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Al-Quds University

"Human Parvovirus B19 Epidemiology, Genotyping, Patients' Hematological Presentation and Clinical Manifestations in Children in Southern Palestine"

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“Human Parvovirus B19 Epidemiology, Genotyping, Patients’ Hematological Presentation and Clinical Manifestations in Children in Southern Palestine”

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Thesis Approval

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Dedication

I dedicate this study to Allah in the hope that it will help patients in need and contribute to scientific research.

To the unsung heroes in my life: Doctor Sheikh Rajab Deeb, Professor George Daher, and Hajeh Suheir for their constant support in every way.

To my beloved, caring and supportive parents, Mr. Munther Abdo and Mrs. Suhad Abdo, who encouraged and supported me immensely with their kindness, support, help and love.

To my family: Mustafa, Akram, Dana, and my grandmother Mrs. Hana Abdo for their constant care.

To my friends for their unending encouragement.

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed: 

Miral Munther Badawi Abdo

Date: 03/09/2023

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Abstract

Background: Human parvovirus (B19V) is a virus that is usually asymptomatic in healthy patients but can sometimes be symptomatic, especially in patients who have a previous underlying disease such as hematological and cardiopulmonary diseases. B19V targets the erythroid progenitor cells by attaching to the P antigen causing slapped cheek syndrome and may cause transient aplastic anemia. The aim of the study was to check the prevalence of B19V, the most common genotypes, its effect on CBC and the clinical symptoms especially transient aplastic crisis for the first time in Palestine.

Methods: B19V was studied in children retrospectively from February 2014 to December 2022 in Caritas baby hospital in Bethlehem. Real-Time PCR for B19V was done on blood EDTA tube for 905 suspected patient samples out of which 28 patients tested positive for B19V (3%). The 28 B19V cases were compared with 32 B19V negative controls. Both groups were checked for social demographics, clinical symptoms, hematological parameters (CBC), and other lab tests (CRP, AST, and ALT). Moreover, genotyping was attempted on all RT-PCR positive B19V cases. This was done by amplifying B19V VP2 gene using VP2 special primers followed by Sanger sequencing.

Results: As for social demographics, being a female or a male does not affect the chance of being infected with B19V, in addition to the age of the patient and the place of inhabitant. Regarding clinical manifestations some variables showed significant statistical association with B19V infection such as respiratory problems (p =0.046), malaise (p =0.005), and neurological issues (p =0.043); however, having fever, rash and anemia did not have any statistically significant association with B19V although 2 patients had severe anemia: one patient was diagnosed with pure red cell aplasia and another patient was diagnosed aplastic anemia. A statistically significant association was also found in certain CBC parameters, including WBC (p =0.003), platelets (p =0.018) and Hb (p =0.019, OD=9.6, 95% confidence interval: 1.45,63.5), notably patients with Hb < 10 mg/dl are 9.6 times more likely to test positive for B19V compared to Hb>10. CRP, AST, and ALT had no significant association with B19V, although all the B19V positive patients had CRP above 6 mg/dl. Of the patients analyzed, 40.7% of the cases had previous underlying disease which was significantly associated with B19V infection (p =0.003, OD=10.3, 95% confidence interval: 2 ,52.3), thus individuals with a previous underlying disease are 10.3 times more likely to test positive for

the B19V compared to previously healthy patients. Moreover, 37% of the cases had coinfections of B19V and another microorganism. As for genotyping, of the 28 positive samples analyzed VP2 fragment was amplified from 5 samples (17.8%) that had high B19V viral load and the phylogenetic tree revealed that the most common genotype of B19V in Palestine is genotype 1.

Conclusion: To conclude, this retrospective case-control study is the first study to check B19V in Palestine which provided valuable insights and more understanding about the virus in the country.

Keywords: Human Parvovirus B19V, Epidemiology, Clinical manifestation, Hematological presentation, CBC, Genotyping, Palestine.

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List of Abbreviations

Abbreviation	Term
Allo-HSCT	Allogeneic hematopoietic stem-cell transplantation
AXL	Tyrosine protein kinase receptor UFO
B19V	Human parvovirus B19
bp	Base pair
CBC	Complete blood count
CMV	Cytomegalovirus
IVIG	Intravenous immunoglobulin
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EPCs	Human erythroid progenitor cells
Epo	Erythropoietin
Hb	Hemoglobin
HHV-6	Human herpes virus 6
HHV-7	Human herpes virus 7
HLH	Hemophagocytic lymphohistiocytosis
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
μL	Microlitres
NF-H2O	Nuclease free H2O

NS1	Nonstructural protein 1
PCR	Polymerase chain reaction
RBC	Red blood cells
RDW	Red blood cell distribution width
RT-PCR	Real-time polymerase chain reaction
SCA	Sickle cell anemia
Tm	Melting temperature
VP1	Viral protein 1
VP2	Viral protein 2
WBC	White blood cells

Chapter 1

Literature Review

1.1 Background

Human Parvovirus B19 (B19V) is a non-enveloped virus that infects only humans. It is known for causing the innocuous childhood rash, the fifth disease (erythema infectiosum or slapped cheek syndrome) that usually affects children. It is most common in young children, but adults can also be infected. It is transmitted by respiratory secretions (Centers for Disease Control and Prevention,2022) (Macri & Crane, 2022).

1.2 Epidemiology of B19V

The prevalence of B19V in developed countries is from 2% to 10% in children under 5 years of age, 40% to 60% in adults over 20 years of age, and over 85% in those over 70 years of age. B19 viral infections are more frequent in late winter, spring, and early summer (Macri & Crane, 2022). In another study, it was found that 31.3% of the B19V patients were between 5 and 9 years old, and 34.4% of the cases were 20 years and older (de Los Ángeles Ribas et al.,2019).

In the Palestinian-occupied territories, it was found that more than 30% of the adult population is susceptible to B19V infection, and it was recommended to observe B19V status so that the specific risk groups such as pregnant women will be protected (Mor et al., 2016).

1.3 Transmission of B19V

Parvovirus B19 is spread through respiratory secretions (saliva, sputum, or nasal mucus) by coughing or sneezing, blood or blood products, and infected pregnant woman to fetus (Centers for Disease Control and Prevention,2022).

1.4 B19V Genotypes

B19V is categorized into three genotypes, genotypes 1, 2, and 3 (Qiu et al.,2017). The genotypes vary from each other by 2-13 percent with genotype 1 being the most common genotype worldwide followed by genotypes 2 and 3 (Jain & Kant,2018) (Hübschen et al., 2009). Both Genotypes 1 and 3 have two subtypes (1A and 1B) and (3a and 3b)

respectively (Jain & Kant,2018) (Figure 1).

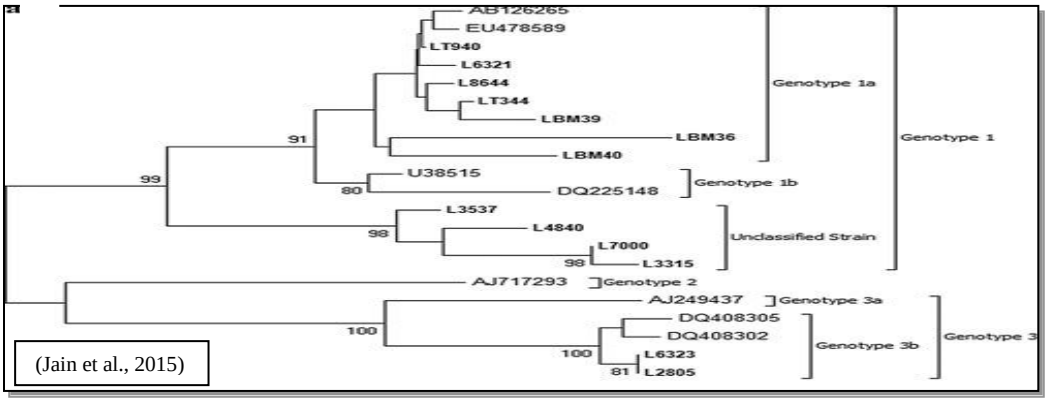

Figure 1.1: Phylogenetic tree of B19V

1.5 B19V Structure

Parvovirus B19 (B19) is a member of the family Parvoviridae, a nonenveloped icosahedral virus that measures ~22 to 24 nm in diameter (Slavov et al.,2011), it is a single-stranded DNA virus and its genome contains 5,596 nucleotides (Heegaard & Brown,2002). B19V has two gene sets: an NS gene and a VP gene (Figure 1.2). The main B19V significant viral proteins are the major nonstructural protein (NS1) and the two structural proteins (VP1 and VP2) (Cotmore et al.,1986) (Figure 1.3).

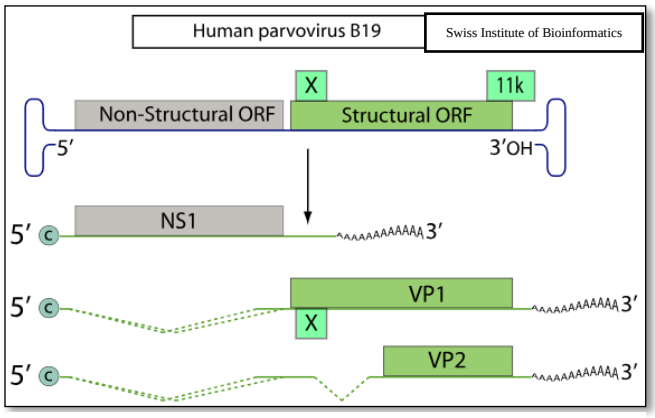

Figure 1.2: B19V genes

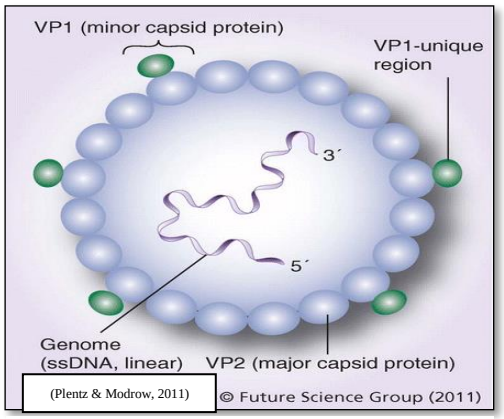

Figure 1.3: B19V proteins

Nonstructural l protein (NS1) is important in DNA replication (Zhi et al.,2006) and is viewed to be the main pathogenic factor that suppresses the differentiation and maturation of erythroid progenitor cells (EPCs) since B19V has a high affinity to bone marrow and fetal liver, the viral DNA suppressive role is accomplished through generating damage responses and cell cycle arrest (Feng et al., 2020).

The capsid surface is made of 60 copies of the VP which plays many functions such as genome encapsidation, specific binding to cellular receptors, recognition, intracellular

trafficking with its phospholipase A2 (PLA2) activity, nuclear entry and exit in addition to the avoidance of the host immune response (Qiu et al.,2017).

The capsid interacts with the P antigen (Brown et al., 1993) , and about 96% of the total capsid protein is made of VP2 which is the major structural component of the protein shell (Ozawa & Young ,1987),VP2 mediates about 55% of the inhibition of the erythropoiesis due to its the lytic or direct cytopathic effect (Kishore & Kishore ,2018).

VP1 differs from VP2 only in 227 amino acids in the N-terminal extension called the unique region (VP1u) (Ning et al., 2023).VP1u helps in the internalization of the virus particles (Leisi et al., 2013).It also plays good role in virus tropism, uptake, and subcellular trafficking. The receptor-binding domain in VP1u is responsible for B19V specific targeting and uptake into cells of the erythroid lineage in the bone marrow (Ros et al., 2020).

1.6 P antigen the cellular receptor for B19V

The P blood group has 3 antigens, P_1, P and a rare P^k. P antigen was known as globoside. P_1 phenotype has both P and P_1 antigens while P_2 phenotype has P antigen alone. $P_1{}^k$ have both P_1 and P^k antigens, therefore $P_1{}^k$ and p phenotypes have no P antigen on their erythrocytes (Marcus,1981). Blood-group p is at risk of huge hemolysis if they are transfused with blood containing P antigen (Sanger,1955).

P antigen is a cellular receptor that is present on erythrocytes, erythroblasts megakaryocytes, endothelial cells, placenta, fetal liver, and heart cells (Von dem Borne et al., 1986) (Rouger et al., 1987). In a lab experiment that was done B19V replicated in bone marrow, peripheral blood, fetal liver, and a few hematopoietic cell lines that have erythroid characteristics (Brown et al., 1993), B19V was not proven to have replicated in megakaryocytes or myocardial cells (Brown et al., 1994), but the entry of B19V into these cells has been indicated from in vitro megakaryocytopoiesis viral inhibition and fetal heart tissue in situ hybridization (Srivastava et al.,1990) (Morey et al,1992).

B19V replicates only in EPCs. The P antigen was proven to be the receptor for the virus by hemagglutination since the RBCs that lacked P (P1 k or p phenotype) did not agglutinate with B19V (Brown et al., 1993) thus they are naturally resistant to it (Brown et al., 1994). Therefore, erythrocyte P antigen also known with glycosphingolipid globoside (Gb4) is the cellular receptor for B19V (Kolmos H. J. ,1994) (Bieri & Ros ,2019). B19 viral proteins

were found in bone marrow cells that also expressed P antigen, that also proved the role of P antigen as the cellular receptor (Kerr et al., 1995).

When B19V attaches to the antigen this leads to receptor-induced structural changes and cell death is triggered by lysis or by apoptosis mediated by the nonstructural NS1 protein (Rogo et al.,2014).

1.7 The requirement of erythropoietin for B19V replication

Erythropoietin (Epo) regulates erythropoiesis, it binds its receptor (EpoR) that is present on EPCs (Lappin et al.,2021). EpoR signaling is required for B19V replication in ex vivo-generated EPCs after initial virus entry and can play a role in the remarkable tropism of B19V for human erythroid progenitors (Chen et al., 2010). When these cells as exposed to hypoxic conditions it closely mimics native infection of erythroid progenitors in human bone marrow and is regulated by the STAT5A and MEK/ERK pathways (Chen et al., 2011).

1.8 Pathogenesis of B19V

B19V binds to host cell receptors in the respiratory tract and enters the cell, then translocation of the genome into the host nucleus occurs, later the cells lyse and the mature virions are released resulting in viremia causing the patient to experience early symptoms in addition to reticulocytopenia for 7 to 10 days (Macri & Crane, 2022).

This occurs because parvoviruses have uncommon requirements for replication, such as rapidly dividing cells since the small amount of DNA contained in the virus does not carry sufficient genetic information to guide its own replication in host cells. The virus replicates in committed erythroid precursor cells in the bone marrow, leading to erythroid aplasia (Pattison & Patou,1996). The high viral load may develop bone marrow suppression in high-risk patients promoting a serious decrease in hemoglobin (Hb) values and probable aplastic crisis (Slavov et al.,2011).

B19V uses the cellular DNA replication machinery for its DNA replication (Zou et al., 2018), the virions then accumulate in the cytoplasm and get out of the cell as membrane-coated vesicles. These properties may play a role in viral escape from the immune responses of the host and in membrane fusion-mediated transmission. B19 viral particles are envisioned to be used as nanoparticles in drug delivery systems (Ishida et al., 2023).

Moreover, modern investigations documented that B19V may infect nonerythroid lineage cells like myocardial endothelial cells (Luo & Qiu, 2015) and that 21.3 % of B19V-infected patients with juvenile chronic arthropathy, recurrent abortions, multi-transfused thalassemia, and leukemia. A study showed that some conditions of B19V infection were fatal, and the patients had pure red cell aplasia, fulminant hepatitis, and haemophagocytic syndrome (Kishore & Kishore,2018).

1.9 Clinical Manifestations of B19V infection

In 1985, an interesting experiment was conducted on healthy B19V seronegative adult volunteers and confirmed the role of B19V , the study was done by intranasally inoculating the volunteers with B19V, after severe viremia developed prodromal symptoms (fever, malaise, myalgia, and itchy skin) were noticed in the first week in addition to viral transmission through the respiratory tract, in the following week complete blood count (CBC) showed a slight decrease in Hb concentration, reticulocytopenia, lymphopenia, neutropenia, and a decrease in platelet count. At seventeen to eighteen days post-inoculation, rash and arthralgia were noticed in three out of four infected volunteers, this second phase of illness lasted three to four days (Anderson et al.,1985).

Then many years later, Heegaard, E. D., & Brown, K. E. (2002) assessed more B19V-related symptoms and categorized the symptoms that may appear according to the infected person's previous medical status:

- Infection in the healthy host who developed symptoms: erythema infectiosum, arthropathy, thrombocytopenia, transient erythroblastopenia of childhood (TEC), neutropenia, neurologic disease, myocarditis, hepatitis, and hydrops fetalis in pregnant women.
- Infection in the immunodeficient host: chronic pure red cell aplasia, virus-associated hemophagocytic syndrome (VAHS).
- Infection in patients with increased red cell turnover: transient aplastic crisis.

1.10 B19V and the development of slapped cheek syndrome

Slapped cheek syndrome (Erythema infectiosum) begins with the early symptoms: headache, myalgias, and fever then after 1/1.5 weeks later rash may develop especially facial erythema (slapped cheek appearance) (Macri & Crane, 2022).

1.11 B19V and the development of transient aplastic crisis

Transient aplastic crisis is a temporary bone marrow erythroid aplasia. These patients experience acute symptoms of severe anemia with very low Hb levels accompanied with reticulocytopenia and sometimes leukopenia in addition to thrombocytopenia. A complete absence of erythroid precursors is seen when bone marrow is examined. The anemia is self-limiting but needs to be supported by transfusion until bone marrow is recovered. Later, reticulocytes are reproduced and reappear in the peripheral blood, and Hb concentrations get back to a stable state (Pattison & Patou,1996), scientists in Northwestern Tanzania observed that B19V decreases Hb level by 1.1 g/dl among children <5 years of age with anemia (Tizeba et al., 2018).

Patients with pre-existing conditions related to red cells are more susceptible to aplastic crisis if they get infected with B19V, conditions such as decreased red cell production (iron deficiency and thalassemia's), or increased red cell destruction (sickle cell anemia, hereditary spherocytosis, pyruvate kinase deficiency, and glucose-6-phosphate dehydrogenase deficiency), in addition to patients with HIV infection and recipients of allogeneic hematopoietic stem cell or solid organ transplants (Macri & Crane, 2022) .

Transient aplastic crisis and pure red cell aplasia may occur respectively in the acute and chronic B19V infection phase (Feng et al., 2020) but cytopenia are more frequent in immunocompromised patients (Jacquot et al., 2022).

1.12 B19V Diagnosis

Diagnosis is primarily done by the detection of specific antibodies by enzyme-linked immunosorbent assay (ELISA) or detection of viral DNA by dot blot hybridization or PCR (Heegaard & Brown,2002)

However, B19V diagnosis can be challenging, for instance, during epidemics in Brazil many clinical conditions were noticed such as erythema infectiosum, arthropathy, transient aplastic crisis, nonimmune hydrops fetalis and B19V related hepatitis, but since there are many

causes of rash and there is a wide circulation of arboviruses that make similar symptoms to erythema infectiosum that led to a more difficult diagnosis (Cnc Garcia et al., 2021).

More investigations are being made about B19V diagnosis, in Nigeria for instance, it was reported that anemic preschool children had a higher prevalence rate of anti-B19V IgM antibodies than non-anemic, and it was recommended to screen for B19V during differential diagnosis of anemia in this age group to exclude other anemia-causing factors (Ashaka et al., 2018).

1.13 Treatment of B19V

There is no specific guideline for chronic parvovirus infection treatment (Gor et al.,2022), however, it was found that intravenous immunoglobulin (IVIG) is a treatment that is used in B19V-induced pure red cell aplasia in an effective way (Heegaard &Brown, 2002), the infused antibodies contain big amounts of anti-HPV-B19 IgG and can neutralize the virus (Gor et al.,2022).

1.14 B19V Coinfections

Many case reports affirmed the mixed infection of B19V and other viruses such as:

1.14.1 B19 and EBV:

- A healthy adult with acute B19 infection and concomitant EBV reactivation had hemophagocytic lymphohistiocytosis (HLH) but surprisingly had spontaneous resolution of it (Larroche et al.,2002). HLH is a syndrome of pathologic immune activation where uncontrolled proliferation of activated lymphocytes and histiocytes happens, thus excessive levels of cytokines (Kim et al.,2022).
- A 19-year-old patient known with Crohn's disease who was on immunosuppressors had mixed B19 infection and EBV since immunosuppression can lead to opportunistic infections (Moreira et al., 2010)
- In 2012**,** a child with hereditary spherocytosis had mixed infection of B19V and EBV developed transient pancytopenia (Cefalo et al., 2012).
- B19V and EBV in an infant lead to a missed deadly case of HLH and it was concluded that early testing of hyperferritinaemia could have saved his life (Kishore & Kishore, 2014).

- A 27 year old male patient had EBV infection and probable B19V reactivation was noticed led to massive splenomegaly, fulminant hepatitis and some symptoms of HLH (Karrasch et al., 2014).
- 20 years old male with EBV and B19V coinfection had typical clinical presentation of Gianotti-Crosti syndrome cutaneous eruption (Stojkovic-Filipovic et al.,2016).

1.14.2 B19V and CMV:

An adult woman had CMV and B19V infection led to splenic infarction, high fever, systemic joint pain, and abnormal liver function but the splenic infarction improved after 7 weeks (Harada et al.,2018).

1.14.3 B19V and Influenza H1N1:

Fulminant myocarditis and pneumonia were seen in a 5-year-old girl who was infected with both B19V and influenza A/H1N1. Viral interactions may result in influenza A/H1N1 viral induced cytokine storm that might be fatal (Callon et al.,2021).

1.14.4 B19V and Covid 19:

Adult patient was coinfected with B19V and SARS-CoV-2 developed bilateral keratitis, anterior uveitis, and ear symptoms (Saturno et al.,2022)

1.14.5 B19V and Hepatitis A & E:

In a child with fatal fulminant hepatic failure had B19V related thrombocytopenia and anemia and was coinfected with hepatitis A and E viruses (Kishore & Sen ,2009)

1.14.6 B19V and HHV-6:

Viral co-infections were in 12% of acute myocarditis in young adults, especially B19V with human herpes virus 6 (HHV-6) (Andréoletti et al.,2009). HHV-6 role in immunosuppression facilitates the dissemination of B19V which led to fatal myocarditis (Rohayem et al.,2001). B19V-coinfection with HHV6 resulted in higher B19V-loads in comparison with B19V-monoinfected endomyocardial biopsy thus it was concluded that HHV6 had transactivated B19V (Bock et al., 2014)

1.14.7 B19V and Syphilis:

Syphilis and parvovirus B19 developed symptoms that mimic nephrotic syndrome biologically and histologically with positive antinuclear antibodies (ANA) and it was recommended to be recognized as a cause of pseudo-lupus nephritis (Jaunin et al.,2019).

1.14.8 B19V and Chlamydia:

Chlamydia pneumoniae and B19V coinfection were found in fulminant myocarditis (Poelzl et al., 2006)

1.14.9 B19V and Measles:

B19V and measles virus co-infections intrafamilial outbreak noted the diagnostic challenges of dual rash-associated infections (Grolhier et al.,2020). It was suggested that B19V, HHV-6, and human herpesvirus 7 should be considered by physicians for the diagnosis of measles-suspected patients (Cho et al., 2023).

1.14.10 B19V and Dengue:

An outbreak of B19V was thought as dengue fever (Di Paola et al.,2019)

1.14.11 B19V and Malaria:

Multiple studies focused on *Plasmodium falciparum* malaria and B19V coinfection (Agarwal et al.,2017) which may be a cause of severe anemia (Gupta & Singh ,2005). Malaria coinfection with B19V may be related to microcytic anemia rather than normocytic normochromic anemia as seen in cases of B19V infection among persons with red cell abnormalities (Duedu et al.,2013).

1.14.12 Multiple viruses:

B19V, CMV, polyomavirus BK and adenovirus DNA sequences were found in adrenal adenoma (Pomara et al., 2006).

1.15 Recombinant B19V vectors

The remarkable tissue-tropism of B19V is being researched for a while to target human hematopoietic cells of the erythroid lineage using hybrid vectors as a possible way for gene therapy and for other medical purposes.

In 1998, recombinant parvovirus B19V vectors on erythroid lineage and non-erythroid lineages were studied since P antigen is expressed in nonerythroid and it was found that there was low-level transduction of nonerythroid cells (Ponnazhagan et al.,1998).

In 1999, B19Vp6 promoter was thought of in recombination, since it is responsible for B19V gene expression and is erythroid cell-specific, it was concluded and suggested that nonpathogenic adeno-associated virus type 2 hybrid vectors may be useful in gene therapy of human hemoglobinopathies especially in sickle cell anemia and beta-thalassemia (Kurpad et al., 1999)

In 2001, the same researchers who found the low transduction in non-erythroid lineages decided to do more research to reevaluate the role of P antigen in viral binding and entry into cells, they concluded that:

a) ∼75% of primary human bone marrow mononuclear erythroid cells and ∼31% of cells in the nonerythroid population were positive for P antigen.
b) P antigen is necessary in binding of B19V to human cells but not sufficient for successful transduction to human hematopoietic cells
c) Viral binding efficiency is not correlated with the amount of P antigen expression.
d) There is a putative undiscovered cellular coreceptor for efficient entry of B19V into human cells (Weigel-Kelley et al.,2001).

Another study in 2002 also affirmed the good transduction of the primary human hematopoietic cells in the erythroid lineage and the low-level of transgene expression in non-erythroid cells by encapsidating B19V with adeno-associated virus type 2 hybrid vectors (Weigel-Kelley et al.,2002).

B19V replication is limited to human EPCs and in some of the erythropoietin-dependent human megakaryoblastoid and erythroid leukemic cell lines, it was noted that B19V DNA replication failure in non-permissive 293 cells can be solved by adenovirus infection if it was needed for gene therapy (Guan et al., 2009)

In 2023, the putative mysterious coreceptor for B19V infection of EPCs was discovered and it is the tyrosine-protein kinase receptor UFO (AXL), this discovery can play an important role in making things clearer and improving the gene therapy research, in this study it was noted that:

a) VP1 (VP1u5-68aa) is the minimal receptor binding domain for B19V to enter EPCs.
b) Genome-wide CRISPR-Cas9 guide RNA screen was done and identified AXL as a proteinaceous receptor for B19V infection of EPCs.
c) AXL gene silencing in ex vivo EPCs expanded remarkably decreased B19V internalization and replication.
d) Polyclonal antibody against it upon infection efficiently inhibited B19V infection of ex vivo expanded EPCs (Ning et al., 2023).

1.16 B19V correlations

1.16.1 B19V and sickle cell anemia:

Sickle cell anemia (SCA) is the most common inherited disease and hemoglobinopathy worldwide caused by a single mutation substituting valine for glutamic acid at position 6 of the β-hemoglobin chain gene of adult hemoglobin A, resulting in hemoglobin S (HbS) (Mbayabo et al.,2022) (Niccoli Asabella et al., 2019).

HbS results in the sickling of the red blood cells under certain circumstances, over the time sickling may cause vaso-occlusive crisis thus acute and chronic abdominal pain, long bone pain due to bone marrow infarction, chronic hemolytic anemia, and splenic sequestration accompanied by fast spleen enlargement, delayed sexual maturation and cholelithiasis (Niccoli Asabella et al., 2019).

Researchers studied B19 viral DNA and antibodies in sickle cell anemic patients, they shared that anti-B19V IgG was in 37.6% of the patients while B19V IgM antibodies were in 2.89%. Anti-B19 IgM samples were positive for viral B19 DNA with symptoms of fever, malaise, and paleness, but no rash, it was suggested that B19V testing in these patients may result in better handling of aplastic attacks (Obeid O. E, 2011). Another study was done and reported a high prevalence of B19V in sickle cell disease patients (Obeid et al.,2019).

A recent literature review about B19V seroprevalence rates in SCA and β-thalassemia patients summed up that B19V infection differs under several conditions: different

epidemiological features, socioeconomic status, overcrowding, age, reinfection, genotypic diversity, the patient's immunological and hematological status, and more abundant blood transfusion (Soltani et al., 2020).

1.16.2 B19V and thalassemia:

Thalassemia are monogenic hematologic diseases that have two main large categories: α-thalassemia or β-thalassemia, they are classified according to the number (quantitative) and the type of adult globin chains of the Hb tetramer (α-hemoglobin chains or β-hemoglobin chains) that got mutated (Zakaria et al.,2022).

Many of the studies about B19V and thalassemia were case reports that were hard to diagnose and later on they were diagnosed with B19V:

- A 15-month-old boy with homozygous beta-thalassemia had an acute, transient aplastic crisis due to a B19V infection (Leblanc et al., 1985)
- A 22-year-old female thalassemia major patient had aplastic crisis due to transfusion-transmitted B19V. Anti-B19V IgM was detected in the acute phase and B19V DNA was detected by PCR and remained detectable up to 4 months after diagnosis. The donor was tested, and it was found that anti-B19V IgM titer and B19V DNA were high. One week after the patient suffered from aplastic anemia the patient had transitory heart failure and acute tricuspid incompetence (Zanella et al.,1995)
- A case of an asymptomatic thalassemia carrier proved that these patients may have serious complications when coupled with an infection, this was concluded when a 14-year-old girl with hemoglobin H (Hb H) had hemodynamic shock due to severe anemia when she got infected with B19V (Slomp et al., 2006).
- A patient with thalassemia minor trait was diagnosed with B19V induced acute aplastic crisis and acquired pure red cell aplasia (Singh et al.,2014).
- 35 years old thalassemic woman developed a serious problem after B19V infection (Bertrand et al.,2017)

1.16.3 B19V and hereditary spherocytosis:

Hereditary spherocytosis is the most common cause of hemolytic anemia where the erythrocytes are unable to maintain their normal biconcave shape due to genetic mutations in membrane/cytoskeletal proteins (spectrin, ankyrin, band 3, and band 4.2) which are

encoded by different genes and play a role in structural morphologic stability resulting in an abnormal red cell membrane with spherocytic shape (EA & CA, 2022)

B19V-infected patients with hereditary spherocytosis can develop virus-associated HLH together with aplastic crisis (Kim et al.,2022).

1.16.4 B19V and hematological malignancies:

Zaki (2012) reported that there is a high incidence of B19V infection among patients with different types of hematological malignancies and recommended testing all patients with hematological disorders for B19V DNA and antibodies.

A study concluded that children with hematological malignancies have a high incidence of B19V infection and long duration of DNA persistence since they found that all the patients in the study were positive for either B19V DNA or B19V antibodies (IgG or IgM) at least once over one year of follow up (Jain et al., 2018).

Moreover, scientists were interested in checking the presence of B19V in children diagnosed with acute leukemia and aplastic anemia, they concluded that this category of patients who use immunosuppressive drugs and take blood products during chemotherapy experience B19V infection in the follow-up period, so it was recommended to detect B19V in patients with acute leukemia and aplastic anemia thus better follow-up and therapy (Colak et al., 2019).

1.16.5 B19V and allogeneic hematopoietic stem-cell transplantation:

Researchers found that B19 viremia is common in pediatric patients receiving allogeneic hematopoietic stem-cell transplantation (allo-HSCT) but without clear clinical relatedness (Rahiala et al., 2013).

However, the clinical presentations associated with B19V disease in adult patients after allo-HSCT are more apparent and researchers concluded them in 3 clinical problems:

- An early rash which is the most common occurs ≤3 months after HSCT.
- Cytopenia may occur later, after HSCT (>3 months).
- Severe organ failure can happen any time after HSCT and mostly it can be accompanied by cytopenia.

But they noted that:

- B19V diagnosis after HSCT should be after excluding other causes such as graft versus host disease, engraftment syndrome, or drug allergy.
- Patients with rash alone had low B19V viral load, compared to cytopenia or organ failure, thus the routine PCR of peripheral blood is an insufficient diagnostic tool.
- Histological examination of the skin and PCR analysis of skin specimens should be performed for suspected cases, especially patients who have also arthralgia or myalgia. (Katoh et al., 2020)

1.16.6 The effect of B19V on the kidneys:

B19V infection was studied in renal transplant donors and recipients to see the frequency of active viral infection in these patients and its role in the development of anemia. It was noticed that only the anemic recipients had active B19V infection thus it could be associated with the development of severe anemia after renal transplantation and it was advised to screen these patients for B19V in plasma (Čapenko et al., 2012).

B19V was also curiously checked in hemodialysis patients, its frequency was significantly high and was associated with lower hematological parameters, thus pointing that it can play an important role in the development of anemia and/or pancytopenia in end-stage renal disease, which can be due to immunosuppression, insufficient erythropoietin, or short lifespan of red blood cells (Mohammad et al., 2022).

1.16.7 The effect of B19V on the skin:

The differential diagnosis of B19V is difficult due to the similarity of the symptoms of erythema infectiosum to other rash diseases and the broad circulation of arboviruses that may develop similar symptoms (Cnc Garcia & Leon ,2021). Skin rash in adult patients was found to be related to B19V and coinfection with: Zika virus (ZIKV), EBV&CMV, ZIKV&EBV, CMV&EBV (Gonçalves Maciel et al.,2022).

1.16.8 The effect of B19V on the heart:

B19V was the most frequently detected cardiotropic virus as mono-infection in adults with clinically suspected myocarditis (Hassan et al ,2022), the phenotypes that were related to B19V in myocarditis and dilated cardiomyopathy were genotype 1 (57.4%) and genotype 2 (36.7%) (Bock et al.,2014). B19V infection can be deadly, a liver transplant recipient female

adult had B19V infection induced myocarditis, the viral genome was detected in the myocardium, and she died from refractory cardiogenic shock (Jonetzko et al., 2005).

1.16.9 The effect of B19V on the liver:

A study was done to check the correlations between infantile hepatitis and B19V in patients who had liver dysfunction of unknown cause, out of 27 patients with ages from 1 month - 2 years B19 viral DNA was present in three babies (11.5%) (Shibata et al., 2005).

A significant correlation between co-infection with hepatitis B virus and B19V, in which there was an increased likelihood to a have more severe hepatitis B-associated liver disease (Toan et al.,2006).

1.16.10 The effect of B19V on the nervous system:

B19V genome may remain in the central nervous system after primary exposure establishing a lifelong latency and it can be involved in the demyelination process (Skuja et al., 2018).

1.16.11 B19V role in autoimmunity:

Scientists assessed the role of B19V in autoimmunity, they summarized that:

- There are many arguments about the role of B19V in autoimmunity; however, there is no clear scientific evidence to support or oppose its straight role.
- B19V can lead to a wide range of autoantibody production, including anti-soluble nuclear antibodies, anti-phospholipid antibodies, anti-native DNA antibodies, anti-lymphocyte antibodies, anti-cardiolipin antibodies, anti-nuclear antibodies, and rheumatoid factor.
- Acute B19V infection can imitate or promote systemic autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Page et al.,2015).

Other researchers also investigated this relationship, and they affixed it in that:

- B19 viral infection plays a big role in rheumatoid arthritis, systemic lupus erythematosus, myasthenia gravis, hashimoto thyroiditis, and other autoimmune diseases.
- The anti-B19V-VP-IgG antibody can increase the activity of cytokines such as interleukin 1, tumor necrosis factor α, and substrate metalloproteinase 9.

- B19 protein NS1 and VP1 can induce interleukin -6 expression.
- B19 can activate T-helper 17 cell-associated cytokines, thus lower interferon-gamma levels and increase plasma interleukin-4 levels (Jia et al., 2020).

Until now there is no causal relationship between B19V and its triggering of autoimmune disease; however, the mechanisms of molecular mimicry, induction of apoptosis and activation of enzymes have been noted thus the production of autoantibodies during infection is more understood (Jacquot et al., 2022).

1.16.12 The effect of B19V on pregnancy:

Ergaz and Ornoy (2006) studied the effect of B19V on pregnancy, they came to know that:

- B19V may affect 1-5% of pregnant women, mostly with normal pregnancy.
- Increased risk on the fetus is seen mainly if the mother is infected during the first two trimesters of pregnancy and it can also happen during the third trimester.
- Due to the shortened half-life of erythrocytes of the fetus, it can cause severe fetal anemia since it infects the fetal EPCs, thus high output cardiac failure resulting in nonimmune hydrops fetalis.
- The P antigen present on fetal cardiac myocytes gives B19V the ability to infect myocardial cells and can result in myocarditis that leads to cardiac failure.
- It is a significant cause of fetal loss throughout pregnancy.

A serologic survey that was done on Iranian women also supports its possible role in abortion since there was a higher frequency of abortion in anti-B19 IgG seropositive pregnant women than seronegative (Khameneh et al., 2014).

Although its potential responsibility for fetal loss, routine screening for parvovirus immunity in low-risk pregnancies is not recommended. However, the pregnant woman who is exposed to B19V or who develop symptoms of B19V should be tested for B19V IgG and IgM to determine if she is immune or not and her susceptibility to the infection. An obstetrician or a maternal-fetal medicine specialist should be considered due to the risks on the fetus, serial ultrasounds should be performed every 1 to 2 weeks, up to 12 weeks after infection (Crane et al., 2014).

Another large-scale seroprevalence research was done in Croatia and supports the previous recommendation but contradicts somehow the study that was done in Iran, as it concluded

that there is no significant difference in seroprevalence between women with an unfavorable obstetric history compared with a normal pregnancy (Vilibic-Cavlek et al., 2021).

1.16.13 Testing blood donors for B19V:

There is a debate about B19V testing of blood donors, as B19V transmission is important to be observed for patients with immune deficiency or hemolytic anemia. Researchers in Turkey used ELISA to test 988 blood donors to check B19V IgM and IgG, they found that IgM positivity was 3.92% and IgG positivity was 58.9% in the blood samples (Göral et al., 2018).

In Korea, plasmapheresis donors were tested for B19V DNA prevalence and it was not high, yet most donors had neutralizing anti-B19V antibodies, accordingly, they did not suggest adding B19V testing to the test panel that is usually done for the Korean donors (Oh et al.,2010).

Chapter 2

Problem statement, Goal of the study and Objectives

2.1 Problem statement

B19V infects mostly young children, it replicates in erythroid precursor cells in the bone marrow and may cause slapped cheek syndrome and sometimes transient aplastic crisis especially in patients who have previous underlying disease. There is no background information about B19V in Palestine either on the epidemiology, genotypes present, or the most common symptoms. Thus, it was decided to conduct this research to study B19V in Palestine.

2.2 Goal of the study

To examine the prevalence and the most common genotypes of B19V for the first time in Palestine, as well as the impact of B19 viral infection on CBC parameters and check the most typical symptoms in B19V positive patients.

2.3 Objectives

- To study the B19V RT-PCR positive samples in Palestine from 2014-2022.
- To compare B19V infected children (cases) and non-infected patients (controls) to check if B19V has any potential associations.
- To determine whether the patients had underlying illness before being infected with B19V such as hematological diseases, heart problems and others.
- To check if any of the patients developed transient aplastic anemia or pure red cell aplasia.
- To genotype all the B19V RT-PCR positive samples by Sanger sequencing.

Chapter 3

Material and Methods

3.1 Study location

The study was conducted at Caritas baby hospital CBH, located in the west bank in Bethlehem southern of Palestine. CBH in general focuses on quality, patient safety, pediatric care requirements and providing the best medical care for the patients. It has one of the best medical laboratories in Palestine it offers many diagnostic tests of infectious diseases for CBH patients, external institutions, and hospitals. The laboratory also contributed to the stressful pandemic period since it was the first lab to test for Covid 19 virus. Moreover, CBH focuses on scientific research, and it is a member in the national antimicrobial stewardship and immunization programs in Palestine.

Further molecular work and sequencing were conducted at Augusta Victoria Hospital (AVH) in Jerusalem which has one of the most advanced services and laboratory in Palestine.

3.2 Study population

Any suspected patient to be infected with B19V was considered in the study, ages from newborns -18 years and a minority were adults from other hospitals. Patient with previous underlying disease before being suspected with B19V were considered in the study.

3.3 Study period

A retrospective case control study was conducted on B19V blood samples from 2014-2022, and during the 9 years a total of 905 patients were suspected to have the virus of which 28 samples were positive.

3.4 Sample collection

The laboratory received 1 ml of blood in EDTA tube from each parvovirus suspected patient, the samples were mainly withdrawn in CBH, and few were sent out samples from other hospitals. Each sample was labeled with the patient's hospital number, full name, sample number and date of collection. The samples were kept in fridge at 4^{o}C until DNA extraction was done then positive B19V samples were frozen for long-term storage in -70C CBH laboratory freezers.

3.5 Ethical approval

The study was approved by the Al-Quds university research committee in addition to the medical research ethics committee of the CBH (Approval number: MRC-48) (Appendix 10).

3.6 Financial fund

Financial support was funded by Caritas baby hospital.

3.7 Patient data collection

3.7.1 Patient privacy:

Patient names were not used instead each patient was coded with a specific code and the patient data was stored in the CBH laboratory and sequencing results in AVH laboratory.

3.7.2 Data collection:

An excel sheet was filled with the patient data from the system of the health information system of CBH for 28 B19V positive patients in addition to 32 patients as a negative control in which patient personal data, CBC results, and clinical symptoms of the patients were filled to study each patient in details.

3.7.3 Control selection criteria:

The number of the total control samples were equals 877 patients, from which 32 patients were chosen to be studied since 877/28 equals about 32, approximately every 27 patients were selected at random from the negative excel sheet of patients since 879/32 = 27.

3.7.4 Statistics:

To provide comprehensive insights and draw meaningful conclusions, the data was statistically analyzed using IBM SPSS v27 software and p-value threshold of less than 0.05 was considered statistically significant. The statistical tests used were: The Chi-Square test and Fisher's Exact Test were used to examine the association between categorical variables. The Independent Sample T-Test and Mann-Whitney U test were employed to compare differences between two independent groups in continuous data, depending on the distributional assumptions of the data. Lastly, binary logistic regression analysis was performed to explore the relationship between a binary dependent variable and a set of independent variables.

3.8 Molecular work

3.8.1 Prevention of contamination:

The laboratory work was done with high care to prevent any possible contamination, the work was done in the clean room which has a UV sterilizing hood, the benches were cleaned with 0.5% Clorox and protective personal equipment were used such as gloves and disposable gowns.

3.8.2 B19V detection:

3.8.2.1 DNA extraction:

DNA extraction was done using blood from EDTA tube using Qiagen QIAamp DNA mini kit (Roche Applied Science, Germany) according to the manufacturer's instructions, in which 200 microlitres (μL) of blood were incubated with binding buffer and proteinase K, then the sample was purified by adding absolute ethanol and transferring it to a silica-based filter column to specifically separate the DNA from other components and then they were washed with alcohol and lastly the DNA was eluted from the filter with 30 μL of nuclease free water (NF-H2O) to release the DNA and the ready samples were stored at 20℃ .

3.8.2.2 Real-time polymerase chain reaction (RT-PCR):

Quantitative RT-PCR was done to detect the presence of B19V using Applied Biosystems 7500 Real-Time PCR system which generates an amplification plot that illustrates the amount of fluorescence detected against the PCR cycle number, the increase in the fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated. By comparing the fluorescence signal to a standard curve generated from known concentrations of DNA the starting quantity of the target DNA in the sample was known.

The cycle threshold (Ct) was registered in this process, since it expresses the PCR cycle number at which the fluorescent signal of the reaction crosses a predefined threshold in which when the amplicons are present in low quantities a higher Ct value is present while when the amplicons are present in high quantities a lower Ct value is seen.

The PCR mix was prepared with 25ml of TaqMan Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan), 5ml of each primer (10 pmol/ml),5ml of template DNA and

0.5ml of TaqMan probe, denaturation was done for 10 min at 95℃, 50 cycles were done and combined primer annealing/extension was performed at 60℃ for 1 min (Table 3.1).

Note: TaqMan probes (hydrolysis probes) are specific, single-stranded DNA probes that contain a fluorescent dye and a quencher molecule. The DNA polymerase cleaves the TaqMan probe that binds to the target DNA sequence during the DNA extension releasing the fluorescent dye from the quencher, leading to an increase in fluorescence signal.

Table 3.1: RT-PCR primers

RT-PCR primers	**Primer sequence**
B19-F	AAGCCGTGTGCACCCATT
B19-R	GTACTGGTGGGCGTTTAGTTACG
B19-fluorogenic probe	FAM-TAAACACTCCCCACCGTGC

3.8.2.3 Quality control of the RT-PCR:

Quality control was done for the test in each run, B19V positive sample was used as the positive control and NF-H2O as a negative control.

3.8.3 B19V sequencing:

3.8.3.1 Primers choosing for PCR and sequencing:

The primers were chosen for the three B19V genes by picking primer sets from numerous B19V genotyping studies using the search engine PubMed then the best primers were chosen. At first, the reference sequences of B19V of all genotypes were retrieved from GeneBank (NCBI) using nucleotide sequence bank and were aligned with all the primers by multiple sequence alignment using molecular biology software program Sequencher program 5.4.6 (Figure 3.1). Then, the optimal set of primers for each gene were selected based on the target gene and the primers with the lowest heterogenicity (Figure 3.2). The lyophilized dried ordered primers were used for PCR and genotyping (Appendix 3.1).

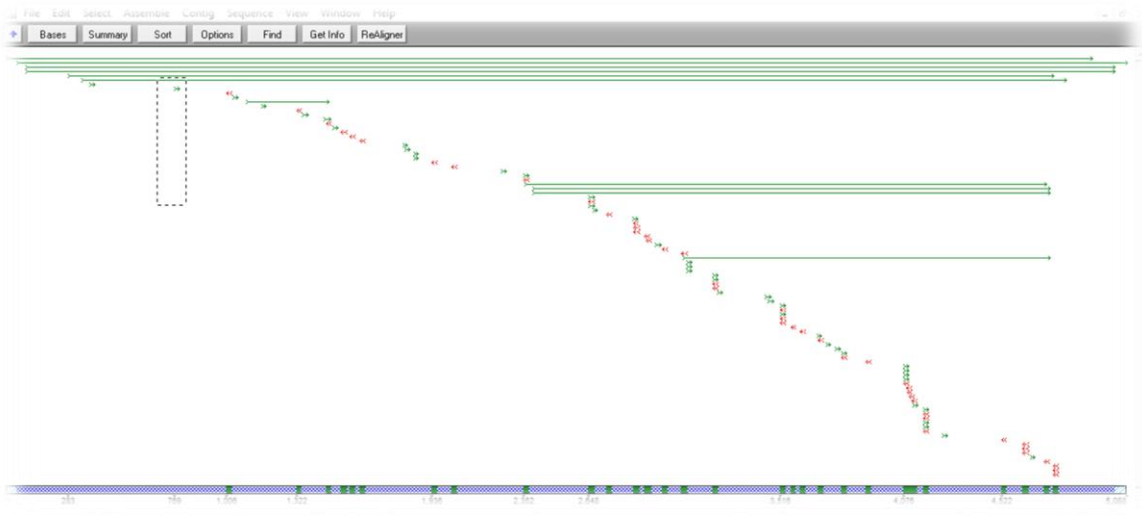

Figure 3.1: Multiple alignment of primers from many studies with B19V reference sequences

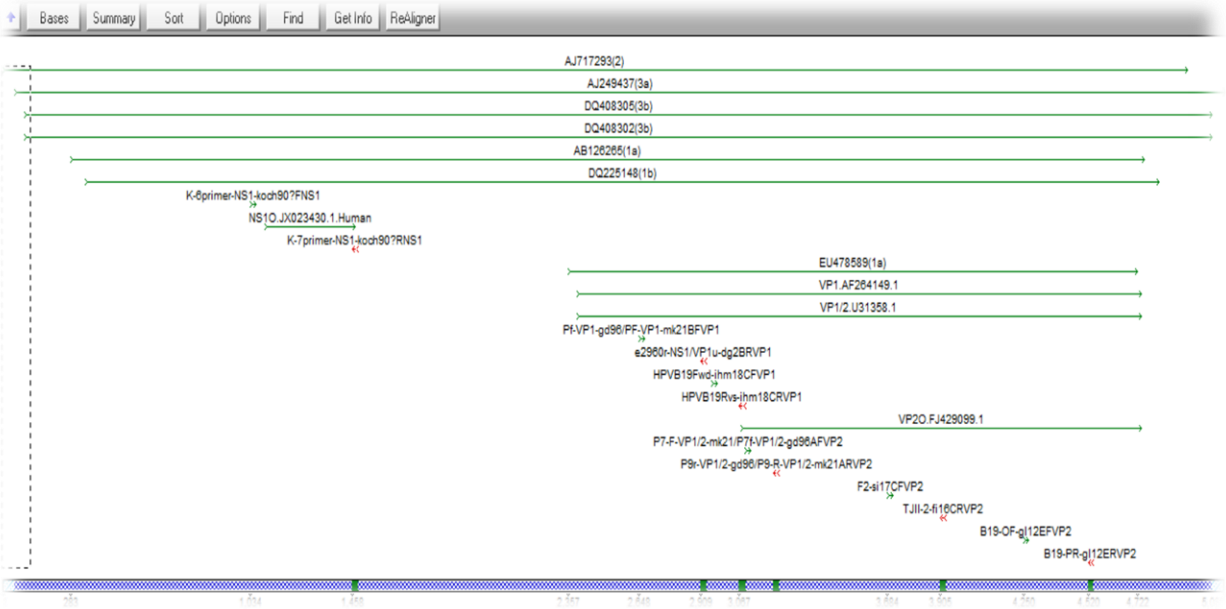

Figure 3.2: Chosen primers

3.8.3.2 Gradient polymerase chain reaction (PCR):

Gradient PCR with 8 temperatures was done with the specific primers for each of B19V genes as seen in tables 3.2, 3.3 and 3.4 , Bio-Rad PCR machine was used (Appendix 3.2) then gel electrophoresis was run (Appendix 3.3) in order to check the optimal temperature of the primer by choosing the best band especially the band that does not have nonspecific binding as seen in figures 3.3, 3.4 and 3.5 ,gradient PCR was done using a control sample that had a Ct that equals 4 which was diluted to Ct 28 which has about 100 copies per reaction and then the best primer annealing temperature was chosen for each gene and compared with the expected calculated melting temperature (Table 3.5) and the company's suggested temperature .

a) Gradient PCR of NS1:

Table 3.2: NS1 primers

	Sequence	Band size	Optimal temperature	Reference
Forward primer for NS1	AAACTATGGTAAACTGGTT	443 bp	45°C	(Koch & Adler, 1990)
Reverse primer for NS1	TGCTACATCATTAAATGGA	443 bp	45°C	(Koch & Adler, 1990)

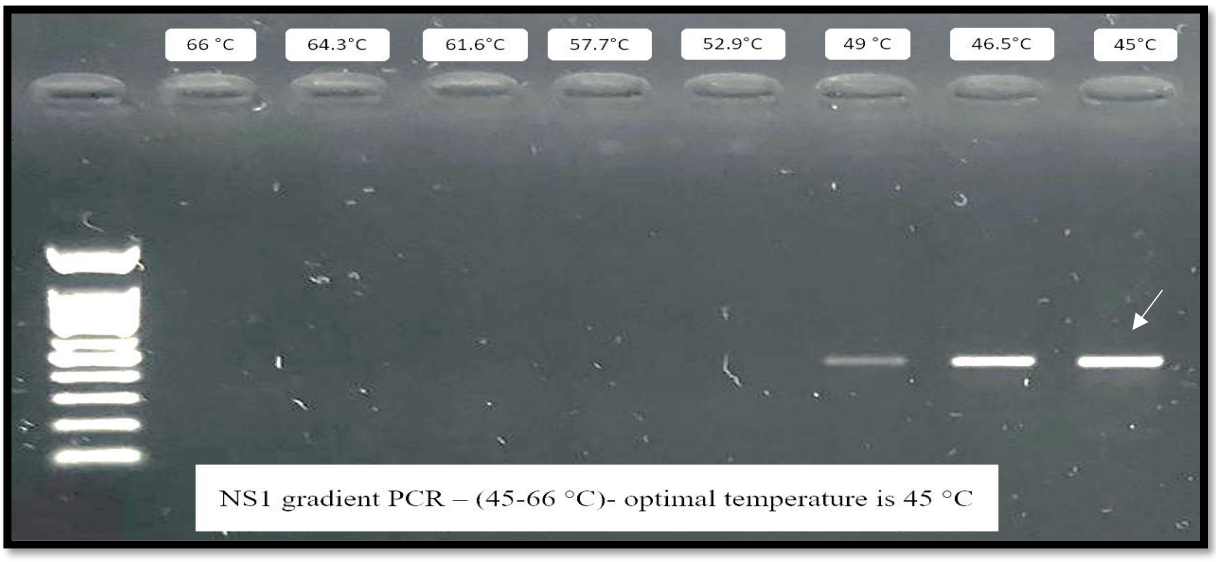

Figure 3.3: NS1 gradient PCR

b) Gradient PCR of VP1:

Table 3.3: VP1 primers

	Sequence	Band size	Optimal temperature	Reference
Forward primer for VP1	CAGTTATCTGACCACCCCCATGC	443 bp	45°C	(Erdman et al., 1996) (Seetha et al., 2021)
Reverse primer for VP1	GARTTAACTGAAGTCATGCTKGGG	443 bp	45°C	(Jain et al., 2018)

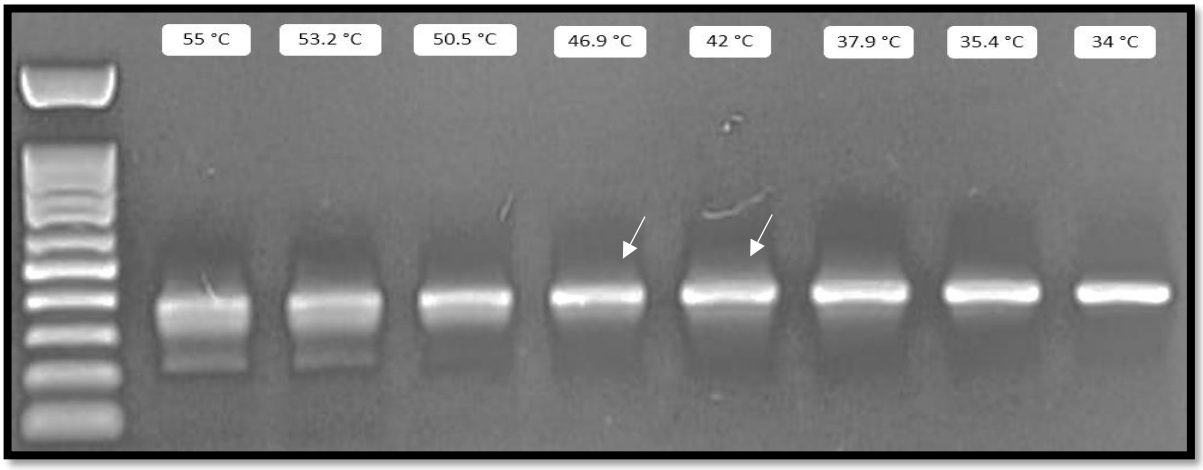

Figure 3.4 : VP1 gradient PCR

c) Gradient PCR of VP2:

Table 3.4: VP2 primers

	Sequence	Band size	Optimal temperature	Reference
Forward primer for VP2	TAGCAAGTGAAGAATCAGC	739 bp	50°C	(Zadsar et al., 2018)
Reverse primer for VP2	ACTGTCATAATTCCCAC	739 bp	50°C	(Molenaar-de Backer et al., 2012)

VP2 Primer melting temperature (TM) calculation:

Tm = [4(G + C) + 2(A + T)] °C

Table 3.5: VP2 primers melting temperature

	Sequence	G+C	A+T	Tm
VP2F2	TAGCAAGTGAAGAATCAGC	32	22	54°C
VP2R3	TACTGTCATAATTCCCAC	28	22	50°C

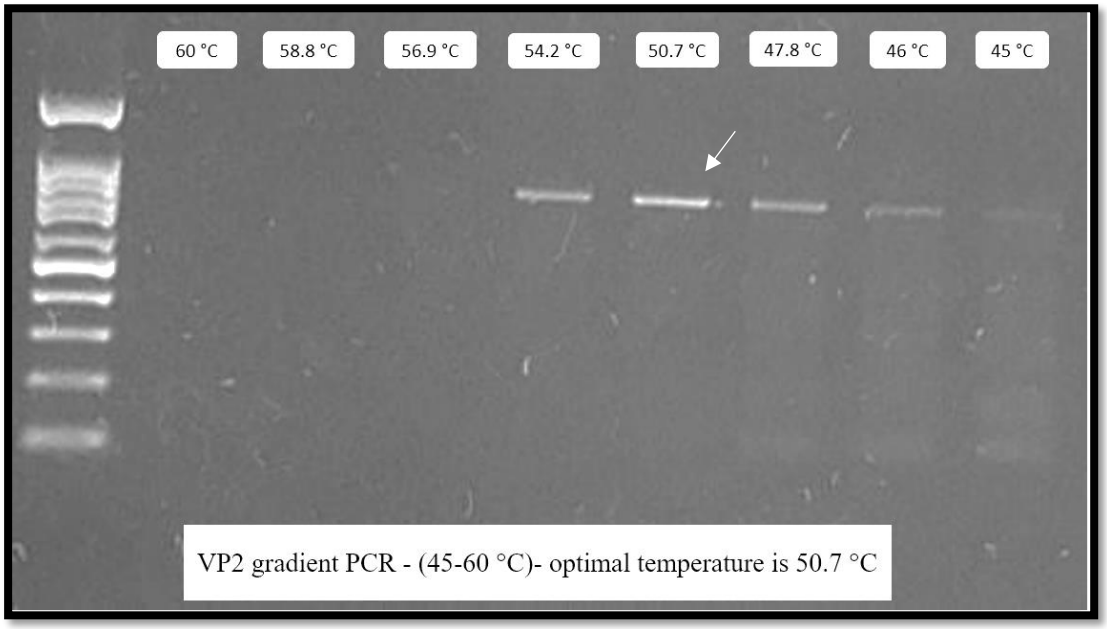

Figure 3.5: VP2 gradient PCR

3.8.3.3 Amplification of all genes:

2 PCR methods were used in the study:

1) GoTaq® Green Master Mix PCR kit (Promega, USA):

After the gradient PCR was done using GoTaq® green master PCR kit, PCR was done only for 2 samples which have the lowest Ct and were run for all genes as a trial: VP2 PCR (50°C), NS1&VP1 PCR (45°C); however, the best bands were seen in the VP2 run (Figure 3.6); thus, it was chosen as the selected gene to use for sequencing since it also has a large 1665 bp area, of which only 739 VP2 bases were sequenced, and most importantly, the 739 bp area covers all the B19V genotypes.

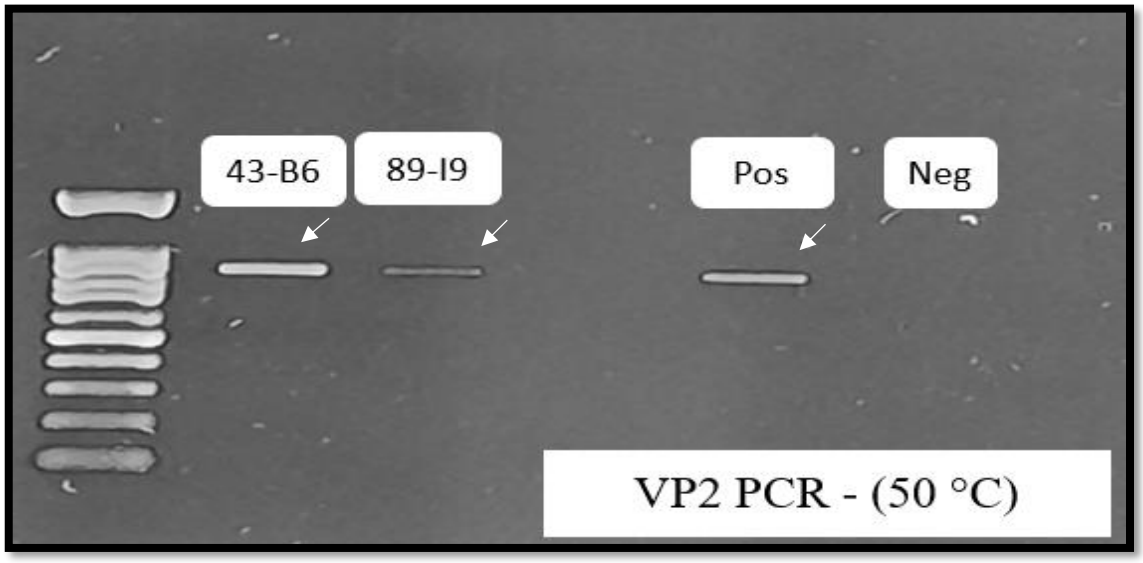

Figure 3.6: Trial of 2 samples with the lowest Ct values

But when the remaining samples were run, only few samples resulted in clear bands (Figure 3.7) (Appendix 3.2), so in order to solve the issue, some adjustments were made. For example, the annealing temperature was changed from 50°C to 54°C but this did not produce any results.Thus, the number of cycles was changed from 32 to 40 cycles and the primer concentration was changed from 1:10 to 1:5 which led to the development of the bands but with nonspecific binding (Figure 3.8) and to solve the nonspecific binding double well method was decided to be done.

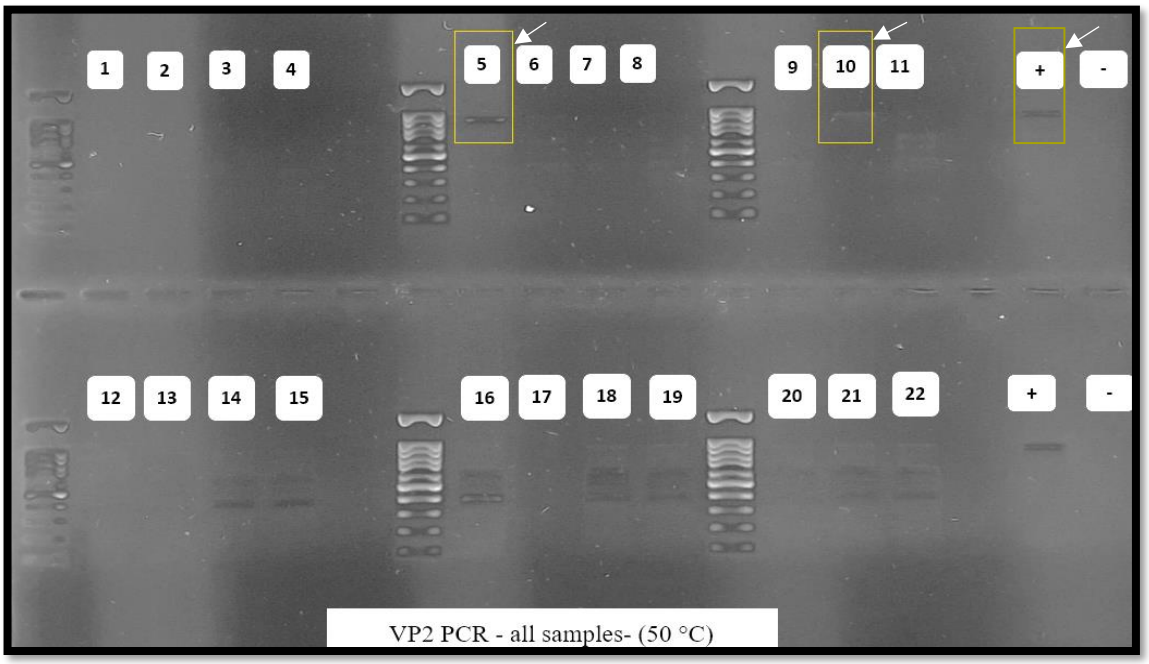

Figure 3.7: Green mix VP2 PCR for the rest of the samples

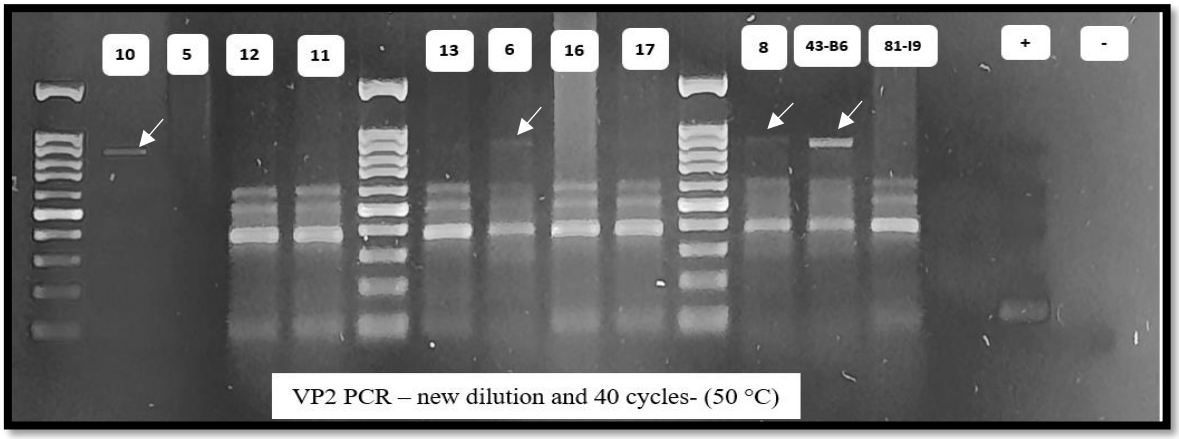

Figure 3.8: Problem solving by changing the dilution and number of cycles

Double well method:

It was done by pipetting 20 µL green mix of the samples that gave a band at 739 bp inside a double well thick 1.1 g gel that was prepared using a double well comb and then gel electrophoresis was run (Figure 3.9). The 739 bp band of each sample was cut and stored in a labeled eppendorf tube in the fridge in order to be purified (Figure 3.10) (Appendix 3.4).

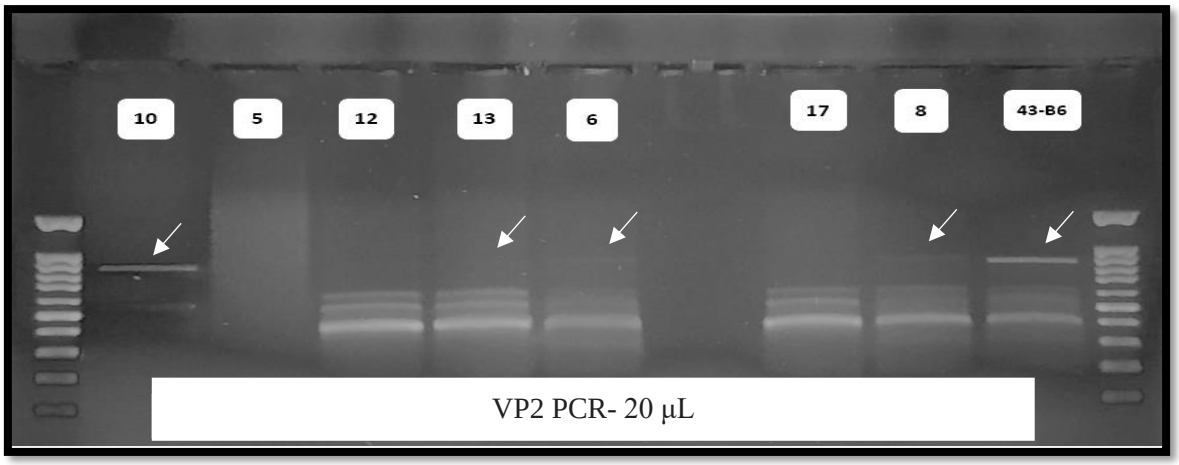

Figure 3.9: Double well gel electrophoresis

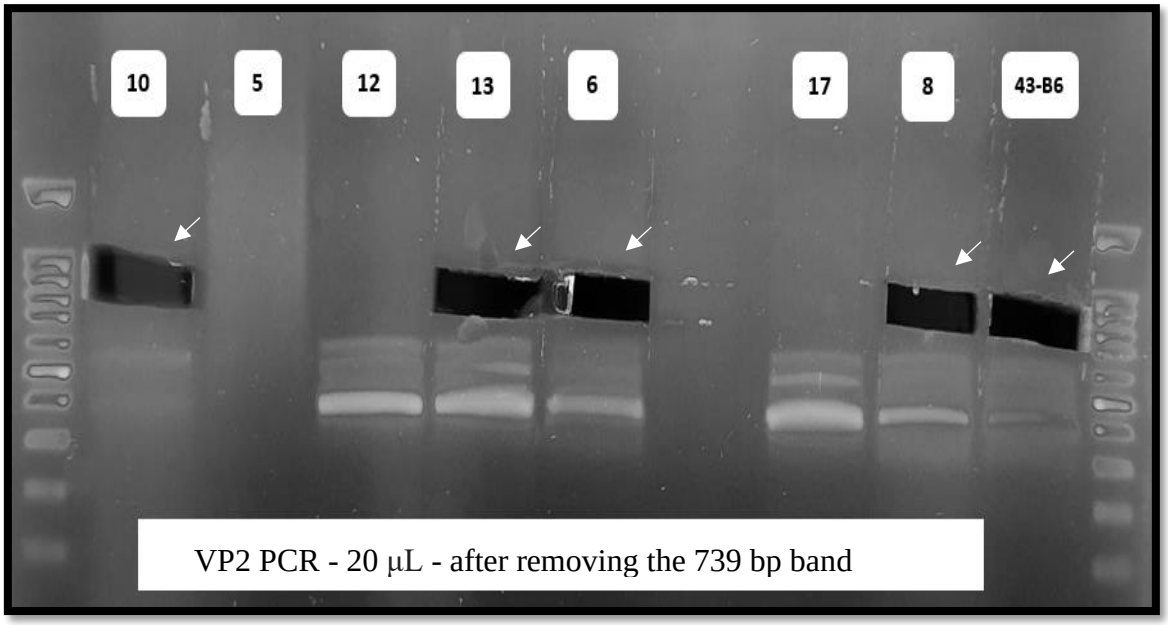

Figure 3.10: Double well 739 bp band cut

2) 5® High-Fidelity DNA Polymerase PCR kit (BioLabs, New England):

Q5® kit was used since it is more sensitive and accurate than the "GoTaq® green master PCR mix", it was used to reamplify the gel purified samples since their DNA content was low which was checked using NanoDrop. Reamplification of the DNA was done on ABI PCR machine then gel electrophoresis was performed (Appendix 3.5); however, non-specific binding showed up again, therefore it was decided to use the double-well method another time to eliminate it and increase DNA yield (Figure 3.11).

Note:

Quality control was done for each PCR run in which a strong B19V sample was used as a positive control and NF-H2O as a negative control.

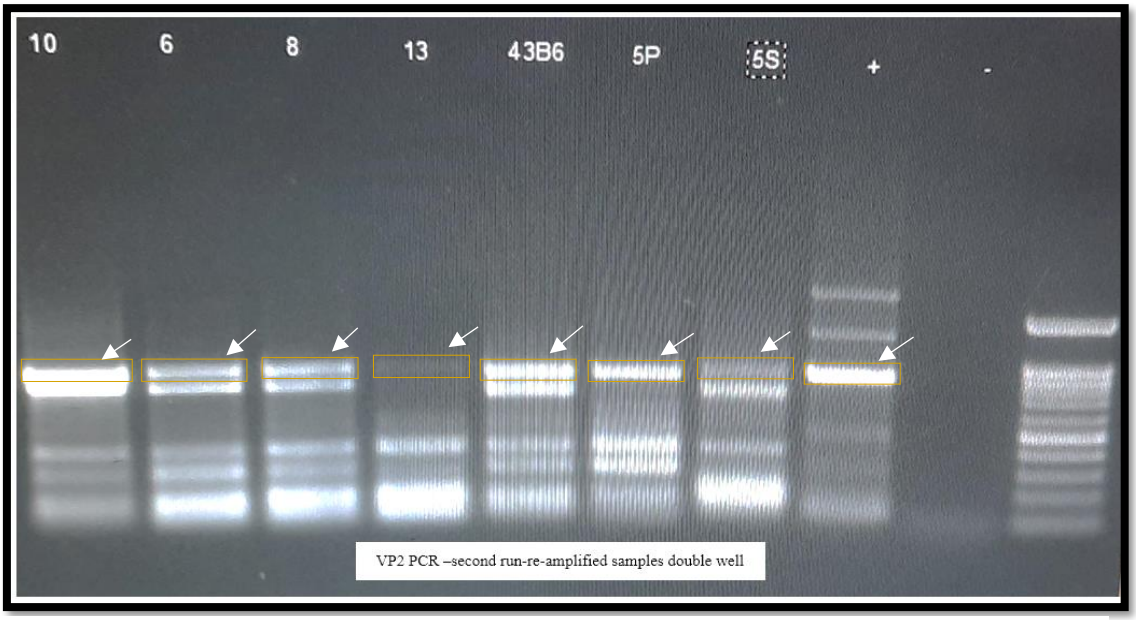

Figure 3.11: Reamplified samples double well method

3.8.3.4 Purification of the PCR product:

3 methods of DNA purification were used in the study, purification was done to remove unwanted dNTPs, primers from PCR products in order to have only the desired gene that was amplified, therefore better sequencing results.

1) QIAquick PCR purification kit (250) (Qiagen, USA) was used first to purify the amplified samples using the green master mix (Appendix 3.6), it was done for some samples but when gel electrophoresis was performed very weak bands were seen (Figure 3.12) thus it was decided to use another method which is ExoSAP-IT.

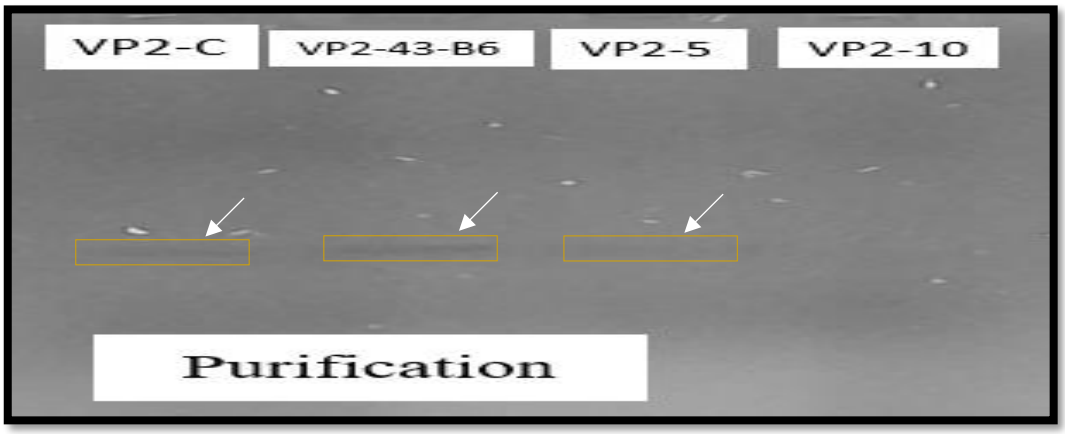

Figure 3.12: Sample purification with QIAquick PCR purification kit (250)

2) ExoSAP-IT single-step PCR cleanup was done for the samples that were amplified using the green master mix and had a strong band (Appendix 3.7). ExoSAP-IT has a formulated buffer that have two hydrolytic enzymes:
    - Exonuclease I which removes the remaining primers and any single-stranded DNA.
    - Shrimp Alkaline Phosphatase (SAP) that removes the remaining dNTPs (Thermo Fisher Scientific, 2022)
3) QIAquick Gel extraction kit (50) (Qiagen, USA) was used after the desired band was removed from the gel using the double well method to purify the DNA from the gel (Appendix 3.8).

3.8.4 Sample sequencing:

Sequencing was done in 3 steps: (Appendix 3.9)

1. Sample PCR: The PCR sequencing mix was prepared by adding the usual PCR components and adding the big dye which is the most important ingredient for sequencing, it is a fluorescent dye that attaches to the DNA, it contains (dNTPs, ddNTPs), after that the mixture was pipetted into each well of the barcoded sequencing plate, then freshly prepared VP2 primers were added and lastly the samples were put and the PCR was done on ABI machine.

   Note: Each sample was run twice, once with the forward primer and once with the reverse primer, this step was useful later on in the sample sequence cleaning process.

2. PCR product purification: which was done in multiple steps: DNA precipitation, washing, dissolving and lastly DNA strand denaturation.
3. Sequencing using ABI3500 machine.

3.8.5 Sample alignment with reference B19V sequences:

The nucleotide sequences of samples were aligned with the main B19V reference gene sequence that was downloaded from NCBI then multiple sequence alignment was done using Sequencher program 5.4.6 ,it was followed with sequence cleaning by trimming the beginning and the ends of the sequences and manually cleaning the ambiguous sequence according to the chromatogram of the forward and the reverse sample sequence (Figure 3.13) then the final sequence of each sample was aligned with the reference sequences of all the B19V genotypes.

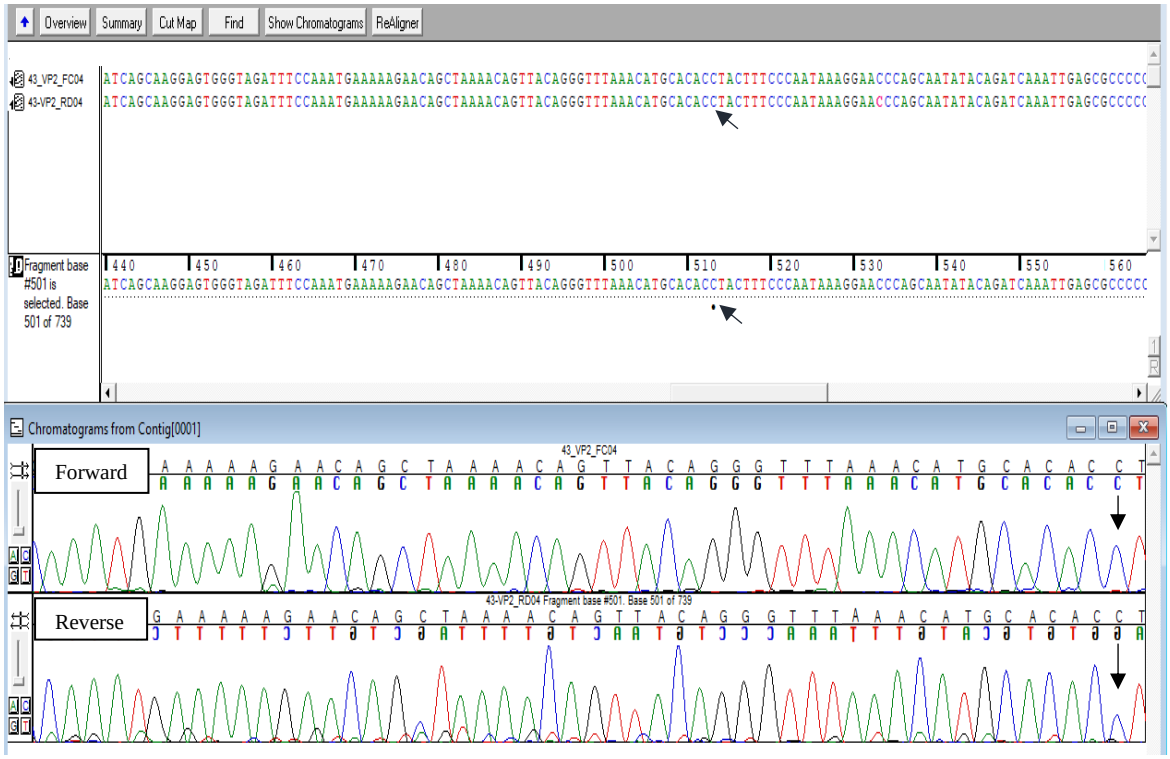

Figure 3.13: Sample manual cleaning using chromatogram of both forward and reverse sequences of the sample

3.8.6 Phylogenetic tree development:

After sequence alignments, a FASTA text file was generated, and the tree was visualized using the program NJ plot then Clustal X program was used to generate the phylogenetic tree using the neighbor-joining method and 1000x bootstrapping.

Chapter 4

Results

4.1 B19V suspected patients

While investigating B19V in Palestine from 2014-2022, 905 patients were suspected to be infected with B19V in which RT PCR was performed for these samples and only 28 patients of the patients tested positive (3%) (Figure 4.1).

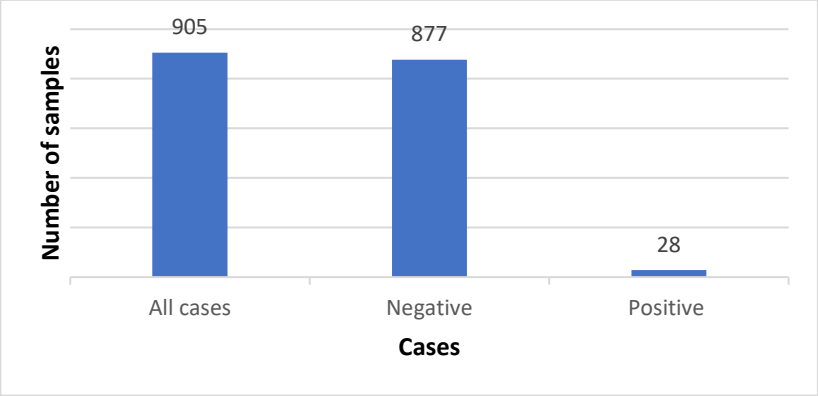

Figure 4.1: Number of suspected B19V samples from 2014-2022

4.2 Positive samples

When the prevalence of the 28 positive B19V samples was checked, it was noticed that the highest number of B19V positive patients were in 2020, accounting for 25%. Following closely behind were 2019 and 2022. When comparing the period from 2014 to 2018 with the period from 2019 to 2022, there was a slight increase in the number of B19 viral infections in which 25% of the patients were during the years 2014 to 2018 while the remaining 75% were during the years 2019 to 2022, as seen in figure 4.2.

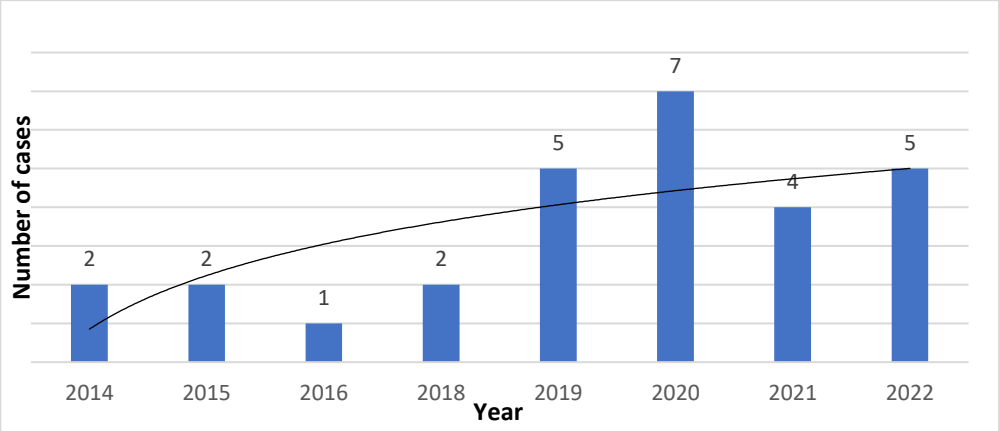

Figure 4.2: B19V infected patients per year (2014-2022)

4.3 Collected data analysis results

4.3.1 Social demographics and B19V:

A comparison between 28 positive B19V patients and 32 controls (suspected B19V negative patients) was done. Starting with patients' demographics it was noted that both groups were more males (55%) than females (45%) with no statistical significance. As for the age of the patients they were grouped as seen in table 4.1 in which 53.6% of the positive cases were less than 5 years old, 42.8% were from 6-15 years old and 3.6% >18 years old, meanwhile 78.1% of the negative patients were less than 5, 21.9% were from 6-15 years old and 0.0% >18 years old. Both groups' ages were mostly from 2-5 years but also with no significant association with B19V. Patient place of inhabitant was also studied, it was found that both groups were from various regions mostly from Bethlehem and Hebron; however, being from these regions does not increase the likelihood of having B19V compared to residents of other cities.

Table 4.1: Social demographics comparison between cases and controls

		Result						
		Positive		**Negative**		**Total**		**P-Value**
		Count	%	Count	%	Count	%	
Gender	Female	12	42.9%	15	46.9%	27	45.0%	0.755
	Male	16	57.1%	17	53.1%	33	55.0%	
Age	<1	0	0.0%	3	9.4%	3	5.0%	0.153
	1	1	3.6%	5	15.6%	6	10.0%	
	2-5	14	50.0%	17	53.1%	31	51.7%	
	6-9	9	32.1%	5	15.6%	14	23.3%	
	10-15	3	10.7%	2	6.3%	5	8.3%	
	>18	1	3.6%	0	0.0%	1	1.7%	
City	Bethlehem	11	39.3%	13	40.6%	24	40.0%	0.496
	Hebron	12	42.9%	15	46.9%	27	45.0%	
	Jerusalem	3	10.7%	4	12.5%	7	11.7%	
	Ramallah	2	7.1%	0	0.0%	2	3.3%	

4.3.2 Clinical manifestation and B19V:

Moreover , many clinical manifestation were studied and compared as seen in table 4.2.In positive patients ,fever was noticed to be present in 89.8 % , rash in 22.2% , anemia in 29.6% ,while surprisingly in negative patients 89.8% had fever, 25% of the patients had rash ,12.5% had anemia thus they all had no association with B19V.However, 2 B19V infected patients

had severe anemia of Hb less than 6 mg/dl: one patient was diagnosed with pure red cell aplasia and another patient was diagnosed with transient aplastic anemia.

Meanwhile, other clinical manifestations were significant including respiratory problems p:0.046 , hypoactivity and malaise p:0.005 and neurological problems p:0.043 and when the degree of association the odd ratio was done it was found that the individuals with respiratory route problems are 2.92 times more likely to test positive for the B19V compared to those with no respiratory problems, also having malaise or being hypoactive are 5.8 times more likely to have the virus and lastly having neurologic problems are 8.5 times more likely to have B19V than B19V negative patients as seen in table 4.2.

Table 4.2: Clinical symptoms comparison between cases and controls

		Result									
		Positive		**Negative**		**Total**		**P-Value**	**Odd Ratio**		
		Count	**%**	**Count**	**%**	**Count**	**%**		**OR**	**95% Confidence Interval**	
Fever	Yes	24	88.9%	29	90.6%	53	89.8%	0.826			
	No	3	11.1%	3	9.4%	6	10.2%				
Rash	Yes	6	22.2%	8	25.0%	14	23.7%	0.803			
	No	21	77.8%	24	75.0%	45	76.3%				
Malaise/ Hypoactive	Yes	14	51.9%	5	15.6%	19	32.2%	**0.005**	**5.815**	**1.722**	**19.634**
	No	13	48.1%	27	84.4%	40	67.8%				
Anemia	Yes	8	29.6%	4	12.5%	12	20.3%	0.12			
	No	19	70.4%	28	87.5%	47	79.7%				
Abdominal tract problems	Yes	15	55.6%	12	37.5%	27	45.8%	0.165			
	No	12	44.4%	20	62.5%	32	54.2%				
Respiratory route problems	Yes	18	66.7%	13	40.6%	31	52.5%	**0.046**	**2.92**	**1.006**	**8.494**
	No	9	33.3%	19	59.4%	28	47.5%				
Lymph Nodes problems	Yes	5	17.9%	4	12.5%	9	15.0%	0.721			
	No	23	82.1%	28	87.5%	51	85.0%				
Neurologic problems	Yes	6	21.4%	1	3.1%	7	11.7%	**0.043**	**8.5**	**0.95**	**75.3**
	No	22	78.6%	31	96.9%	53	88.3%				
Heart problems	Yes	6	21.4%	7	21.9%	13	21.7%	0.967			
	No	22	78.6%	25	78.1%	47	78.3%				
Liver problems	Yes	4	14.3%	2	6.3%	6	10.0%	0.404			
	No	24	85.7%	30	93.8%	54	90.0%				

4.3.3 The effect of B19 viral infection on CBC parameters:

The association between B19V and CBC parameters (WBC, RBC, HB, MCV, MCH, MCHC, RDW and reticulocytes percentage) was also analyzed among cases and controls. In admission, the first CBC (CBC 1), second CBC (CBC 2), and the last CBC (CBC 3) were studied. There was a significant association between positive cases and the controls in the second CBC of admission in the WBC (p=0.003) and platelets (p =0.018); moreover, there was an association in the last CBC of admission in the HB (p=0.043) and WBC (p=0.046), in other words B19V is associated with lower values in WBC, platelets and Hb. The first CBC done had no significant association between positive cases and the controls. Also, all other CBC markers like MCV, MCH, MCHC, RDW were not significantly associated with B19 viral infection. Besides, reticulocytes were noted and there was an association between being infected with B19V and having reticulocytopenia (Table 4.3).

Table 4.3: CBC comparison between cases and controls

Result							
	Positive		**Negative**		**Total**		**P-Value**
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
WBC-1	10.56	8.13	13.45	8.69	12.10	8.49	0.113
WBC-2	7.72	3.80	12.89	7.06	10.65	6.37	**0.003**
WBC-3	7.47	3.42	11.29	5.37	9.79	5.00	**0.046**
RBC-1	4.25	0.85	4.38	0.65	4.32	0.74	0.391
RBC-2	4.15	0.81	4.40	0.42	4.30	0.62	0.165
RBC-3	3.98	0.66	4.43	0.49	4.26	0.59	0.055
HB-1	10.57	2.40	11.07	2.10	10.84	2.24	0.362
HB-2	10.66	2.12	11.20	1.71	10.96	1.90	0.315
HB-3	10.05	2.34	11.39	1.05	10.88	1.75	**0.043**
MCV	75.8	7.8	77.6	5.7	76.7	6.8	0.767
MCH	25.6	2.7	25.7	2.0	25.7	2.3	0.846
MCHC	33.0	1.7	33.3	1.2	33.2	1.5	0.413
RDW	13.10	2.25	12.87	1.70	12.98	1.96	0.791
PLT-1	229.4	132.8	289.3	129.8	261.4	133.5	0.053
PLT-2	208.9	108.2	294.5	138.9	257.4	132.5	**0.018**
PLT-3	245.2	154.2	383.1	210.1	328.9	199.2	0.073
Reticulocyte %	0.016	0.019	1.449	1.840	0.812	1.505	**0.019**

4.3.4 Hb comparison between cases and controls:

To understand the effect of B19V on hemoglobin it was grouped into 2 groups , Hb <10 mg/dl and Hb >10 mg/dl since it statistically binned at about 10 mg/dl , thus when positive cases were compared with the negative cases, a significant association was noticed in the last CBC in admission and the p was 0.019 with an odd ratio of 9.6 thus giving an indication that individuals with lower hemoglobin levels are 9.6 times more likely to test positive for the B19 virus compared to those with higher hemoglobin levels (Table 4.4).

Table 4.4: Hb comparison between cases and controls

HB (Binned about 10 mg/dl)		**Positive**		**Negative**		**Total**		**P-Value**	**Odd Ratio**		
		Count	**%**	**Count**	**%**	**Count**	**%**		**OR**	**95% Confidence Interval**	
HB-1	<10.00	5	17.9%	4	12.5%	9	15.0%	0.564			
	10.00+	23	82.1%	28	87.5%	51	85.0%				
HB-2	<10.00	7	30.4%	4	13.3%	11	20.8%	0.137			
	10.00+	16	69.6%	26	86.7%	42	79.2%				
HB-3	<10.00	6	54.5%	2	11.1%	8	27.6%	**0.019**	**9.6**	**1.451**	**63.5**
	10.00+	5	45.5%	16	88.9%	21	72.4%				

4.3.5 The effect of B19 viral infection on CRP, ALT, and AST:

To better understand the virus, other laboratory tests were studied ALT, AST, and CRP (Table 4.5) and they all had no association with B19V but it was noticed that all the patients had CRP more than 6 mg/dl, the positive cases were mostly >100 mg/dl with a percentage of 34.6% while the negative patients were mostly from 6-50 mg/dl with a percentage of 43.3% (Table 4.6).

Table 4.5: AST and ALT comparison between cases and controls

	Result					
		N	**Mean**	**Median**	**SD**	**P-Value**
AST	Negative	28	53.2	29.9	56	0.905
	Positive	24	252	39.4	989	
ALT	Negative	29	29.3	11.8	46.1	0.645
	Positive	23	131	11.8	495	

Table 4.6: CRP comparison between cases and controls

		Result				
		Positive		Negative		P-Value
		Count	%	Count	%	
CRP	<6	6	23.1%	6	18.8%	0.414
	6-50	6	23.1%	14	43.8%	
	51-100	5	19.2%	5	15.6%	
	>100	9	34.6%	7	21.9%	

4.3.6 Previous underlying diseases and B19V:

Since B19V may be symptomatic in patients with previous illness, it was decided to be inspected and it was found that 40.7% of the positive cases had previous underlying disease while only 6.3% of the controls thus a significant association between having previous underlying disease and being B19V positive was found (p=0.003); moreover, odds ratio was done and it was found that the individuals with previous illness are 10.3 times more likely to have B19V compared to those with no problems with being 95% confident that the odds ratio would be between 2 and 52.3 (Table 4.7).

Table 4.7: Having a previous underlying disease comparison between cases and controls

		Result									
		Positive		Negative		Total		P-Value	Odd Ratio		
		Count	%	Count	%	Count	%		OR	95% Confidence Interval	
Previous underlying disease	Yes	11	40.7%	2	6.3%	13	22.0%	**0.003**	**10.3**	**2**	**52.3**
	No	16	59.3%	30	93.8%	46	78.0%				

4.3.7 Coinfection and B19V:

Surprisingly, 37% of the cases had coinfection with different microorganisms but p-value could not be done since none of the controls had coinfections (Table 4.8). There were many types of microorganisms that were coinfected with B19V (Figure 4.3) with EBV being the most common coinfection in the study (25.9%) which was proven to be positive with RT-PCR.

Table 4.8: Coinfection comparison between cases and controls

		Result					
		Positive		Negative		Total	
		Count	%	Count	%	Count	%
Coinfection	Yes	10	37.0%	0	0.0%	10	37.0%
	No	17	63.0%	0	0.0%	17	63.0%

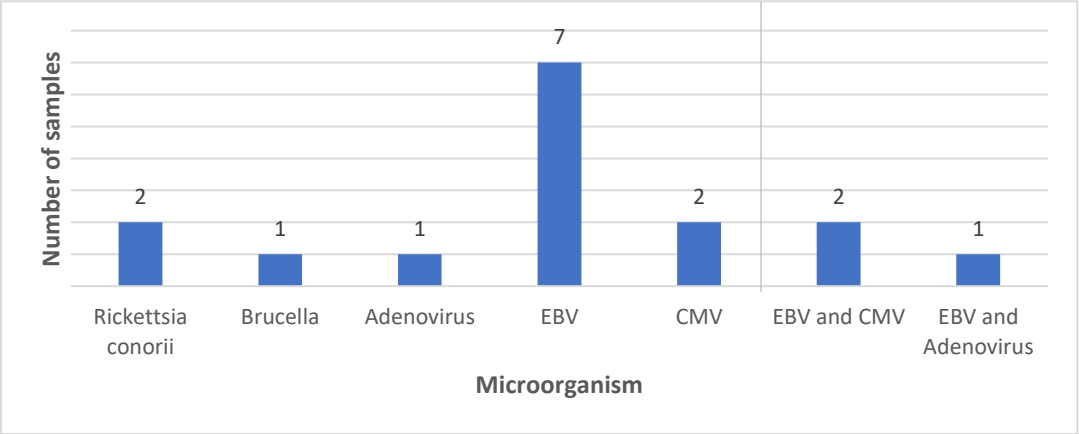

Figure 4.3: Microorganism coinfected with B19V

4.4 Molecular results:

4.4.1 PCR for sequencing:

Since one of the goals of the study is genotyping, sequencing was decided to be done for the 28 positive samples, but 3 samples were missing (Figure 4.4), thus PCR was done only for 25 samples with different Ct values (Figure 4.5); however, only the samples with Ct less than 30 gave good bands in gel electrophoresis and the rest were unclear as a result only 5 samples (17.8%) were sequenced (Figure 4.4).

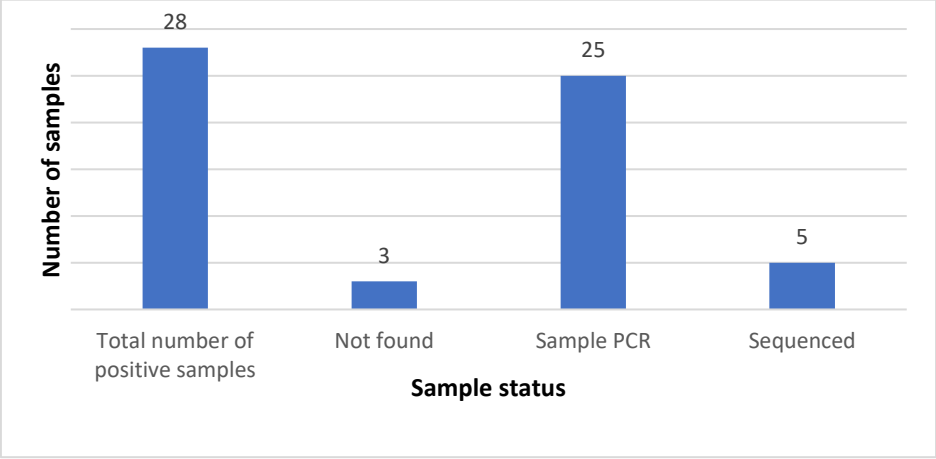

Figure 4.4: Samples status

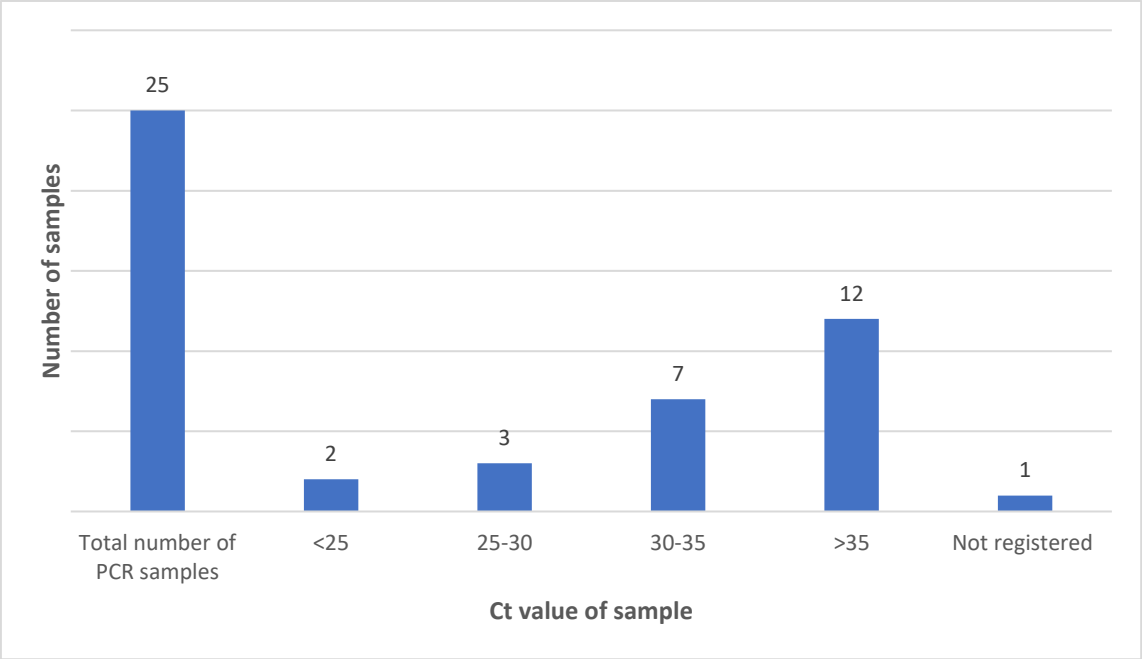

Figure 4.5: Ct of samples

4.4.2 Genotyping:

5 samples were successfully sequenced then genotyped by building a phylogenetic tree, all the samples were genotype 1 of which 3 samples were genotype 1a and the other 2 were genotype 1b. It was noticed that years 2016-2020 were mainly genotype 1a, while 2021 and 2022 were genotype 1b. (Table 4.9).

Table 4.9: B19V genotypes in Palestine

Ct	Genotype	Year
25-30	Genotype 1a	2016
<25	Genotype 1a	2018
25-30	Genotype 1a	2020
<25	Genotype 1b	2021
25-30	Genotype 1b	2022

4.4.3 Phylogenetic tree:

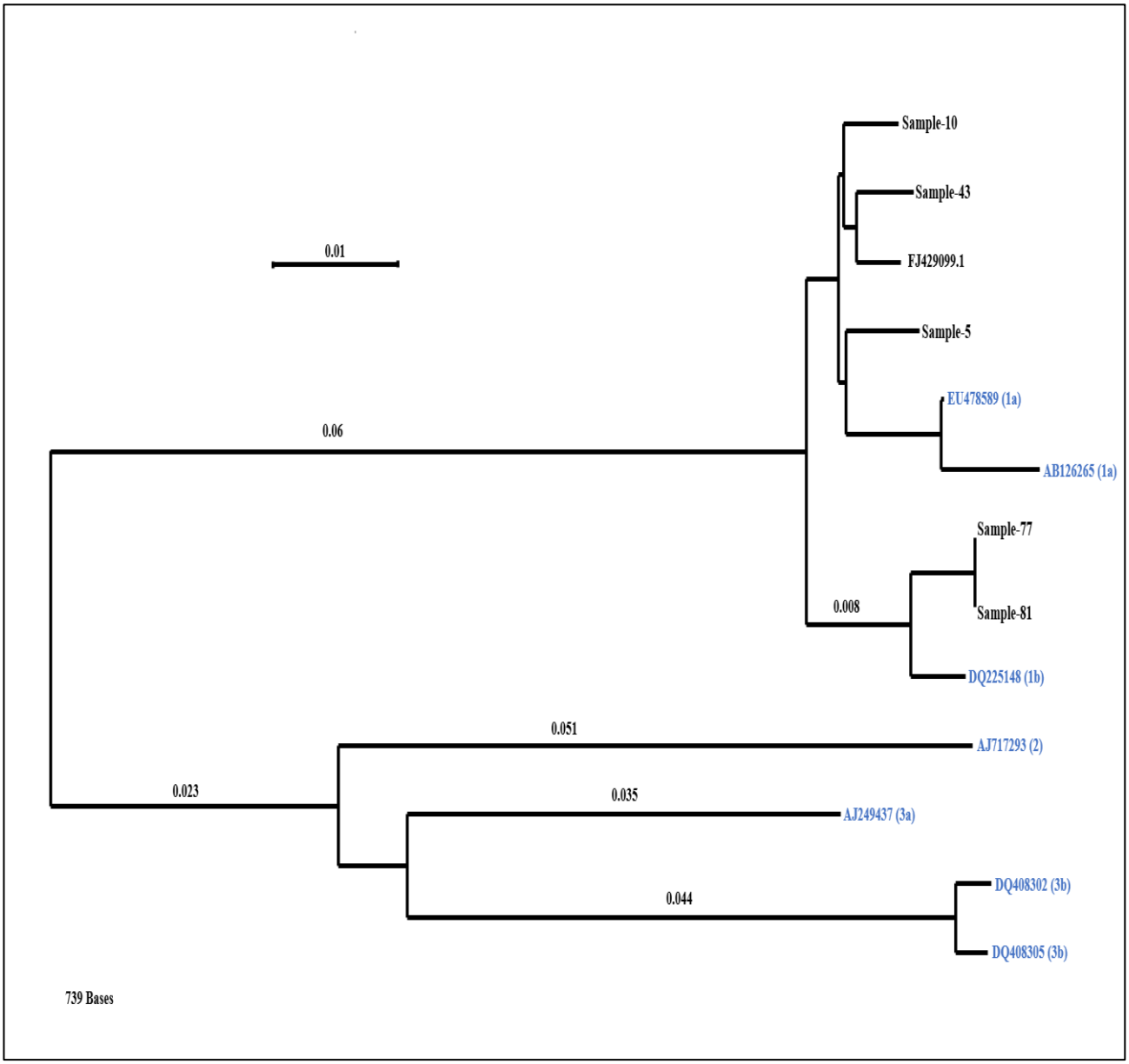

Figure 4.6: Phylogenetic tree

4.5 Case presentation

Out of all the 28 positive cases, there was one important special case that is worth to be mentioned in which a 7 years old female child with no previous underlying disease was admitted to a hospital in Ramallah. The patient presented with fever that began before 3 days of admission, she was hypoactive and had malaise, she also had rash , myalgia, respiratory problems (cough) ,abdominal problem (vomiting) and most importantly pancytopenia and reticulocytopenia, she was admitted in hospital for 2 weeks during which she was transfused: blood (3 times) and platelets (4 times) and she had bone marrow biopsy to check for aplastic anemia where it showed hypocellular bone marrow; however , her case was not well understood and it was decided to transfer the patient to another hospital to check the cause of the pancytopenia, in the other hospital she had platelet transfusion and had another bone marrow biopsy but B19V was suspected and when the sample was tested for B19V where it gave weak positive, therefore the viral load was low meaning that the patient was tested in the last stage of the infection with B19V after that the patient was discharged .Therefore, this 7 years old child patient was having transient aplastic crisis which only needed IVIG instead of doing 2 painful bone marrow biopsies.

Chapter 5

Discussion

Human parvovirus B19 is a virus that is usually asymptomatic but symptomatic in some patients especially those who have previous underlying disease, in Palestine there is no data about it thus this study was decided to be done and it was divided into two parts: first by filling patients' data in Excel program for both cases and controls and second by sequencing and then genotyping B19V RT-PCR positive samples.

When the prevalence in Palestine was investigated, 3.1% of the suspected patients were found to be positive using sensitive RT-PCR during the 9 years period which was a higher percentage than surrounding regional countries, for example, the prevalence in Qatar was 1.4% (Abdelrahman et al., 2021) and 0% in Sudan (Adam et al., 2015). However, the prevalence in developed countries is from 2% to 10% in children under 5 years old and 40% to 60% in adults over 20 years old (Macri & Crane, 2022), this variation can be attributed to the differences in the study design, the sample size and the diagnostic methods that were used.

As for the distribution of positive B19V samples, it was noticed that it varied across the different years, the majority of positive samples (75%) were collected during the years 2019 to 2022 while 25% were in 2014-2018, indicating a higher prevalence during the recent years but when compared with a British study, 2017 had the highest percentage of positivity and it began to decline in 2020 till it reached zero % in 2021 (Williams et al ., 2022), similarly in Netherlands more cases were seen from 2013 to 2019 than 2020 and the first 6 months of 2021 in which 2021 also had zero cases (Molenaar-de Backer et al .,2021).

When the patients social demographics was checked it was found that both cases and controls groups had a higher proportion of males than females; however, in a study that investigated the seroprevalence of B19V (anti-B19V IgG & IgM) in the Palestinian-occupied territories females were more than males but similarly had no statistically significant association between males and females (Mor et al., 2016). As for the age distribution, most of the samples were young children which was different than the previous mentioned study in which B19V IgM active infection positivity was present more in patients with 20-29 years of age (Mor et al., 2016). As for the city, both groups were mainly from Hebron and

Bethlehem but since this is the first study in Palestine patient city could not be compared to other studies.

Regarding clinical manifestations and symptoms, fever was present in high percentage in both positive and negative patients (89.8%) which was a higher percentage than another research that showed that 40.8% of B19V positive patients had fever (Rodríguez Bandera et al., 2015). But since there is no significant association in fever in this research this affirms that fever alone cannot be used as a reliable indicator of B19V infection in addition to rash and anemia which had also no differences between the case and the controls, but this was different than the literature that explained that these symptoms are common in patients with B19 viral infection (Macri & Crane, 2022).Although B19V is not significantly associated with anemia, 2 patients in this study had severe anemia: one patient was diagnosed with pure red cell aplasia and another patient was diagnosed aplastic anemia. However, other clinical manifestations such as respiratory problems, malaise, and neurological problems showed significant associations with B19V infection. But there is contradictory results about respiratory problems and B19V, in which a case report stated that B19 viral infection is associated with respiratory distress (Morris & Smilack ,1998), another case report study stated that acute lung injury was developed due to B19V pneumonia (Wardeh & Marik,1998) , also a research concluded that acute respiratory diseases with obstructions were developed in the respiratory airways in children with a particular innate tendency (Wiersbitzky et al., 1991), but a research that was done in Iran found that there was low frequency of B19V among patients with respiratory infection (Tavakoli et al.,2018). As for malaise, it was found that it was present in all sickle cell patients who had B19V active infection (Obeid O. E, 2011). Moreover, neurological problem in this research was like a study that was done in India which suggested that B19V is an agent associated with neurological disease (Pattabiraman et al., 2022).

As for the effect of B19V on CBC parameters, there were differences between cases and controls in which it was configured that B19V affects WBC count, platelets, Hb levels, and reticulocyte percentage in some CBC tests. However, there was no differences in markers such as MCV, MCH, MCHC and RDW. But most importantly individuals with Hb levels < 10 were more likely to test positive for B19V in comparison with Hb levels >10 which was similar to literature that stated the effect of B19V on lower Hb (Slavov et al.,2011) (Anderson et al.,1985) , also a research found a significant association between B19V IgG seropositivity past infection and lower Hb levels (Gasim et al., 2016), another study in

Tanzania found that B19V is significantly associated with the decrease in Hb in children <5 Years with anemia (Tizeba et al., 2018). Moreover, the low results of WBC count and platelet count were similar to other studies (von Landenberg et al., 2007) (Rogers et al., 1996) in addition to low reticulocytes count (Macri & Crane, 2022).

In terms of other laboratory tests, B19V positive patients had CRP above 50 in 53.8% of the patients with no significant difference from controls, the percentage was higher than a retrospective study which showed that CRP levels were mildly to moderately elevated in 30.6% of patients (Rodríguez Bandera et al., 2015), but differently there was a significant increase in CRP in a case control study of sickle cell anemic patients (Iwalokun et al.,2012). As for liver enzymes, a low percentage of patients had raised transaminase levels which was similar to another research (Rodríguez Bandera et al., 2015).

The study also investigated the presence of previous underlying diseases among B19V positive cases, the previous illness in the study were : heart problems, anemia, neurological problems, cancer, Familial Mediterranean Fever, and problems with growth ; however, heart problems and anemia were more common, likewise a study that focused on B19 viral infection in patients with or without underlying diseases noticed that the patients with underlying diseases were immunologic diseases, hematologic diseases, and cardiopulmonary diseases (Sim et al.,2019). Moreover, none of the patients in the study had previously diagnosed hematological malignancies or hematological hereditary diseases such as thalassemia or sickle cell disease; however, a one-year-old child that had very low Hb was tested with B19V RT-PCR and Hb electrophoresis in the same hospital admission and the patient was strongly positive for B19V with very low Ct value and was diagnosed at the same time with beta thalassemia minor (trait).

Concerning B19 virus and coinfection, surprisingly there was a high prevalence (37%) of coinfections with other microorganisms. But when investigations were done, interestingly there were many case reports that had B19 viral infection and coinfections such as EBV (Stojkovic-Filipovic et al.,2016) (Karrasch et al., 2014), CMV (Harada et al.,2018), Malaria (Duedu et al.,2013), and others, but up to the knowledge this study was the first study to have B19V coinfection with Brucella or Rickettsia conorii.

As for the second part of the study PCR and sequencing were performed on a subset of positive samples and a phylogenetic tree was done which revealed that all sequenced samples belonged to genotype 1 including the two subtypes (genotype 1a and 1b), this result was

similar to other B19V genotyping studies that had a predominance in genotype 1 (Zadsar et al 2018) (Abdelrahman et al., 2021) (Williams et al.,2022).

Moreover, it was noticed that the percentage of samples with Ct value of > 30 was 77.7% which means low viral load, likewise a study that was done in England found that most of the positive samples of blood donors had low viral loads (Williams et al.,2022). However, it was noticed that the 5 genotyped samples had a Ct value of <30 and a high viral load , also it was noticed that 4 of the 5 samples had previous illness before being infected with B19V and the previous illness were: heart problems, beta thalassemia trait, cancer, and growth problems.

Chapter 6

Conclusion, Recommendations, and Limitations

6.1 Conclusion

In conclusion, this study highlights B19V for the first time in Palestine. It discovered that the prevalence of B19V had risen in the recent years and noticed some associations between B19 viral infection and certain clinical symptoms, Hb levels, CBC parameters and underlying illness. It provided information about the most prevalent B19V genotype in Palestine which was genotype 1. Additionally, to the best of our knowledge this study was the first to show B19V coinfection with Brucella or Rickettsia conorii.

6.2 Recommendations

Since the majority of B19 viral infections in healthy individuals are self-limiting and there are other microorganisms and diseases that can produce fever, rash, or anemia, it is advised to increase physician awareness of B19V as it may not be recognized by the medical community. For instance, aplastic anemia may be only treated as a symptom in patients with hematological disorders, without further thinking about the B19V as the causative agent thus mismanaging and misdiagnosing the patient's status. Therefore, for faster, easier, and better diagnosis, it is recommended that patients with fever and/or rash, respiratory problems, hypoactivity and hemoglobin <10 to be suspected for B19V, especially if the patient has pancytopenia or has previous underlying disease.

6.3 Limitations

- Sample problems such as missing or insufficient sample or even having samples with low viral load.
- The absence of a clear clinical history in the health information system.
- Small number of samples may have affected the statistical significance.
- Lack of prior data in Palestine since it's the first research about B19V.
- Low Ct of the samples led to genotype only few samples.
- Unavailability of some resources as only the abstract is available.

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Appendix

Appendix 1.1: Ethical approvals

Al Quds University
Faculty of Health Professions
Jerusalem –Abu Dis

جامعة القدس
كلية المهن الصحية
القدس – أبو ديس

Research Ethics Subcommittee of Faculty of Health Professions
Letter of approval

30/11/2022
Ref. No.: RESC/2022-21

Dear Applicants, (**Dr. Musa Hindiyeh, Ms. Miral Abdo**)
Program: **MSc Medical Labs Department**

The Research Ethics subcommittee of Faculty of Health Professions has recently reviewed your proposal entitled (**Human parvovirus B19 epidemiology, genotyping, patients' hematological presentation, and clinical manifestations in children in southern Palestine**) submitted by (**Dr. Musa Hindiyeh**).
Your proposal is deemed to meet the requirements of research ethics at Al-Quds University, but further assessment is required by the Central Research Ethics Committee of Al-Quds University. We wish you all best for the conduct of the project.

Hussein ALMasri
Research Ethics Subcommittee Chair
Faculty of Health Professions

Hussein ALMasri

CC: File
CC: Committee members

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Medical Research Agreement of Principles

Date: 20-1-2023

This is to certify that Caritas Baby Hospital represented by: __**Dr. Musa Hindiyeh**__ will be collaborating with ____ **Alquds University** _____ represented by: _ **Al-Quds University**___ to conduct a Medical Research Project entitled: _ **Human parvovirus B19 epidemiology, genotyping, hematological effects, and clinical manifestations in children in southern Palestine** ___. The research project will be conducted by: _____**Miral Munther Abdo** _____. Ms Abdo will have access to all the data that is related to this project.

The above research project was reviewed by memebrs of Caritas Medical Research Committee and was approved on____**20-9-2022**_____ and given MRC-Project Number _____**MRC-48**_____.

After the fruitful accomplishment of the project both parties agree to publsih the work in peer reviewed journal and the authorship location in the manuscript will be as follows:

First author: **Miral Munther Abdo**
Second author:
Third author:
Before last author:
Last author: **Musa Hindiyeh**

Caritas Baby Hospital
Medical Research Committe Representative
Dr. Musa Hindiyeh

Appendix 3.1: Primer lyophilization

Primers were lyophilized into 100 picomol stock primers, using the method advised by the primer company:

1. Forward and reverse primers were spined down.
2. Primers were thawed by NF H2O to prepare the 100 picomol primer stock, the amount of NF H2O added was as recommended in the primer company paper, for example: if the primer concentration is 22, 22.0*10 =220 µL of NF H2O should be added.
3. Vortex and spin was done 2 times.
4. Primers were incubated in fridge for 20 minutes, then vortex and spin were done twice for both of the primers.
5. Aliquoting of the 100 picomol thawed primer was done which was put in labeled eppendorf tubes.

Appendix 3.2: Gradient PCR with GoTaq® Green Master Mix PCR kit

Gradient PCR was done for NS1, VP1 and VP2 genes, using primers that were chozen specifically for each area.

1. The working eppendorf was prepared with a dilution of 1:10 (10 picomol) by adding 5 μL of the stock to 45 μL of NF H2O.
2. Vortex and spin 2 times was done.
3. PCR mix was prepared ((per sample:12 μL of the green PCR master mix which contains the dNTPs, MgCl2, DNA Polymerase, PH buffer and NF H2O), 1 μL of the forward primer, 1 μL of the reverse primer and 6 μL of NF H2O) Note: they were multiplied by the number of samples that will be worked on.
4. Vortex and spin 2 times was done
5. 20 μL of the pre-PCR mix was pipetted in 8 wells
6. 5 μL of the positive control was added in each well in the PCR room.

Gradient PCR program:

1. Number of cycles of repeats :32X
2. Volume :25 μL
3. Lid temperature:95C
4. PCR temperatures were:
    - Preparation :95C,5min
    - Denaturation :95C,30 seconds
    - Annealing: gradient temperatures for example:(45-66 C), 30 seconds
    - Elongation :72C,1 minute
    - Step 6: 72C ,7 minutes
    - Step 7: 4C

Appendix 3.3: Agarose gel electrophoresis in CBH

1.1g of the gel powder was added to 100 ml of 1XTBE and were dissolved in the flask in the microwave after that one drop of the tracking dye (ethidium bromide) was added then the gel was poured in the gel tray to cool down then the tray was put in the gel electrophoresis machine that was filled with 1XTBE and the comb was removed after that the samples were inserted in the gel and electrophoresis was run on voltage at 87 for one hour.

Appendix 3.4: Double well PCR, gel electrophoresis and gel band removal

1. The PCR strips that were done from the previous PCR runs were thawed.
2. 1.1 gel was prepared and poured in the small gel rack and double well comb was used.
3. The 20 μL PCR product was inserted in the gel.
4. Gel electrophoresis was run.
5. The 739 bp band was cut.
6. Each sample was saved in a labeled eppendorf tube and were saved in the fridge.

Appendix 3.5 : PCR with Q5® High-Fidelity DNA Polymerase PCR kit

The following method was performed according to the manufacturer:

1. The working primer tube was prepared with primer concentration of 1:10 (10 picomol).
2. Vortex and spin was done 2 times.
3. Preparation and calculations for the PCR mix were performed (each sample had :5 μL of the Q5 reaction buffer, 0.5 μL of dNTPs,0.25 μL of DNA polymerase,1.25 μL of the forward primer ,1.25 μL of the reverse primer ,11.75 μL of NF H2O, and 5 μL of the sample)
4. Vortex and spin were performed twice.
5. 20 μL of the pre-PCR mix was pipetted in the wells.
6. Samples were added.

ABI machine PCR program:

1. Number of cycles of repeats :40X
2. Volume :25 μL
3. Lid temperature:105C
4. PCR temperatures were:
    - Preparation :96C,30 seconds
    - Denaturation :98C,30 c
    - Annealing:54C, 30 seconds
    - Elongation :72C,1 minute
    - Step 6: 72C ,7 minutes
    - Step 7: 4C

Agarose gel electrophoresis in AVH:

Gel was prepared by adding 1.5 g of the agarose with 100 ml of TBA buffer, the voltage was set at 100 and for 50 minutes.

Appendix 3.6: Purification using Qiagen PCR purification kit

The following steps were followed according to the manual instruction:

1. The previously frozen 20 μL PCR sample wells were thawed.
2. 100 μL of the binding buffer PB was added to the sample (the DNA attaches to the binding buffer) and then the mix was withdrawn and was pipetted in the spin column that was labeled with the same sample name.
   Note: The binding buffer PB was prepared by adding 150 μL of the PH indicator to 600 ml of the binding buffer, the color should be yellow.
3. Centrifugation for 1 minute at 130000(130 RPM) was done so that the DNA will set on the filter.
4. Sample washing was done with the wash buffer PE:
   a) The filter was removed and put it in a normal collection tube.
   b) 640 μL of the wash buffer PE was added to the samples.

   Note: this buffer should be prepared by adding 220 ml of ethanol to the PE buffer, the ethanol precipitates the DNA but if the ethanol is not added to the buffer and is directly put on the samples this will result in DNA washing thus the sample will be discarded.
5. Centrifugation for 1 minute at 130000(130 RPM) was done twice until the filter is dry.
6. Elusion was done to gather the DNA from the filter since the DNA melts in the elution buffer EB.
   a) The spin column was put in the conical eppendorf tube.
   b) 30 μL of the elusion buffer was pipetted to the spin column in middle of the column in a vertical mode.
   c) They were incubated with the elusion buffer for 1 minute.
7. Centrifugation for 1 minute at 130000(130 RPM) was done.
8. Purified samples were prepared for gel electrophoresis to make sure that the purified samples were present and had no problems.
   a) 2 μL of the loading dye with a concentration of 5X was pipetted on a parafilm.
   b) 3 μL of the purified sample was added and mixed together (the loading dye colors the DNA thus the presence of purified DNA can be confirmed by the band appearance after gel electrophoresis is performed).

Appendix 3.7: PCR ExoSAP-IT clean

2 μL of the ExoSAP-IT enzyme mix was added to the empty PCR wells then 5 μL of green sample was added, after that they were run on BioRAD PCR machine in which the wells were incubated for 30 minutes on 37C to enhance the enzyme performance then they were incubated on 80C for 15 minutes to degrade the enzyme.

Appendix 3.8: Purification using Qiagen quick gel extraction gel extraction kit

The following steps were followed according to the manufacturer instruction:

1. Each sample was weighed.
2. Solubilization: the solubilizing reagents QG buffer was added to dissolve the gel, the amount added was: the weight of the gel multiplied by 3.

Sample number	Weight(g)	Weight(mg)	Amount of QG buffer to add = Weight * 3
10	0.03	30	90
6	0.07	70	210
8	0.079	80	240
13	0.061	60	180
43B6	0.125	125	375
5P	0.1	100	300
5S	0.1	100	300
Positive control	0.05	50	150

3. Vortex and spin.
4. The tubes were incubated on 50C for 10 minutes, every 2 minutes the tubes were well mixed.
5. In the hood, isopropanol was added to each sample to precipitate the DNA, the amount added was the same amount of the weight (for example :30 µL of isopropanol was added to sample 10).
6. Vortex and spin down were done.
7. The samples were pipetted in spin columns.
8. Centrifugation of the spin column for 1.5 minute at 130000(130 RPM) was done.
9. The excess fluid was discarded.
10. 500 ml of the QG buffer was added to the spin columns.
11. Centrifugation for 1.5 minute at 130000(130 RPM) was performed.

12. The excess fluid was discarded.
13. Washing: 750ml of the washing solution (buffer PE) was added to the samples
    a. Incubation for 5 minutes.
    b. Centrifugation for 1.5 minute at 130000(130 RPM).
    c. Discard of the excess fluid.
    d. Re-centrifugation of the spin column for 1.5 minute at 130000(130 RPM).
    e. The spin column was put inside eppendorf tubes and then was incubated for 5 minutes on a hot plate to evaporate the alcohol.
14. Elusion: this step was done to gather the DNA from the filter since the DNA melts in the elution buffer.
    a) The spin column was put in a labeled conical eppendorf tube.
    b) 30 μL of the elusion buffer was pipetted inside the spin column in the middle of the column in a vertical mode.
    c) Incubation for 5 minutes.
    d) Centrifugation for 1.5 minute at 130000(130 RPM).

Appendix 3.9: Sequencing

A. Sample preparation for sequencing:

1. Primer preparation:

From the 100 picomol primers stock working primers were freshly prepared with a concentration of 5 picomol (38 µL NF-H2O+2 µL primer stock).

2. Mix preparation:
    a) The sequencing mix was prepared in the clean room by adding 11.5 µL NF-H2O, 3.5 µL buffer and 1 µL of the most important ingredient for sequencing the big dye which contains (dNTPs, ddNTPs).
    b) 16 µL of the prepared mix was added to each well of the barcoded sequencing plate.
    c) 2 µL of the 5 picomol primers were added to the wells.
3. Sample adding to the sequencing plate:
    a) 2 µL of the samples were added to their position, except for 2 samples in which 4 µL was added.
    b) The plate was sealed with the plastic stick sealer and was spined down for 1 minute.
4. PCR was done using ABI machine:

- Number of cycles of repeats :40X
- Volume :25 µL
- Lid temperature:105C
- PCR temperatures were:
    - Preparation :96C,1 minute
    - Denaturation :96C,0.1 minute
    - Annealing:50C, 0.05 minute
    - Elongation :60C, 2 minutes
    - Last step: 4C

B. Purification steps:

1. DNA precipitation was done by adding 5 μL of freshly prepared EDTA to each well.
2. Washing was done:

   First:

   a) 60 μL of 100% ethanol was added to each well using the multichannel pipette.
   b) The plate was firmly sealed with a sticky sealer.
   c) Plate mixing and well vortex was done for 4 minutes.
   d) Plate centrifugation for 30 minutes on the highest speed 2272 g on 4 C since the cold temperature increases the precipitation.
   e) After the plate was removed from the centrifuge it was flipped down on tissues to remove the excess fluid.
   f) The plate was centrifuged upside down for 5 seconds.

Second:

60 μL of 80% ethanol was added to the wells with the same previous steps however it was put in centrifuge for 15 minutes and 1600 g for speed.

3. The plate was kept open for 10 minutes in a dark drawer after the plate was removed out of the centrifuge.
4. Then it was put on the hot plate for 5 minutes to make sure everything has evaporated.
5. DNA dissolving:
   a) 10 μL of formamide was pipetted in each well to melt the DNA.
   b) The plate was properly covered with a clean septa.
   c) Spin down was done for 1 minute.
6. DNA strand denaturation was done by putting the plate on the thermal block plate on 95C for 3 minutes.
7. The plate was put for 3 minutes on ice to ban the strands from reannealing.
8. The plate with the septa was put in the machine for sequencing.

بارفو فيروس البشري الحالة الوبائية, نوع الفيروس الجيني ,التأثير على فحص الدم الشامل و الاعراض السريرية في الأطفال في جنوب فلسطين

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ملخص

الخلفية: بارفوفيروس البشري هو فيروس لا يظهرأعراض في أغلب حالات المرضى ، ولكنه قد يسبب أعراضاً في خاصة في المرضى الذين يعانون من أمراض سابقة مثل امراض الدم. البارفو يصيب لخلايا الدم الام بعض الحالات في نخاع العظم في عن طريق الارتباط ب البي انتيجن وقد يتسبب في أعراض مثل الحرارة والطفح و التعب الجسدي و ممكن ان يسبب فقر الدم البلازمي المؤقت. تم إجراء هذا البحث لدراسة البارفوفيروس لأول مرة في فلسطين وفحص مدى انتشاره وأكثر الأجناس شيوعًا و اكثر الأعراض ظهورا.

منهجية البحث: تم اجراء الدراسة بشكل رجعي من ٢٠١٤ الى ٢٠٢٢ في مشفى الكاريتاس للأطفال في بيت لحم, وتم إجراء اختبارتفاعل البلمرة المتسلسل لـ ٩٠٥ عينة من المرضى المشتبه بهم، وثبتت إيجابية ال فيروس لدى ٢٨ مريضًا (٣ %). تمت مقارنة الحالات الـ ٢٨ مع ٣٢ مريض ممن لم يصابوا بالفيروس، وتم فحص كل منهما للخصائص الاجتماعية والأعراض السريرية و فحص دم شامل والاختبارات المخبرية الأخرى , و أيضًا تم اجراء فحص التصنيف الجيني للحالات الإيجابية الذي يعمل على مضاعفة عينات المرضى لجين ال في بي ٢ و قد تمت إجراء التسلسل الجيني ل ٥ عينات (١٧.٨%))التي كانت تحتوي على كمية عالية من الفيروس.

النتائج: لم تظهر الخصائص الاجتماعية (الجنس، الفئات العمرية والمدينة) أي أهمية احصائية بين المصابين بالفيروس و غير المصابين ,اما بالنسبة للأعراض السريرية ,تم ايجاد علاقة مهمة في بعض الاعراض مع الفيروس منهم مشاكل الجهاز التنفسي ,ظهور تعب على المريض و مشاكل في الجهاز العصبي و لكن بعض الأعراض لم يكن لديها أي أهمية مع فيروس البارفو بالنسبة لظهورها مثل الحرارة و الطفح الجلدي و فقر الدم مع العلم انه اثنين من المرضى حصل لهم فقر دم حاد بسبب الفايروس حيث اصيب احدهما بفقر دم الخلايا الحمراء البلازمي ومريض اخر اصيب بفقر دم النخاع المؤقت. و بالنسبة لفحص الدم الهيمولوجي الشامل تم ايجاد ارتباط مهم في بعض الفحوصات الهيماتولوجية يتضمن كريات الدم البيضاء,الصفائح الدموية و نسبة الهموجلوبين و قد تم تصنيف المرضى اللذين لديهم هيموغلوبين اقل١٠ ملغ/د من بأن لديهم بأنهم اكثراحتمالية من باقي المرضى الذين لديهم هيموغلوبين اعلى أن يكونوا مصابين بالفيروس ,اضافة الى ذلك تم ايجاد علاقة مهمة بين نقص الخلايا الشبكية و بين الاصابة بالفيروس ,اما بالنسبة للفحوصات المخبرية الاخرى للكشف عن حدوث التهاب أو فحوصات الكبد لم يتم ايجادعلاقة مهمة مع الفيروس . تم ايجاد انه ٣٧ بالمائة من المرضى كانت مصابة بمرض قبل الاصابة بالبارفو و كان هناك علاقة مهمة حيث ان المرضى المصابين بمرض سابق اكثراحتمالية ب ٨.٨ مرة ان يكونوا مصابين بالفيروس و ايضا تم ايجاد أن ٣٧ بالمائة من المرضى كانوا مصابين بشكل متزامن مع الفيروس بكائن حي دقيق اخر. لمعرفة نوع الفيروس الجيني تم عمل الشجرة الفيلوجينية(شجرة النشوء والتطور) التي تبين منها ان كل العينات تنتمي للبارفوفيروس النمط الجيني رقم ١.

الاستنتاج: في الختام ، هذه الدراسة تعد أول دراسة لفحص البارفوفيروس في فلسطين و التي قدمت نتائج قيمة وفهمًا أعمق حول الفيروس في البلاد.

الكلمات الرئيسية: بارفو فيروس البشري, فلسطين,الحالة الوبائية ,الاعراض السريرة ,فحص الدم الهيماتولوجي الشامل, التصنيف الجيني.