوان

بكالوريوس كلية العلوم / قسم علوم الحياة / جامعة بغداد 2002 م

ماجستير علوم حياة / كلية التربية للعلوم الصرفة / جامعة ديالى 2015 م

بإشراف

أ. د. محمد عبد الدايم صالح **أ. م. د. نادرة سلمان محمد**

2020 م

1442 هـ