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Molecular Surveillance of *Varicella-Zoster Virus* among School Age Children in Diwaniyah Province

A Thesis

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بسم الله الرحمن الرحيم

((وما أوتيتم من العلم إلا قليلاً))

صدق الله العلي العظيم

سورة الإسراء : الآية ٨٥

Dedication

TO

MY WIFE AND SONS

Ahmed Salim

2019

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Summary

Varicella (chickenpox) is the primary infection of *Varicella-Zoster Virus* (VZV), it is a mild self-limiting infection, but it is also highly contagious and can cause severe complications among high-risk group of individuals. It is usually a childhood infection providing lifelong immunity, but adults without varicella history are also susceptible to infection. High-risk group of individuals is more likely to develop serious complications.

The aim of the present study was to figure out the genetic strains of *Varicella Zoster Virus* (VZV) that are common in Al Diwaniyah governorate. A total of 4545 children were enrolled and data about those children obtained form 10 primary schools in Al-Diwaniyah governorate in the Mid-Euphrates region of Iraq whereas Infection documented in 800 out of 4545 children making the prevalence rate (17.6 %). No significant difference was obtained of VZV infection with respect to age and gender.

A Forty-Three Samples selected randomly for molecular diagnosis and identifying genetic strains in laboratory, by conventional PCR showed that all the 43 children were positive for VZV. The mean age of the 43 children selected for molecular study was 8.44 ±1.65 years and the age range was 6 – 10 years. The study sample included 23 male children (53.5 %) and 20 female children (46.5 %). The slight difference in male proportion from that of female proportion was statistically insignificant ($P = 0.647$).

Three single nucleotide polymorphism (SNP) alleles were estimated and these were ORF38, ORF54 and ORF62. All the cases were positive for ORF34 (100.0 %) and the number of cases that were positive for ORF54 were 31 out 43 (72.1 %) whereas, none of the cases positive for ORF62 (0.0%). Accordingly virus

genotype strains were identified. Cases which were positive for ORF34 and ORF54 SNPs were considered genotype B whereas cases which were positive for ORF34 only were labeled as genotype A. However, no case was labeled as genotype C (ORF62). Therefore, the most prevalent VZV genotype in Al Diwaniyah governorate was the B (ORF34 & ORF54) genetic subtype (wild type) accounting for 31 out of 43 (72.1 %) whereas, the A genetic subtype accounted for 12 out of 43 (27.9 %). Genotypes A and B were also neither correlated significantly to age of children nor gender of children.

Over expression of VZV-MCP was seen in (65.1 %) of cases; over expression of VZV-ICP4 was seen in (34.9 %) of cases and over expression of VZV-ICP22 was seen in (44.2 %) of cases. So far, gene expression of VZV-MCP was the highest. In conclusion the most prevalent VZV genotype in Al Diwaniyah governorate was B genetic subtype (wild type) than other.

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List of Abbreviations

CD4+	Cluster of Differentiation 4
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CDC	Centers for Disease Control
cDNA	Complimentary DNA
CMI	Cell-mediated immunity
CNS	Central Nerves System
CT value	Computer Technology
DNA	DeoxyriboNucleic Acid
DRG	dorsal root ganglia
dsDNA	Double stranded DNA
DTT	DiThioThreitol
E Genes	Early Genes
EM	Electron microscope
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gB	Glycoprotein B
GCC	Gulf Cooperation Council
gH	glycoprotein H
gL	glycoprotein L
GP	general practitioner
HHV	Human herpesvirus
HSV	Hepres simplex virus
HZ	Herpes zoster

ICP	Internal Capsid Protein
IE	immediate-early protein
IF	immunofluorescence
IgG	Immunoglobulin G Antibody
IQ	Iraq Country
IR	internal repeat
kbp	kilo base pairs
L genes	Late genes
MCP	Major Capsid Protein
NCBI	National Center for Biotechnology Information
ng/mL	Nanograms per Milliliter
Oka Vaccine	Okinawa, Japan Vaccine
ORF	Open Reading Frame
OriS	Origin of Replication
PA-gold	protein A-gold
PCR	Polymerase Chain Reaction
PHN	post herpetic neuralgia
RFLP	Restriction Fragment Length Polymorphism
RNA	RiboNucleic Acid
SCID	severe combined immunodeficiency
SNP	Single Nucleotide polymorphism
Sp1	specificity protein 1
SPSS	Statistical Product and Service Solutions
TBE buffer	Tris Borate Edta buffer
TG	Trigeminal Ganglia
TGN	trans-Golgi network

TR	terminal repeat
UAE	United Arab Emirates
UL	unique long
US	unique short
VZV	Varicella zoster virus
VZV -VLT	VZV latency-associated transcript
WHO	Word Health Organization

Chapter One

Introduction and Literatures Review

1. Introduction and Literatures review

1.1. Introduction

Varicella-Zoster virus (VZV) is one of the human herpes viruses (HHVs); it's official name known as (*Human Herpes Virus -3*) HHV-3, a member of DNA-containing *Herpesviridae* family. VZV causes varicella (chickenpox) as a primary infection and herpes zoster (HZ) after the reactivation of a latent VZV. Varicella is a childhood illness with highest incidence between 1 and 9 years of age, characterized by fever and a generalized pruritic vesicular rash. It is usually a mild-to-moderate illness in immunocompetent individuals but sometimes can cause serious complications such as central nervous system (CNS) involvement, pneumonia, secondary bacterial infections, and death. The severity of varicella is highly associated with pregnancy, infancy, elderly, and among immunocompromised individuals, however, in immunocompromised individuals, the reactivation of latent VZV results in more serious painful illness (Steiner *et al*.,2007)

Varicella is a worldwide infection, is more prevalent in temperate climates than tropical ones, and often occurs in late winter and spring seasons; furthermore, varicella transmitted by the respiratory aerosols from infected individuals and by direct contact with skin lesions of individuals affected by VZV. (Mueller *et al*., 2008)

Universal vaccination against primary VZV infection first introduced in the United States in 1995, a live-attenuated varicella vaccine, and then in the same year, the World Health Organization (WHO) adopted a mass vaccination against varicella in most developed countries. (Marin *et al*., 2007)

In developing countries generally and in the Middle East particularly, the status of varicella infection and vaccination is unclear. The Middle East is a transcontinental region centered on Western Asia and Egypt and includes the Gulf Cooperation Council (GCC) countries (Saudi Arabia, Kuwait, Bahrain,

Qatar, Oman, and United Arab Emirates [UAE]), in addition to Iraq, Yemen, Syria, Jordan, Lebanon, Palestine, Iran, Turkey, and Cyprus. Some Middle East countries have reported on varicella burden, seroprevalence, complications, and eventually the cost of medical care of hospitalization. To develop a vaccination protocol and appropriate preventive health care measures against a disease in different countries. (Kanra *et al*., 2002)

Varicella in unvaccinated persons characterized by the rash is generalized and pruritic. It progresses rapidly from macular to papular to vesicular lesions before crusting. Lesions are typically present in all stages of development at the same time. The rash usually appears first on the chest, back, and face, and then spreads over the entire body. The lesions are usually most concentrated on the chest and back. In healthy children, varicella is generally mild, with an itchy rash, malaise, and temperature up to 39^0 C for 2 to 3 days. Infants, adolescents, adults, pregnant women, and immunocompromised people are at risk for more severe disease and have a higher incidence of complications. Recovery from primary varicella infection usually provides immunity for life. In otherwise healthy people, a second occurrence of varicella is uncommon. Second occurrence of varicella may be more likely to occur in people who are immunocompromised. As with other viral infections, re-exposure to natural (Wild-Type) varicella may lead to re-infection that boosts antibody titers without causing illness or detectable viremia. (Kasabwala *et al*., 2018)

Symptoms begin ten to 21 days after exposure, but the average incubation period is about two weeks. Chickenpox is a worldwide, airborne disease that spread by coughing and sneezing, and by contact with skin lesions. It may start to spread one to two days before the rash appears until all lesions crusted over. Patients with shingles may spread chickenpox to those who are not immune through blister contact. (Shrim *et al*., 2018).

In this study a sample of children clinically affected by chickenpox are going to be included and then random sample will be subjected to skin scraping of the crusted lesion which will be used for viral DNA extraction followed by conventional PCR to confirm diagnosis, real time PCR to identify MCP, ICP22 and 4 virulence factors gene expression, RFLP for identification of viral ORF38, 54 and 62 and then finally DNA sequencing to identification of viral genetic strains.

1.1.1Aim of the study:

The aim of the present study was to figure out the genetic strains of *Varicella Zoster Virus* (VZV) that are common in Al Diwaniyah governorate. To executing this aim, the following objectives were conducted.

Objectives:

1. Confirmative identification of (*mcp*) gene of*Varicella-Zoster virus*by using conventional polymerase chain reaction (PCR).
2. Gene expression of VZV-MCP, VZV-ICP4 and VZV-ICP22genes by Real-time PCR.
3. Genotyping of *Varicella Zoster Virus* of ORF38, ORF54 and ORF62 in Chickenpox Patients using Restriction Fragment Length Polymorphism (RFLP) Technique.
4. Analyze the partial nucleotide sequences of VZV genome to compare them with the published sequences (Phylogenetic tree).

1.2 Literature Review

1.2.1 Classification and Historical perspective of VZV

The earliest reports of vesicular rashes of herpes simplex and zoster date to the ancient civilizations. It was not until 1888, however, that a relationship between herpes zoster and chickenpox was suggested. Establishing this link represented one of the major hurdles in the history of varicella zoster virus. There was no animal host and this meant that much of the evidence needed to be obtained by clinical and epidemiological observation. Since the link was proven, in the 1950s, the advent of the live attenuated vaccine virus, in 1974, and aciclovir in the 1980s, has had a huge impact on prevention and treatment, respectively. The complete DNA sequence of VZV was established in 1986 (Wood, 2000).

Group: dsDNA virus

Order: *Herpesvirales*

Family: *Herpesviridae*

Subfamily: *Alphaherpesvirinae*

Genus: *Varicellovirus*

Species: *Human herpesvirus 3*(HHV-3) **(**McGeoch, 2009)

1.2.2 Structure of the VZV

1.2.2.1 Structure and Genomic Organization

Varicella-Zoster Virus particles are pleomorphic to spherical in shape, ~150–200 nm in diameter and are composed of three proteinous layers: a nucleocapsid containing the viral double stranded DNA (dsDNA) genome,a tegument layer, consisting of numerous proteins of both viral and host origin

surrounding the nucleocapsid, and an envelope comprising a host-derived lipid bilayer inserted with viral glycoproteins facing outwards (Storlie *et al*., 2008; Arvin, *et al*., 2013). Upon entry of a VZV virion into the host cell, tegument proteins are released into the newly infected cell, altering the host environment, thereby inhibiting antiviral responses and influencing the fate of the virus program, i.e., a lytic or latent infection (Penkert *et al*., 2011). The VZV dsDNA genome is about 125 kilo base pairs (kbp) in size and has a G + C content of46%. It is composed of two unique segments, termed unique long (UL) and unique short (US), that are flanked by inverted terminal repeat (TR) and internal repeat (IR) structures with high G + C contents(68% for the TRL/IRL and 59% for the IRS/TRS) (Depledge *et al*., 2018). The very short (~88 bp) TRL andIRL sequences flank the UL region, while the long (7319 bp) IRS and TRS sequences flank the US region. The composite structure generally allows for two isomeric configurations (Dumas *et al*., 1981) that differ only in terms of whether the US region is inverted. While up to 5% of virions are reported to contain structural isoforms with an inverted UL region (Kinchington *et al*., 1985), their relative scarcity is attributed to a unique DNA sequence at the extreme 50 end of the UL region that is required for viral DNA cleavage during packaging (Kaufer *et al*., 2010) i.e., these virions may not contain functional genomes. Five regions of the genome contain tandem direct reiterations (R1, R2, R3, R4, and R5) of short repeat sequences, one of which (R4) is located in the IRS/TRS. All except R5 are G + C rich, and all are subject to length and structural polymorphisms that vary both within and between strains. Three of these reiterative regions (R1, R2, and R3)are located within the coding portion of VZV genes (open reading frame (ORF) 11, ORF14, and ORF20,respectively) and may therefore exert an effect on protein function. Two copies of R4 are present in the IR and TR, neighboring the origin of

replication (OriS), while R5 is located between ORF60 and ORF61. (Sauerbrei *et al*., 2006; Depledge *et al*., 2014)

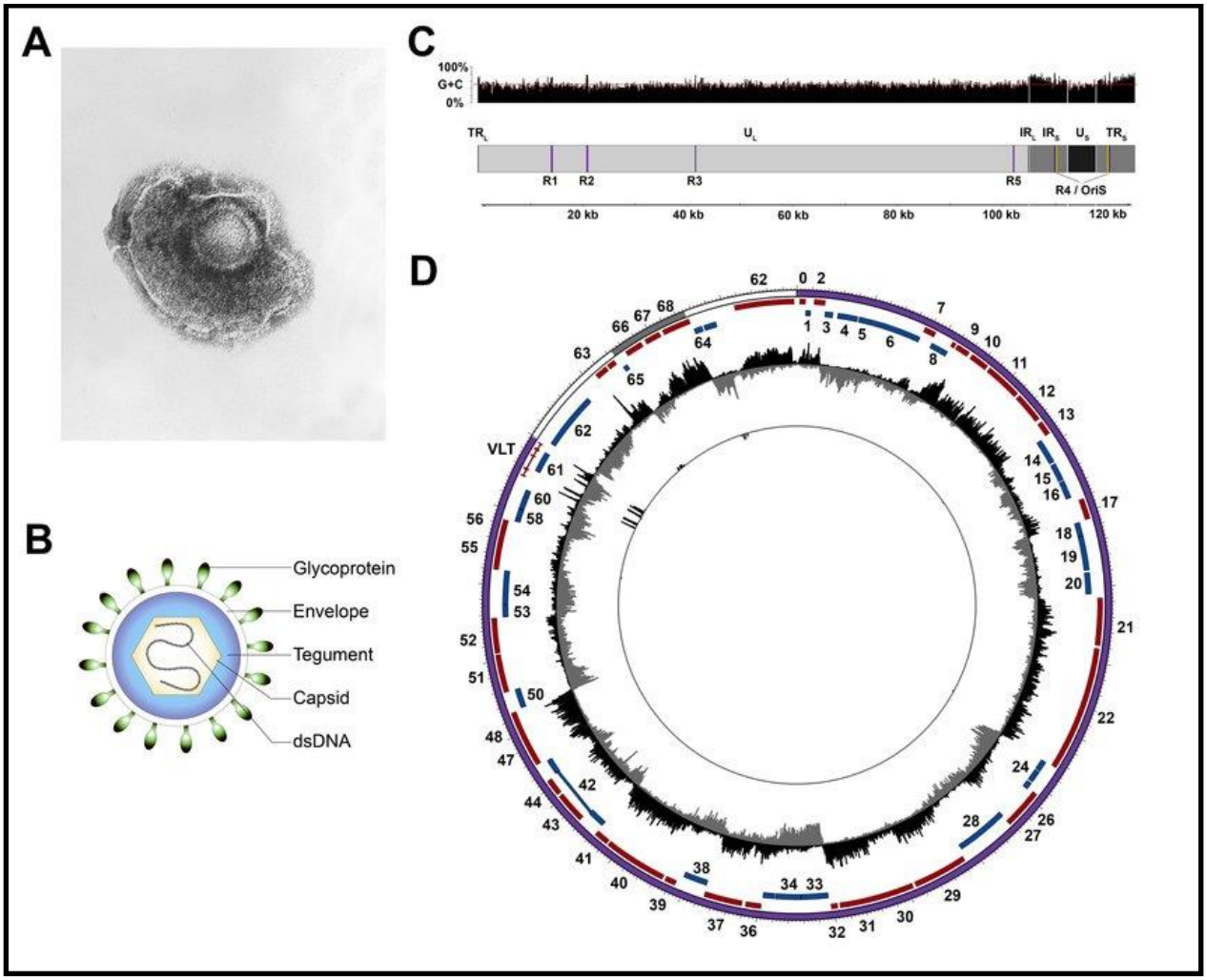

Figure 1.1: Structure of varicella-zoster virus (VZV) particles and genome. (**A**) Electron microscopy image of VZV (obtained from Centers for Disease Control and Prevention (CDC)/Dr Erskine Palmer; B.G. Partin. (Depledge *et al*., 2018).

1.2.2.2 Coding Potential of the VZV Genome

The VZV genome was originally reported to encode 65 unique viral genes, three of which are located in the duplicated IRS/TRS region (Depledge *et al*., 2018). Four additional VZV genes have since been identified including ORF0 (Kemble *et al*., 2000), ORF9A (Ross *et al*., 1997), ORF33.5 (Preston *et al*., 1997), and the newly discovered VZV latency-associated transcript (VLT) (Depledge *et al*., 2018). An underappreciated feature of VZV is that transcription of several genes, including ORF0, ORF42/45, ORF50, and VLT, require the host-splicing machinery to remove introns from pre-mRNA and have also shown evidence of alternative splicing, resulting in the synthesis of alternative proteins (Peters *et al*., 2012). It thus seems likely that the full transcriptional potential of VZV has yet to be revealed, and we predict that the latest technological advances (e.g., full length sequencing of native RNA) will yield further novel discoveries. It is also worth noting that the encoding of additional RNA types, including micro RNAs and small non-coding RNAs, is still an area of active study with contrasting results (Markus *et al*., 2017).

1.2.3 Replication cycle

Like those of other herpesviruses, VZV particles are presumed to enter cells by fusion of the virion envelope with the plasma membrane or by endocytosis followed by the transport of capsids and associated virion tegument proteins to the cell nucleus (Reichelt *et al*., 2009). The major VZV transactivating protein, referred to as immediate-early 62 (IE62) is a tegument component, as are other VZV regulatory proteins, including IE4, ORF10, IE63, and the viral kinases ORF47 and ORF66 (Kinchington *et al*., 1995). As has been demonstrated in cells infected with HSV and other herpesviruses, VZV gene transcription is believed to occur in a cascade that leads to the synthesis of viral proteins that are classified as

immediate-early, early, and late, based on the time course of their expression after virus entry (Cohen *et al*., 2007). Studies using VZV-infected cells to inoculate uninfected cells in conjunction with metabolic pulse-labeling of newly synthesized proteins and Western blot analysis have indicated that viral proteins are expressed by 4 to 6 h after infection (Reichelt *et al*., 2009). However, because VZV is so highly cell associated in cultured cells, experiments that reveal the timing of gene transcription or the spatiotemporal characteristics of VZV protein expression in single cells within one infectious cycle have not been performed (Cohen *et al*., 2007). Achievable titers of cell-free VZV are too low to permit synchronous infections of cultured cells, as is done to define the kinetics of viral mRNA and protein synthesis for HSV-1 and other herpesviruses (Chen *et al*., 2004). Therefore, information is lacking, and there is some controversy about when and where VZV proteins are expressed in newly infected cells, how the assembly of VZV nuclear replication compartments is orchestrated, the time required to complete one infectious cycle, and the role of cell-cell fusion in VZV propagation, which is of interest, given the extensive syncytium formation that characterizes VZV replication (Cole and Grose, 2003).

Varicella Zoster virus experiments are usually done by adding an infected-cell inoculum of human fibroblasts or melanoma cells to a monolayer of uninfected cells. Initial events during replication are assessed by using low numbers of infected inoculum cells as a means to enrich for newly infected cells. Infection is then monitored for 24 to 72 h to demonstrate viral spread within the monolayer and to allow enough new VZV protein synthesis for detection by Western blotting, confocal microscopy, or other methods. Since VZV is not released into media, secondary plaque formation does not occur during the 72-h interval. Many important parameters of VZV genome replication, protein expression, and virus-

host cell interactions have been defined by using this approach, these experimental conditions are not compatible with generating an accurate time-resolved analysis of events in the VZV replication cycle because the infected cells are a mixed population that reflects different stages of viral infection (Cohen *et al*., 2007).

Overcoming the experimental challenges to studies of the VZV replication cycle requires a strategy that permits the use of high numbers of infected inoculum cells so that enough cells are infected to evaluate the earliest time points while allowing unequivocal discrimination of the inoculum and newly infected cells at sequential time points. To address these challenges, we used methods to label either the input cells (VZV-infected inoculum cells) or the output cells (uninfected cells) and investigated the VZV infectious cycle, including the VZV genome and viral protein synthesis, by confocal immunofluorescence (IF) or by standard electron microscopy (EM). Analyses using confocal microscopy were done by labeling VZV-infected inoculum cells with fluorescent cell dyes. In EM experiments, the cells in the uninfected monolayer were preincubated with protein A-gold (PA-gold) beads before being inoculated with VZV-infected cells, allowing their identification as newly infected cells based on detection of the PA-gold particles in the cytoplasm (Möbius *et al*., 2003).

Varicella Zoster virus infects the human host when virus particles reach mucosal epithelial sites of entry. Local replication is followed by spread to tonsils and other regional lymphoid tissues, where VZV gains access to T cells. Infected T cells then deliver the virus to cutaneous sites of replication. VZV establishes latency in sensory ganglia after transport to neuronal nuclei along neuronal axons or by viraemia. Reactivation from latency enables a second phase of replication to occur in skin, which typically causes lesions in the dermatome that is innervated by the affected sensory ganglion. (Zerboni *et al*., 2014)

Enveloped VZV particles attach to cell membranes, fuse and release tegument proteins. Uncoated capsids dock at nuclear pores, where genomic DNA is injected into the nucleus and circularizes. On the basis of events that have been documented in herpes simplex virus 1 (HSV-1) replication, immediate-early genes are expressed, followed by early and late genes. Nucleocapsids are assembled and package newly synthesized genomic DNA, move to the inner nuclear membrane and bud across the nuclear membrane. Capsids enter the cytoplasm, and virion glycoproteins mature in the *trans*-Golgi region and tegument proteins assemble in vesicles; capsids undergo secondary envelopment and are transported to cell surfaces, where newly assembled virus particles are released (Zerboni *et al*., 2014).

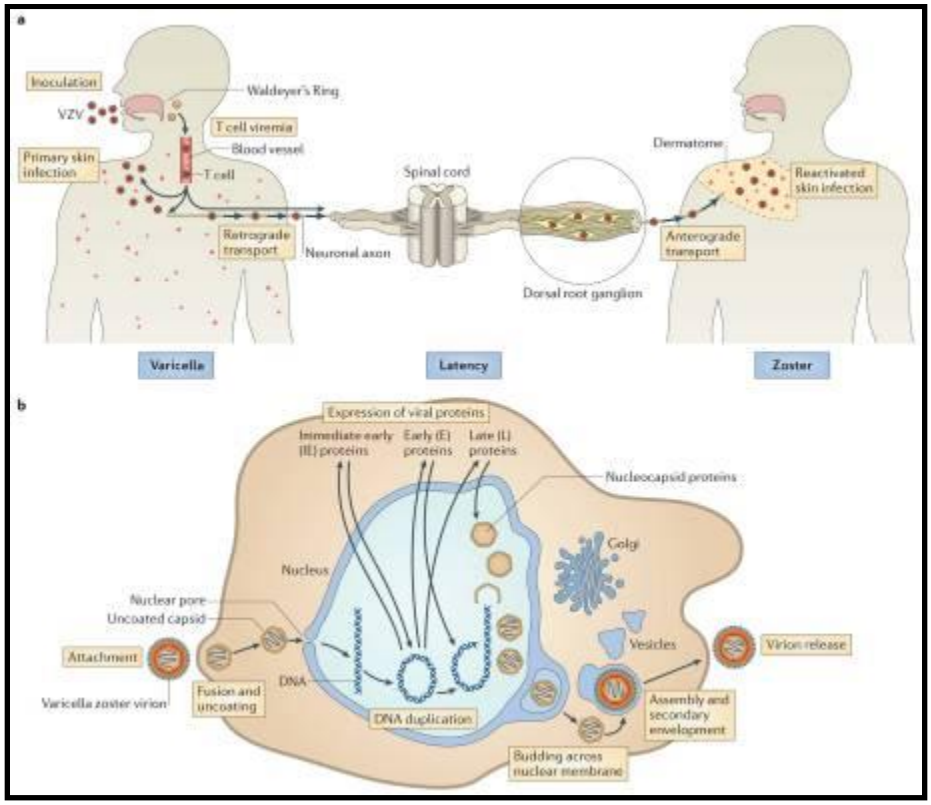

Figure 1.2a | Model of the varicella zoster virus (VZV) life cycle.(Zerboni *et al*., 2014).

1.2.4 Epidemiology of VZV

Epidemiology of varicella is apparently different according to the climate: while in temperate regions, up to 90% of children are infected before the age of 10, only small proportions of children are seropositive in tropical and subtropical regions. Thus, the susceptibility to varicella is more common among adults living in tropical regions than in temperate climate (Hoseini *et al*., 2016).

Varicella is a highly contagious disease caused by Varicella Zoster virus (VZV). The disease is usually benign in immunocompetent children but can be life-threatening in adults and immunocompromised individuals, with an attack rate approaching >85% after exposure (Singh *et al*., 2011). Humans are the only known hosts for this virus, which exists as only one recognised serotype. Viral shedding occurs from the nasopharynx via droplets and aerosols and also from the skin lesions. The incubation period of the disease is usually 10-21 days. The contagious period starts 1-2 days before the appearance of the exanthem and lasts till all the vesicles have crusted, usually within 5-7 days (Singh *et al*., 2011).

The incidence of Varicella in temperate climates is 13-16 cases per 1000 people per year, and is highest in children aged 1-9 years old, although an increased incidence has been observed in children younger than 5 years due to attendance at child care centers. By contrast, in tropical countries like India, the incidence of Varicella is higher in adults (Balraj and John, 1994). Authors have postulated the concept of epidemiologic interference by the high prevalence of certain childhood viruses in the developing countries; the interference is said to postpone the age of Varicella infection. In both temperate and most tropical climates, the incidence of Varicella shows pronounced seasonality, with peaks occurring in the cooler months during winter or spring (Lee, 1998). In temperate climates, epidemics of Varicella have been reported to occur every 2-5 years. The

overall case fatality rate in developed countries is 2-4 per 100,000 cases, with the risk of death being highest at the extremes of age. The rate of hospital admission for all ages is 2-6 per 100,000 population, with most admissions occurring in children (Singh *et al*., 2011).

In Saudi Arabia, the overall seroprevalence of VZV was 68%. The study also showed a progressive increase of immunoglobulin G (IgG) antibodies against VZV with age as 90% of adults showed the presence of antibodies. In addition, the varicella seroprevalence was reported to increase with age, with 20% in those aged 1 year, 40% in those aged 4 years, 60% at age 6 years, 80% at age 8 years, 85% at age 10 years, and 85%–90% in those over 10 years (Al-Turab *et al*., 2018).

A seroepidemiological study has been conducted to study the pattern of exposure and immunological responses to VZV infection among 92 children from hospitals and clinics between 2007 and 2008. The overall VZV antibody prevalence was 53.3% for both serologic and cellular responses. It was also found that a difference in the immune responses to VZV infections between male and females and the rates of exposure to VZV infections was higher in urban areas than in rural areas (Yassien and Hasony, 2012).

1.2.5 Pathology and Pathogenesis of VZV

Varicella zoster virus (VZV), which is a human alphaherpesvirus of the genus *Varicellovirus*, causes varicella (also known as chickenpox) and zoster (also known as shingles)(Arvin and Gilden, 2013). Epidemiological evidence suggests that primary VZV infection begins with replication in epithelial cells of the upper respiratory mucosa, which is followed by the widely distributed vesicular rash that is typical of varicella after an incubation period of 10–21 days. This pattern probably reflects viral spread to the tonsils and other local lymphoid tissues, from

where infected T cells can transport the virus via the bloodstream to the skin (Ku *et al.*, 2004). During primary infection, virions presumably gain access to the sensory nerve cell bodies in ganglia by retrograde axonal transport from skin sites of replication or by T cell viraemia, and latent infection is established (Gilden *et al*., 1983). When viral replication is reactivated, VZV reaches the skin via anterograde axonal transport to cause the symptoms of zoster, which is characterized by a vesicular rash in the dermatome that is innervated by the affected ganglion. Both varicella and zoster skin lesions contain high concentrations of infectious virus and are thus responsible for transmission to susceptible individuals. Varicella epidemics occurred annually in the United States until a varicella vaccine (which is a live attenuated form of the VZV Oka strain) was introduced in 1995, but epidemics continue among children in countries that do not have immunization programs (Gershon *et al*., 2010).

Varicella zoster virus genome has at least 71 known or predicted ORFs (Cohen *et al*., 2010). Similar to all herpesviruses, VZV has a lipid-rich envelope, which is acquired from cellular membranes and into which viral glycoproteins are inserted. Within the envelope, a tegument layer that is predominantly composed of viral regulatory proteins surrounds an icosahedral nucleocapsid core that contains the linear double-stranded DNA genome (Arvin and Gilden, 2013). The viral life cycle begins with VZV entry, which is a poorly understood process that is presumed to involve either direct fusion of viral particles with the plasma membrane or endocytosis . Viral envelope proteins are predicted to interact with cell surface molecules, such as mannose-6-phosphate receptor or myelin-associated glycoprotein (Suenaga *et al*., 2010). VZV glycoprotein B (gB), gH and gL function as the core fusion complex, but other envelope glycoproteins probably contribute as accessory proteins (Suenaga *et al*., 2010). After entry, the virions undergo

uncoating, and tegument proteins, including the immediate-early protein 62 (IE62) which is the major viral protein that functions as a transcription factor (that is, as a viral transactivator) (Arvin and Gilden, 2013) are released and might be transported to the nucleus before *de novo* protein synthesis occurs. Nucleocapsids anchor at nuclear pores, where viral genomes are injected into the nucleus. VZV genome replication and viral gene expression depend on virus-encoded and host cell transcription factors and cellular translation systems (Ruyechan, 2010). Furthermore, the tegument proteins ORF47 and ORF66 are important serine/threonine kinases that autophosphorylate and phosphorylate viral transcription factors and other VZV proteins .IE62 forms regulatory complexes with cellular factors, such as transcription factor specificity protein 1 (Sp1), which has binding sites in many viral promoters (Ruyechan, 2010), to transactivate VZV genes. Similarly to other herpesviruses, nucleocapsids undergo primary envelopment, fusion with nuclear membranes and de-envelopment during transfer to the cytoplasm. Secondary envelopment occurs in the cisternae of the *trans*-Golgi network (TGN), where the capsids acquire tegument proteins and glycoprotein-containing membranes. Nascent virus particles then move to the cell surface in post-Golgi compartment vesicles; the first enveloped progeny virions are detected 9 hours after infection and many are present on cell surfaces within 12 hours of infection (Reichelt, 2009). VZV differs from other herpesviruses in that assembled virions typically remain highly cell-associated. The same viral glycoproteins that are predicted to mediate entry are expressed on cell membranes and induce fusion of infected and uninfected cells, producing syncytia and multinucleated polykaryocytes, which further contribute to virus spread (Oliver *et al*., 2013).

Investigating VZV pathogenesis is challenging as VZV is a highly human-specific virus that has little or no capacity to infect other species. This obstacle can

be overcome by using human tissue xenografts in mice with severe combined immunodeficiency (SCID). Infecting foetal thymus-liver T cell, skin and dorsal root ganglia (DRG) xenografts enables studies of the three major tissue tropisms of VZV: T cell-, skin- and neuro-tropism (Zerboni *et al*., 2005). In these models, innate responses that modulate infectious processes can be assessed independently of adaptive immunity, which is absent in SCID mice. VZV-specific T cells are necessary to clear primary infection and prevent symptomatic reactivation from latency, but the xenograft models show the importance of intrinsic responses of differentiated cells in the absence of an adaptive immune response. Such studies can be done in knockout mouse models that have defects in adaptive immunity, but VZV does not infect mice. Furthermore, the xenograft models have the advantage of investigating infection in the various human tissue microenvironments that are targeted by VZV. Inoculating human tissue xenografts with mutant VZV can show functions of viral genes that are dispensable in tissue culture but necessary under the more stringent conditions that are present in intact tissues and fully differentiated human cells *in vivo*. To investigate which host cell factors are required during infection, small-molecule inhibitors or antibodies that block cell functions can be administered (Zerboni *et al*., 2014).

1.2.5.1 *Varicella Zoster virus* Gene Expression during Productive Infection

Based on limited experimental data and mostly by analogy to other alphaherpes viruses, VZV genes have been loosely designated as immediate-early (IE) genes, early (E) genes, and late (L) genes, with each ordered wave of expression thought to be dependent on the protein products of previous classes. Proteins encoded by IE genes act as transcriptional regulators, while those produced by E genes are mainly involved in DNA replication, and L genes encode

structural proteins that are crucial forvirion formation and egress.(Roviš *et al*., 2013).

Varicella Zoster virus encodes at least five transcriptional regulatory proteins specified by four putative IE genes, ORF4, ORF61, ORF62 and ORF63, and one L gene, ORF10. All except the ORF61protein, IE61, are part of the VZV virion (Kinchington *et al*., 1995). Our understanding of the transcriptional regulation of VZV genes remains incomplete, in part due to the high cell-associated nature of VZV that precludes synchronized infections using cell-free viruses. The dominant transcriptional regulator and possibly only true immediate-early protein encoded by Varicello virus is homologous to VZV IE62(Yang *et al*., 2006; Khalil *et al*., 2016).Consistent with this idea, the VZV IE62 major viral transactivator protein can activate all three kinetic classes of VZV genes in the absence of other viral proteins, including all IE genes, ORF4, ORF61, ORF62,and ORF63, while IE4, IE61 and IE63 either do not or minimally stimulate the ORF61 promoter (Wang *et al*., 2009; Ruyechan *et al*., 2010). Host transcription factors, either by themselves or through interactions with viral transcriptional regulatory proteins, also contribute to viral gene expression (Ruyechan *et al*., 2010). VZV virion proteins delivered into newly infected cells upon entry are not absolutely required to initiate VZVgene expression, as evidenced by the resulting VZV replication upon transfection of cells with viral DNA (Cohen, 2010). Notably, near identical VZV transcriptomes are detected during productive infection of diverse cell types, including neurons, suggesting a prominent role for either commonly expressed cellular transcription factors or viral proteins in coordinating VZV gene expression (Jones *et al*., 2014; Baird *et al*., 2014; Markus *et al*., 2014; Sadaoka *et al*., 2016).

The MCP stands for the major capsid protein which is the basic unit for the synthesis of the icosahedral structure necessary for viral assembly. Therefore over

expression is detected at maximum viral replication during acute stage (Cohen, 2010). On the other the two internal capsid proteins encoded by genes ICP-4 and ICP -22 are necessary for viral replication and transition for acute into more latent phases, respectively (Reichelt *et al*., 2009). Internal capsid protein ICP-4 is mandatory for viral replication since it functions through blockage of the antigen presentation associated transporter by binding to it and by this way it prevents the cell from viral antigen presentation and gets protected against cytotoxic T cell recognition and cell elimination (Erazo *et al*., 2008); whereas, ICP-22 is necessary for viral replication since its product regulates viral genes that are necessary to transmission form early acute phase into more latent stages (Cohen, 2010).

The virulence factors MCP, ICP4 and ICP22 have been mentioned in the section concerning viral genomic structure.

1.2.5.2 Molecular Genetics and Pathogenesis of VZV

Molecular epidemiological tools have also provided insights into VZV pathogenesis. Restriction enzyme analyses to genotype VZV causing varicella and zoster in the same patient proved Hope Simpson's hypothesis that zoster is due to reactivation of latent VZV originally acquired from chickenpox. This finding was confirmed in 3 additional patients. All of these patients were immunosuppressed, with primary varicella and zoster occurring close together. In 2006, we made use of SNP genotyping to investigate a case of recurrent zoster in an immunocompetent man. This young man, who had a self-reported history of a single varicella episode at 5 years of age, presented as an adult with 2 episodes of zoster 3 years apart, with the first case in the ocular division of the left trigeminal nerve and the second in the left thoracic region. (Norberg *et al*., 2015)

Genotyping showed the first strain to be a clade 5 strain and the second to be a clade 4 strain. Microsatellite typing of human DNA associated with the strains proved that the host was identical in both cases, thus ruling out laboratory contamination. This suggested that infection with 2 different viruses had occurred and that both had established latency and reactivated. The result overturned previous dogma that zoster is always caused by the strain of virus that caused primary varicella. Furthermore, the data corroborated findings from earlier studies, which, using *Bgl*1 genotyping, had shown that zoster due to *Bgl*1-negative viruses (clades 1 and 3) occurred in 30% of adults who had immigrated to the United Kingdom from areas where *Bgl*1-positive (clade 2/4/5) viruses circulate [19]. None of these adults had a history of varicella while in the United Kingdom. Together with studies of healthcare workers who received vOka vaccine and developed proven infection due to wild-type virus after having seroconverted to the vaccine strain, these findings confirm that asymptomatic reinfection of latently infected individuals is part of the natural history of VZV, with establishment of latency plus reactivation, at least in some cases, by the second strain. These findings have been further corroborated by studies showing that 86% of immunized children have wild-type VZV within their enteric neurons despite lacking a history of varicella. Importantly, most reinfections are asymptomatic with no evidence of skin rash, supporting the likelihood that hematogenous transfer of virus to ganglia is possible. (Gershon *et al*., 2012)

The evidence that VZV is highly recombinant provides conclusive evidence that coinfections must be occurring, although where in the body the process of viral recombination takes place remains a matter for conjecture. Intriguingly, genotyping of viruses from varicella outbreaks in the United Kingdom and Guinea Bissau showed that multiple strains can co-circulate within a single outbreak. In

the former outbreak, there were strains from different clades, and in the latter there were 4 distinct strains from clade 5. We have also demonstrated co-infection with 2 different viruses within the same individual. In that study, limiting dilution PCR analysis allowed genotyping of single molecules, providing evidence that a swab of vesicle fluid from a child with chickenpox contained clade 1 and clade 3 viruses in a ratio of 3:1 (Norberg *et al*., 2015)

1.2.6 Clinical features of VZV

Varicella-zoster virus (VZV) is a pathogenic human alpha-herpesvirus that causes chickenpox (varicella) as a primary infection, which usually occurs in children in locales where vaccination is not practiced (Gershon *et al*., 2015). Following the primary infection, this neurotropic virus becomes latent, primarily in neurons in peripheral autonomic ganglia throughout the entire neuroaxis including dorsal root ganglia (DRG), cranial nerve ganglia such as the trigeminal ganglia (TG), and autonomic ganglia including those in the enteric nervous system(Nagel and Gilden, 2007).

Symptoms of VZV begin with a rash, low-grade fever, and malaise, all of which are more severe in adults. Some patients develop a prodrome before the vesicular rash. Skin lesions progress over hours from erythematous macules and papules to vesicles and then to crust (scabs). As a result, vesicles at various stages of evolution are found on the skin. New batches of lesions appear every few days, starting on the face and trunk, and spread to the extremities in a so-called "centripetal spread." Adults tend to have more numerous, larger lesions, while immunocompromised individuals often develop lesions with more prominent hemorrhagic bases that take longer to heal (Leonid and Evelyn, 2009).

Varicella zoster virus is a double-stranded DNA virus with a genome of just under 125,000 base pairs and it contains 68 unique open reading frames (ORF) (Kennedy and Cohrs, 2010). The mechanisms of VZV latency are slowly being unravelled, but several issues remain to be clarified. It is known that during ganglionic latency, VZV DNA is located predominantly, if not exclusively, in neurons in which it is present in a nonintegrated form, probably as endless episomes of unit or concatemeric length (Kennedy *et al*., 2010). It has been known for some time that viral transcription during latency is highly restricted, with transcripts for VZV gene 63 being the most commonly detected transcript, and previous work using different techniques has also reported transcription of VZV genes 21, 29, 62, and 66 (Cohrs and Gilden, 2007). However, a problem with many previous reports is that the ganglia obtained at autopsy have been studied only after 12–48 h after death, at which time the process of viral reactivation may well have already started. Indeed, when human ganglia were analyses at less than 9 h after death, no transcripts for VZV were detected, though VZV ORF63 transcript levels in human TG increased with longer postmortem intervals (Ouwendijk *et al*., 2012). This study suggested that expression of other VZV genes previously detected was probably a reflection of viral reactivation, a view that is supported by the detection by multiplex polymerase chain reaction (PCR) of several VZV ORFs, including those other than those just corresponding to immediate-early or early transcripts(Nagel *et al*, 2011). On the other hand, studies of human enteric ganglia removed during gastrointestinal surgery from children immune to varicella and placed immediately in "RNA later" solution revealed transcripts for ORFs 63, 4, and 66(Gershon *et al*., 2012). One possibility is that when analyzing human ganglia for VZV latency, both true latent transcripts and those indicating a degree of low-level viral reactivation are being detected unless the ganglia are studied prior to 9 h postmortem. Very recently, a unique spliced latency-associated VZV

transcript was detected in human TG neurons which maps antisense to the viral transactivator gene 61. Since the latter ganglia studied had been obtained at about 6 h after death, it is clear that this could not have been detected due to viral reactivation. Given the inconsistent results in various laboratories, the molecular status of VZV during latency needs further study (Depledge *et al*., 2018)

1.2.6.1 Varicella Disease "Chickenpox"

The primary infection with VZV is varicella, commonly known as chickenpox. Varicella is highly contagious; it is most commonly seen in children under the age of 10 years in countries where live attenuated varicella vaccine is not routinely administered; the major feature of the illness is a vesicular pruritic rash that occurs mainly on the trunk, head, and face. The extremities are somewhat spared; skin vesicles are full of infectious, well-formed virus which are aerosolized and serve to transmit VZV to others who have not had the disease previously. The skin lesions commonly occur in crops and progress from papules to vesicles to crusts over a few days. There may be anywhere from a few to many hundreds of vesicles, with an average of about 500. More severe cases manifest more severe rashes and take longer to heal. Concomitant symptoms include malaise, fever, and fatigue, and the illness usually lasts about a week. Complications include bacterial super infection of the skin, encephalitis, and pneumonia. Adults and immunocompromised patients are more prone to severe infections than healthy children (Gershon *et al*., 2015).

Individuals who have received live attenuated varicella vaccine may still develop varicella after an exposure to the virus (either a person with varicella or one with zoster). Patients with zoster can transmit varicella to others but with a lower attack rate of chickenpox than those with primary infection (Gershon*et al*., 2013).Vaccinees who nevertheless develop varicella usually have mild cases with

fewer vesicles and complications. This situation is termed "breakthrough varicella" and is less contagious than primary varicella. Vaccinees who have had only one dose of vaccine are much more likely to transmit the virus to varicella susceptible who are exposed than those who have had two doses of vaccine. When a person develops varicella despite receiving two doses of vaccine, the disease is often very minor and may be difficult to diagnose as varicella both clinically and in the laboratory (Kennedy and Gersho, 2018).

The diagnosis of VZV infection is usually made clinically by the appearance of the skin rash. In confusing or unusual appearing cases, the diagnosis may be made by identifying VZV DNA in skin lesions by PCR. Culture of VZV from skin lesions may also be used, but it is more expensive, takes more time, is poorly available, and is less sensitive than PCR (Gershon *et al*., 2013). In patients with suspected meningitis or encephalitis and other complications due to VZV, the viral DNA may be demonstrable in cerebrospinal fluid and/or saliva (Gershon *et al*., 2015).

The infants of women with varicella in the first 20 weeks of pregnancy are at about a 2% risk of developing the congenital varicella syndrome. These infants often have a variety of severe abnormalities of their brain, eyes, extremities, and skin and most succumb in infancy or early childhood. They frequently experience recurrent VZV reactivation and may have multiple cases of clinical zoster. Fortunately the syndrome is unusual in that only about 2% of women who develop varicella in pregnancy give birth to an infant with the congenital varicella syndrome.Adults are more likely to experience severe varicella than children. Severe and even fatal varicella, moreover, often occurs in patients who are immunocompromised due to disease or medications such as corticosteroids or cancer chemotherapy (Gershon *et al*, 2013)

. These patients manifest extensive, often haemorrhagic rashes and may also develop complications such as pneumonia, hepatitis, and/or encephalitis all due to VZV. They are likely more prone to develop severe bacterial infections as well. Severe varicella may be prevented to some extent by administration of passive immunization with VariZig, a form of immunoglobulin containing high titers of antibodies to VZV; passive immunization should be administered as soon as possible after a recognised close exposure to VZV in a high-risk person who has never had varicella (Kennedy and Gershon, 2018).

Routine treatment of varicella in otherwise healthy children is not uniformly recommended, although an oral form of the antiviral acyclovir is available. Otherwise, healthy adults and immunocompromised patients who develop varicella should receive treatment. If it develops or seems to be developing, severe varicella should be treated with intravenous acyclovir. For the best outcome, antivirals should be given as soon as possible to immunocompromised individuals and to anyone who seems to be developing severe varicella (Kennedy and Gershon, 2018).

Patients who develop zoster should be treated as soon as possible with acyclovir, famciclovir, or valacyclovir, which are administered orally. If zoster is severe, especially in immunocompromised patients, intravenous acyclovir can be administered, especially at the start of treatment. Although generally well tolerated, adverse effects of antiviral therapy for which clinical monitoring is necessary include gastrointestinal, neurological, and renal toxicity (Arvin and Gilden, 2013).

An important feature of varicella is the development of a viremia that just precedes the appearance of the rash. The virus is carried to the skin in T lymphocytes, where the rash develops. Latency in DRG, Cranial nerve ganglia CNG, and also to autonomic ganglia may be established by two mechanisms

during the viremia and as VZV travels directly from skin to ganglia by anterograde transport to DRG and CNG (Arvin and Gilden, 2013).

1.2.6.2 Herpes zoster Disease "Shingles"

Reactivation of VZV in neurons occurs with unknown frequency but is possibly very common. Over 50 years ago, Hope Simpson postulated that reactivation was frequent and could occur with or without symptoms(Kennedy and Gershon, 2018). The reality of subclinical reactivation was demonstrated when it was determined that one-third of astronauts developed reactivation of VZV transiently during space travel. The diagnosis was made by finding VZV DNA in saliva; the astronauts had no symptoms of zoster and the viral DNA disappeared within a few weeks after return to Earth (Mehta *et al*., 2004). Importantly, it is very rare to isolate infectious VZV from saliva of patients with active or subclinical VZV infections (Cohrs *et al*., 2008).

When symptomatic reactivation of VZV occurs, the condition is termed herpes zoster, often referred to as “zoster”. Although it is now recognised that zoster may occur in the absence of rash, the classical presentation is appearance of a unilateral, dermatomal rash that is painful, pruritic, or both. The causes and mechanisms of reactivation remain unclear, but zoster is associated with a preceding decrease of cellular immunity (CMI) to VZV. Vaccination against zoster is aimed at restoring CMI to VZV to prevent zoster from occurring (Kennedy and Gershon, 2018).

Since the process of reactivation is not fully understood, the incubation period of zoster is unknown. Characteristically, zoster presents with a unilateral vesicular rash on the face, head, or trunk, although it can also occur on the extremities. The vesicles are full of infectious virions that can become airborne and

infect nearby varicella susceptible as chickenpox, although zoster is only about half as contagious as varicella. The zoster rash may be mild and heal quickly or it can be severe with extensive lesions that may last for weeks. The latter possibility is more likely to occur in elderly patients or immunocompromised individuals than others (Gershon *et al*., 2015).

1.2.7 Diagnosis of Chickenpox

Standard laboratory diagnosis has been obtained by culture of the virus in diploid fibroblasts seeded into shell vial cell cultures or directly by immunostaining of viral antigens in infected cells collected by swabs of vesicles from patients. In the past, several studies have demonstrated the superiority of detection of VZV antigens by immunologic techniques or, more recently, by molecular amplification of viral DNA by PCR assays compared with serologic assays for immunoglobulin G or A class antibodies or the cultivation of this virus in cell cultures. Sauerbrei *et al*., 2006 reported that the laboratory diagnoses of VZV infections of 100 patients by culture (20%), antigen detection by immunofluorescence (82%), and serology (48%) were inferior relative to those by PCR (95%) (Espy *et al*., 2000)

There are various methods to differentiate wild type (WT) and vaccine VZV strains, including single stranded conformational polymorphism analysis, long PCR, PCR-RFLP analysis, and Light Cycler Real-Time PCR; although Restriction Fragment Length Polymorphism (RFLP) is the original method and PCR-RFLP widely used (Roycroft *et al*., 2012).

There are three salient single nucleotide polymorphisms (SNPs) in VZV vaccine surveillance and epidemiological studies that could be distinguished by the

RFLP analysis, like open reading frames (ORFs) 38, 54, and 62, each of which had a specific silent base mutation at position 943 (A to G), 744 (C to T), and 2872 (C to T) alleles; these were used to determine the digestion pattern profiles by *Pst*I (for ORF 38), *Bgl*I (for ORF 54) and *Sma*I (for ORF 62) restriction enzymes, respectively (Sauerbrei and Wutzler, 2007).

*Pst*I positive strains (*Bgl*I/*Sma*I negative) were dominant in the Europe and the North America in that wild type strains, *Pst*I and *Bgl*I positive strains (*Sma*I negative) contain the Oka like strains and African or Asian strains. The specific restriction site of the varicella vaccine strains (vOka) was *Sma*I that located on ORF 62. Thus, RFLP could be used to distinguish strains from WT, vaccine, and or their geographical dominance (Loparev *et al*., 2007).

As a function of geographic distribution, VZV has been separated into the five major clades 1 to 5 confirmed by full-genome sequencing (Garcés-Ayala *et al*., 2015).

1.2.8 Complications of Chickenpox

Complications include pneumonia, brain inflammation, and bacterial skin infections. The disease is more severe in adults than in children (Shrim *et al*., 2018).

Varicella is generally mild, but there is an increased risk of complications in immunocompromised individuals and neonates if maternal varicella is temporally close to birth. Nevertheless, severe complications can occur even in previously healthy children, including secondary bacterial infections, central nervous system manifestations and death. Approximately 90% of UK varicella cases occur in children less than 15 years of age, with the highest incidence in the 1–4-year-old age group (Cameron *et al*., 2007).

1.2.9 Vaccination of VZV

The live attenuated varicella vaccine was first developed in Japan by Takahashi. Initially, the vaccine was solely used to protect high-risk leukemic children. In 1989, the vaccine was first introduced to healthy children in Japan and Korea and in 1995 the US Food and Drug Administration (FDA) approved the vaccine for children aged at least 12 months with a negative varicella history (Papaloukas *et al*., 2014).

Luckily, varicella-zoster virus is a vaccine-preventable disease, and the FDA approves the use of the live varicella Virus Vaccine to provide immunity for the prevention of varicella in individuals 12 months and older. The varicella vaccine is used routinely in children with two doses. The first dose is given to children between 12 to 15 months of age, and the second dose is given to children between 4 to 6 years old (Walker *et al*., 2018).

The varicella-zoster vaccine contains live attenuated varicella-zoster vaccine (Oka strain). This vaccine produces an IgG humoral immune response in individuals, and it is well tolerated. There is also a cell-mediated immune response by the mechanism of the varicella-zoster-specific activation of both CD4+ T-helper and CD8+ T-lymphocyte cells. The mechanisms of contributions made by these immunities for protection against varicella is not currently known. The duration of protection is also currently still unknown; however, there is evidence shown in some efficacy trials that the vaccine can offer continued protection for up to ten years after vaccination (Malaiya *et al*., 2015).

The varicella vaccine is contraindicated in individuals who have a severe allergy or have had an anaphylactic reaction to neomycin or gelatin, which are

components of this vaccine, or to the previous dose of a varicella-containing vaccine (Dolan *et al*., 2015).

1.2.9.1 Molecular Genetics of the Vaccine and VZV Natural History

The unique opportunity posed by having a live vaccine that results in varicella-like and zoster-like rashes prompted us to compare the genome sequences of the infecting virus (ie, the vaccine virus) with that of the virus recovered from rashes. This provided a model for the natural history of VZV, a human-restricted virus for which there are no tractable animal models that accurately reproduce its natural history. Using statistical genetic analyses, we established that the live vaccine is highly stable from batch to batch (Depledge *et al*., 2014); that, following inoculation, all viruses within the vaccine are capable of establishing latency and reactivating [45]; and that there were no neurotropic variants within the vaccine and no evidence of selection acting on any of the vOka variants. (Weinert *et al*., 2015)

Mathematical modeling of the results yielded 3 important findings. First, the vaccine virus evolves rapidly, at a rate of approximately 10^{-3} substitutions/site/year. This is about 10 times faster than has previously been calculated from dated-tips calculations for short-term evolution of double-stranded DNA viruses. A rate of 10^{-6}–10^{-5} substitutions/site/year was calculated from circulating VZV transmitted in varicella outbreaks. Conversely, a rate of 10^{-9} substitutions/site/year was based on a small subset of highly conserved genes and calculated over long periods. (McGeoch *et al*., 2009)

The new estimates of VZV mutation rates date the origins of the clades to between 20 and 50000 years ago, which is earlier than hitherto supposed but fits with other estimates and with evidence for considerable rates of recombination and

of global spread of African and Asian strains associated with population migrations (Norberg *et al.*, 2015).

Second, that most individual VZV vesicles arise from a single virion, with a few apparently originating from up to 3 virions. Individual vesicles within the same zoster rash harbored different varicella strains. Third, we were able to show that mutation rates for viruses recovered from varicella rashes were several logs higher than for viruses recovered from zoster rashes. In the former, the mutation rate remained constant with time, while in the latter, the mutation rate declined over time. The best explanation for these findings is that viruses reactivating to cause herpes zoster are not replicating during the time that they are latent in neurons and therefore do not acquire mutations. When the mutation rates derived from replicating viruses that had caused varicella were applied to the zoster-associated viruses, that the latter viruses had replicated for a mean of 13 days during the period of establishing latency and reactivating to cause the zoster rash. These findings provide a unique insight into VZV natural history in its cognate host. (Weinert *et al*., 2015)

Chapter Tow

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Instruments and Equipment:

The instruments and equipment used in this work with remarks are listed in table (2-1) below:

Table (2-1): Instruments and equipment used with their remarks

Equipment & instrument	Company
High Speed Cold centrifuge	Eppendorf /Germany
Incubator	Mammert/Germany
Microwave	Argose/Germany
Sensitive Balance	Sartorius/Germany
Water Bath	Mammert/Germany
Vortex	CYAN/ Belgium
Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
Refrigerator	Concord /Lebanon
Thermocycler PCR	Mygene /Bioneer
Exispin centrifuge	Bioneer/ Korea
Eppendorf tubes	Biobasic/ Canada
Disposable syringe 5 ml	Sterile EO. / China
Sterile test tube	Superestar/ India

UV Transilluminator	ATTA/ Korea
Gel electrophoresis	Bioneer/ Korea
Digital camera	Samsung/ china
Miniopticon Real Time PCR	Bio-Rad/ USA
Micropestle	Geneaid/ USA

2.1.2 Molecular Diagnosis Kits:

The kits used in this study with their remarks are listed below

Table (2-2): The kits used in this study with their companies and countries of origin

Kit	**Company**	**Manufacture**
gSYNC™ DNA Extraction Kit	**Geneaid Biotech Ltd.**	**USA**
Lysis (GSB Buffer) 25 ml Proteinase K 25 mg 2 vial		
W1 buffer		
Wash buffer Elution buffer		
Spin column		
Collection tube 2ml		

AccuPower™ PCR PreMix	**Bioneer**	**Korea**
Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP)		
Tris-HCl pH 9.0		
KCl $MgCl_2$ Stabilizer and Tracking dye		
Total RNA Extraction Kit AccuZol™	**Bioneer**	**Korea**
Trizol reagent 100ml		
DNase I enzyme kit	**Promega**	**USA**
Dnase I enzyme		
10x buffer		
DEPC water		
Stop reaction		
AccuPower® RocketScript™ RT PreMix 96 plate	**Bioneer**	**Korea**
RocketScript Reverse Transcriptase (200 u)		
X Reaction Buffer (1 x 5)		
DTT (0.25 mM)		
(dNTP (250) µM each		

RNase Inhibitor (1 u)		
AccuPower ® Greenstar ™ qPCR PreMix 96 plate	**Bioneer**	**Korea**
SYBER Green fluorescence		
Taq DNA polymerase		
dNTPs (dATP, dCTP, dGTP, dTTP)		
DEPC water		

2.1.3 Primers

The PCR detection primers and Real-Time PCR gene expression primers were designed in this study by using NCBI-Genbank data base and primers 3 plus, whereas, , RFLP-PCR (ORF) genotyping primers were designed by (**Liu *et al.*, 2009**) and these primers were provided by (Bioneer company, Korea) as following tables:

Table (2-3): The PCR detection primers with their sequence and amplicon size

Primers	**Sequence (5'-3')**		**Amplicon**
Mcp	F	TGACAAATGCTAGGCGGGTT	520bp
	R	CGACGCAACGATTCGGTAAC	

Table (2-4): The Real-Time PCR gene expression primers with their sequence and amplicon size:

Primers	Sequence (5'-3')		Amplicon
qPCR-MCP	F	AACGTTACCGAATCGTTGCG	146bp
	R	ATGGCCACAAACTCACACAC	
qPCR-ICP4	F	TTGTACGCCAGTCAATCTGC	120bp
	R	TAACACAATGCCGTGGTTGC	
qPCR-ICP22	F	AGCACCGATTCTTGTGAACC	132bp
	R	ATATTCCGCGGTTTCTGCAC	
Human Internal control gene (GAPDH)	F	AATTCCATGGCACCGTCAAG	104bp
	R	ATCGCCCCACTTGATTTTGG	

Table (2-5): The RFLP-PCR genotyping primers with their sequence and amplicon size

Primers	Sequence (5'-3')		Amplicon
ORF54	F	CGTAATGCATAACAGGCCAACAC	-
	R	AAACCTGGCGTCAAACATTACA	
ORF38	F	AAGTTTCAGCCAACGTGCCAATAAA	-
	R	AGACGCGCTTAACGGAAGTAACG	
ORF62	F	TTCCCACCGCGGCACAAACA	-
	R	GGTTGCTGGTGTTGGACGCG	

2.1.4 Restriction enzymes

Table (2-6): The restriction enzymes were used in RFLP-PCR assay with their company and country of origin

Restriction enzymes	Target gene	Company/Country
*Bgl*I	ORF 54	New England Biolabs. UK
*Pst*I	ORF 34	New England Biolabs. UK
*Sma*I	ORF 62	New England Biolabs. UK

2.1.5 Chemicals

Table (2-7): All the chemicals materials that used in this with their company and country of origin

Chemical	Company and Origin
Absolute ethanol	Scharlau (Spain)
Agarose	BioBasic (Canada)
TBE buffer	BioBasic (Canada)
Ehidium Bromide	BioBasic (Canada)

Ladder 2000-100bp	Bioneer (Korea)
Free nuclease water	Bioneer (Korea)
Isopropanol	BDH (England)
DEPC water	Bioneer/ Korea

2.2 Methods

2.2.1 Samples collection

Out of the many primary schools in Al Diwaniyah governorate we randomly selected 10 schools including rural and urban areas. The study was approved by the Ethical Approval Committee of College of Medicine/ University Of Al-Qadisiyah, after formal agreements were obtained from the Directorate of the Ministry of Education. Samples from skin were obtained from forty-three children (infected with Chickenpox) after verbal consent has been obtained from parent of each child.

A total of 4545 children were enrolled with an age range of 6 to 10 years, 800 were diagnosed clinically to have VZV infection, then information about age, gender and residency about each child were introduced into an excel spread sheet. After that 43 children were selected for genetic assessment of VZV.

Skin crusted lesion was taken for purpose of analysis. The samples of crusted obtained by crust lever then transferred into a tube (Texwipe's Absorbond® Swab) which is made of polyester (hydroentangled) nonwoven material. Then samples transferred into freeze at -20 C°. Then collection of 43

samples was accomplished we started with lab work according to the steps clarified in the below study design. Figure 2.1

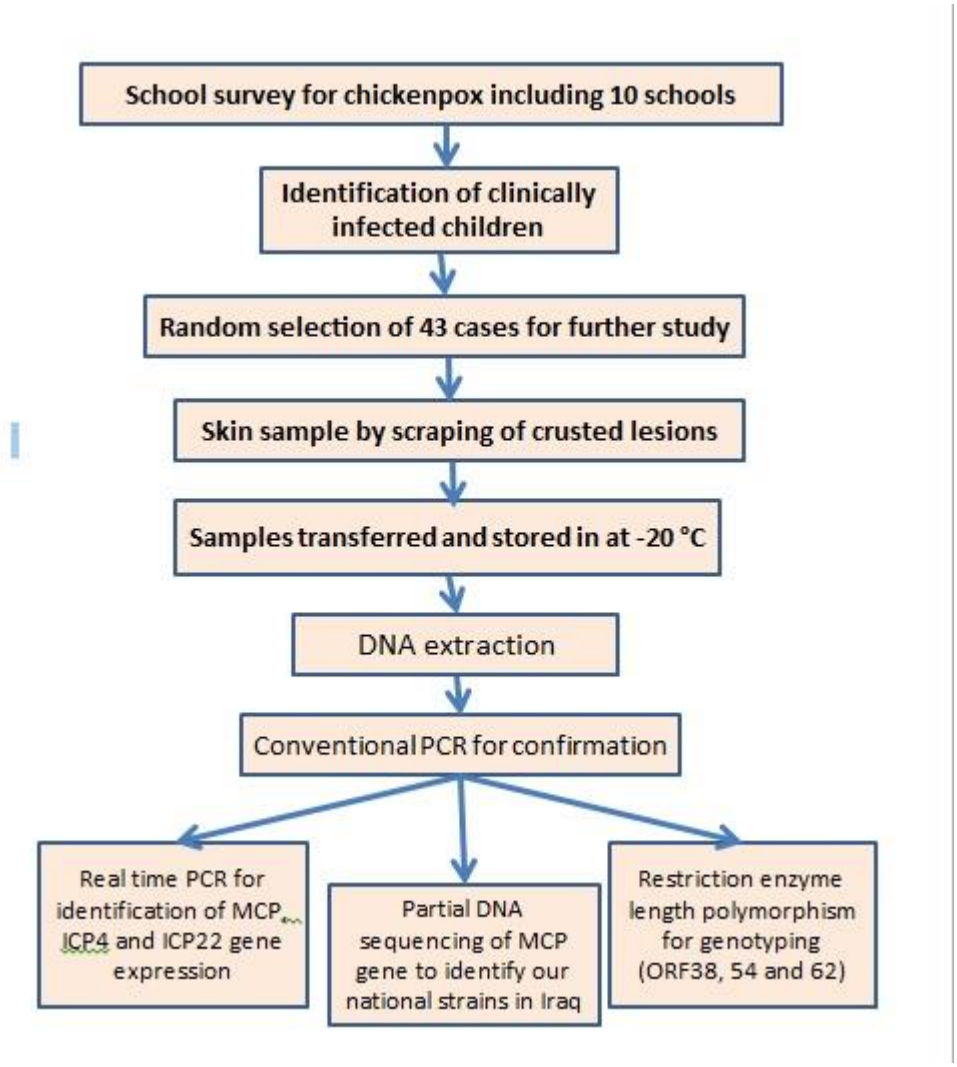

Figure 2.1: Study Design of present study

2.2.2 Polymerase Chain Reaction (PCR)

PCR technique was performed for direct detection of *Varicella Zoster Virus* (VZV) based on amplification of **Major Capsid Protein** gene (*mcp*) in VZV from skin crusted lesions samples. This technique was done according to method described by (Loparev *et al*., 2000) as following:

2.2.2.1 Viral DNA Extraction

Viral DNA was extracted from transport media of skin lesions scrap samples were extracted by using Genomic DNA mini kit extraction tissue, and done according to company instructions as following steps

1. A 250µl transport media of skin lesions scrap sample was transferred to sterile 1.5ml microcentrifuge tube, and then added 30µl of proteinase K and mixed by vortex and incubated at 60℃ for 15 minutes.

2. After that, 200µl of GSB lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃ for 15 minutes, and inverted every 3 minutes through incubation periods.

3. A 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.

4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow through discarded and placed the column in a new 2 ml collection tube.

5. A 400µl W1 buffer was added to the DNA filter column, then centrifuge at 10000 rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.

6. A 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.

7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.
8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated elution buffer were added to the center of the column matrix.
9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.2.2.2 Genomic DNA estimation

The extracted DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry clean wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O and place on the measurement pedestal and click blank.
3. After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

2.2.2.3 PCR master mix preparation

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as following table

Table (2-8): Preparation of Multiplex PCR master mix used in the study

PCR Master mix	Volume
DNA template	5 µl
VZV mcp gene forward primer (10pmol)	1.5µl
VZV mcp gene reverse primer (10pmol)	1.5µl
PCR water	12 µl
Total volume	20 µl

After that, these PCR master mix components that mentioned above were placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, then placed in PCR Thermocycler (Mygene. Bioneer. Korea).

2.2.2.4 PCR **Thermocycler Conditions**

PCR thermocycler conditions were done by using convential PCR thermocycler system as following table

Table (2-9): PCR Thermocycler Conditions used in the study

PCR step	Temp.	Time	Repeat cycle
Initial Denaturation	94°C	5min	1
Denaturation	94°C	30sec.	30 cycle
Annealing	58°C	30sec.	
Extension	72°C	1min	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

2.2.2.5 PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- (1%) Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left in 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 10µl of (2000-100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour. Then PCR products were visualized by using UV Transilluminator.

2.2.3 RFLP-PCR Genotyping Technique

Restriction fragment length polymorphism-PCR technique was performed for genotyping of positive *Varicella Zoster Virus* (VZV) in direct PCR. The VZV genotyping depend on amplification of ORF54, ORF38, and ORF62. This method was carried out according to (Safarnezhad *et al*., 2016) as following steps:

2.2.3.1 RFLP-PCR master mix preparation

Restriction fragment length polymorphism-PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as following table:

Table (2-10): RFLP-PCR mater mix preparation used in the study

PCR Master mix	Volume
DNA template	5µl
ORF Forward primer (10pmol)	1.5µl
ORF Reveres primer (10pmol)	1.5µl
PCR water	12µl
Total volume	20µl

After that, these PCR master mix component that mentioned in table above placed in standard AccuPower PCR PreMix Kit that contains all other components which

needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes then that placed in PCR Thermocycler (Mygene. Korea).

2.2.3.2 RFLP-PCR Thermocycler Conditions

Polymerase chain reaction thermocycler conditions were done for each gene independent as following tables:

Table (2-11): RFLP-PCR Thermocycler Conditions used in the study

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	38cycle
Annealing	58°C	30 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

2.2.3.3 RFLP-PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 10ul of (100bp Ladder) in First well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- A 204 bp PCR products were visualized by using UV transilluminator.

2.2.3.4 RFLP-PCR mix preparation

RFLP-PCR mix was prepared by using *Bgl*I, *Pst*I, and *Sma*I restriction enzymes for ORF54, ORF38, and ORF62 respectively. and this master mix done independent according to company instructions as following table

Table (2-12) RFLP-PCR mix used in this study

RFLP-PCR Master mix	Volume
PCR product	10µl
Restriction enzyme buffer 10X	2 µl
Restriction enzyme (10 unit)	1 µl
Free nuclease water	7 µl
Total volume	20 µl

After that, this master mix placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°C for overnight. After that, RFLP-PCR product was analysis by 3% agarose gel electrophoresis methods that mention in PCR product analysis.

2.2.4 Quantitative Real-Time PCR of VZV

Quantitative Reveres Transcription Real-Time PCR technique was performed for estimation relative gene expression analysis for some virulence factors genes (VZV major capsid protein, **ICP4** tegument protein transactivator, **ICP22** tegument protein transrepresser genes). These genes were normalized by using human housekeeping gene (GAPDH). This technique was done according to method described by (Tipples *et al.*, 2003) as following steps:

2.2.4.1 Total RNA extraction

Total RNA were extracted from transport media of skin lesions scrap samples by using (TRIzol® reagent kit) and done according to company instructions as following steps:

1- A 250µL transport media of skin lesions scrap samples were homogenized by using micropestle in 1 ml of TRIzol® reagent.

2- A 200µl chloroform was added to each tube and shaken vigorously for 15 seconds.

3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.

5- Supernatant was transferred into a new eppendorf tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and

incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm , 4C° for 10 minutes.

8- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes.

9- The supernatant was discarded and the RNA pellet was left to air to dry.

12- 50µl Free nuclease water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -20.

2.2.4.2 Estimation of extracted total RNA yield

The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA), There are two quality controls were performed on extracted RNA. First one is to determine the quantity of RNA (ng/µL), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine as follow:

1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

2- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipeted 2µl of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.

3- After that, the pedestals are cleaned and pipet 1µl of total RNA sample for measurement.

2.2.4.3 DNase I Treatment

The extracted total RNA was treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using (DNase I

enzyme kit) and done according to method described by Promega company, USA instructions as follow:

Table (2-13) The mixture to remove the trace amounts of genomic DNA from the eluted total RNA used in this study

Mix	Volume
Total RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total	20ul

After that, the mixture was incubated at 37C° for 30 minutes. Then, 1µl stop solution was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

2.2.4.4 cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as following table:

Table (2-14) RT PreMix used in this study

RT master mix	**Volume**
Total RNA 100ng/ul	10ul
Random Hexamer primer (50 pmol)	1ul
DEPC water	9ul
Total	20ul

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then dissolved completely by vortex and briefly spinning down.

The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Table (2-15) Thermocycler condition to converted RNA to cDNA used in this study

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.2.4.5 Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using AccuPower™ Green Star Real-Time PCR kit based SYBER Green dye detection of gene amplification in Real-Time PCR system and include the follow:

Table (2-16) qPCR master mix used in this study

qPCR master mix	volume
cDNA template (100ng)	3µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
DEPC water	15 µL
Total	20 µL

After that, these qPCR master mix component that mentioned above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system .

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table:

Table (2-17) Thermocycler protocol for qPCR loaded

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	15 sec	45
Annealing\ Extension Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

2.2.4.6 Data analysis of qRT-PCR

The data results of qRT-PCR for target and housekeeping genes were analyzed by the relative quantification gene expression levels (fold change) ΔCT Livak method that described by (Livak and Schmittgen, 2001).

Table (2-18) Target and housekeeping genes used in this study

Gene	Test (Treatment group)	Cal. (Control group)
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (control) = CT (ref, control) – CT (target, control)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

ΔΔCT= ΔCT (test) – ΔCT (control)

Fold change = 2-ΔΔCT

Ratio (reference/target) = 2CT (reference) – CT (target)

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

2.2.5 DNA sequencing method

DNA sequencing method was performed for Phylogenetic relationship analysis study of local VZV virus positive isolates with NCBI-Genbank Global VZV virus isolates. The sequencing of the PCR product of *MCP* gene, where the 520bp PCR product was **purified** from agarose gel by using (EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). As the following steps:

1. The specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube.

2. A 400µl Binding Buffer II was added to gel fragment. Then, incubated at 60ºC for 10 minutes and shaked until the agarose gel is completely dissolved.

3. Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000rpm for 2 minutes and discard the flow-through in the tube.

4. A 750µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Then, solution discarded.

5. After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.

6. The column was placed in a clean 1.5ml microcentirfuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and store at -20ºC.

After that, the purified VZV mcp gene PCR product samples were sent to Macrogen Company in Korea for performed the DNA sequencing by AB DNA sequencing system.

Phylogenetic analysis was performed based on NCBI-Blast Alignment identification and Neighbor Distance Phylogenetic tree analysis (Mega version 4).

2.2.6 Statistics analysis

Data were summarized, analyzed and presented using two software programs; these were the statistical package of social sciences (SPSS version 23) and Microsoft Office Excel 2010. Categorical variables were presented as number and percentage whereas numeric variables were expressed as mean,

median, standard deviation inter-quartile range and range. Independent samples t-test was used to compare means between control and study groups in case of normally distributed data whereas Mann Whitney U test was used in case of not normally distributed data and Wilcoxon sign test was used to study changes in level of non parametric variable level before and after treatment. Chi-square test was used to study association between categorical variables, Pearson and Spearman correlations were done to evaluate correlation coefficient (r) between numeric variables. The level of significance was considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Chapter Three

Results &Discussion

3. Results and Discussion

3.1. Results

3.1.1. Frequency rate of Varicella Zoster Virus (VZV)

The present study enrolled 4545 children; data about those children were obtained form 10 primary schools in Al Diwaniyah governorate in the Mid-Euphrates region, Iraq. Infection was documented clinically in 800 out of 4545 children making the Frequency rate (17.6 %) as shown in figure (3-1), also the Frequency rate according to school is shown in table (3-1).

Table (3.1) The Frequency rate of VZV infection according to primary schools

No. of primary School	Clinical observation positive	Total	Total Frequency %
1	147	541	27.2
2	88	369	23.8
3	109	461	23.6
4	127	583	21.8
5	83	409	20.3
6	63	356	17.7
7	66	508	13.0
8	53	420	12.6
9	21	208	10.1
10	43	690	6.2
Total	800	4545	17.6

Schools were arranged according to the Frequency rate so that the highest rate was seen in school number one and the lowest Frequency rate was observed in school number 10. The first school included 147 infected children out of 541 with a Frequency rate of 27.2 %. The second school included 88 infected children out of 369 with a Frequency rate of 23.8 %. The third school included 109 infected children out of 461 with a Frequency rate of 23.6 %.The fourth school included 127 infected children out of 583 with a Frequency rate of 21.8 %.The fifth school included 83 infected children out of 409 with a Frequency rate of 20.3 %.

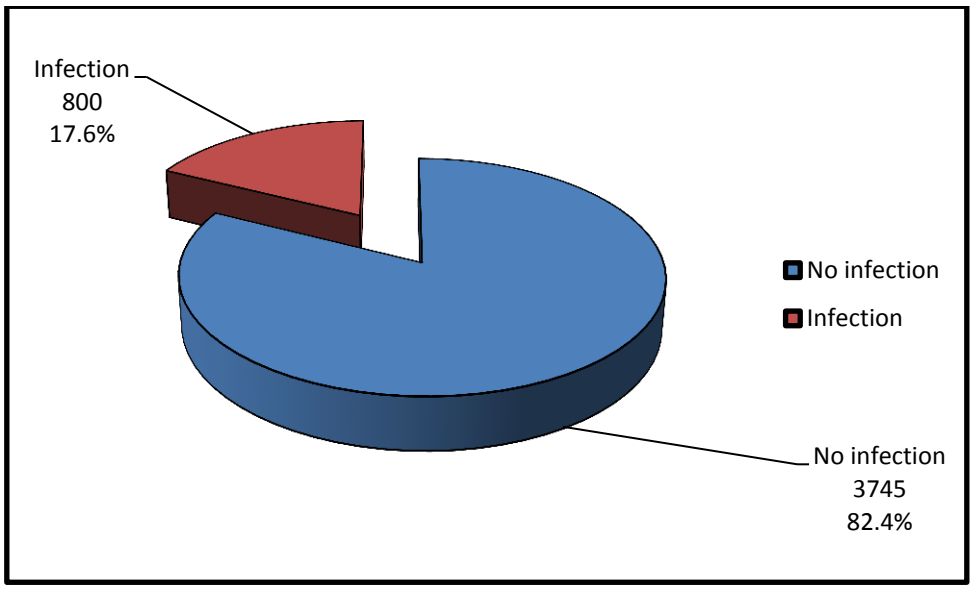

Figure 3.1: Pie chart showing the Frequency rate of Varicella Zoster Virus (VZV) infection among children in 10 primary schools in Al Diwaniyah governorate

The sixth school included 63 infected children out of 356 with a Frequency rate of 17.7 %. The seventh school included 66 infected children out of 508 with a Frequency rate of 13.0 %. The eighth school included 53 infected children out of 420 with a Frequency rate of 12.6 %. The ninth school

included 21 infected children out of 208 with a Frequency rate of 10.1 %. The tenth school included 43 infected children out of 690 with a Frequency rate of 6.2 %. So far, the Frequency rate of VZV infection rate ranged from 6.2 % to 27.2 %.

3.1.1.1Association between child residency and Frequency rate VZV infection

According to residency, the Frequency rate of VZV infection in urban areas was 16.5 % and that of rural areas was 18.1%. Despite some difference in the Frequency rate between rural and urban areas, the difference was statistically not significant (P = 0.207), as shown in table (3.2)

Table (3.2) Association between child residency and Frequency rate VZV infection

Infection with VZV according to clinical findings	**Total** ***n*** **= 4545**	**Urban** ***n*** **= 1299**	**Rural** ***n*** **= 3246**	χ^2	***P****
Positive, *n* (%)	800 (17.6 %)	214 (16.5 %)	586 (18.1 %)	1.594	0.207 NS
Negative, *n* (%)	3745 (82.4 %)	1085 (83.5 %)	2660 (81.9 %)		

n: number of cases; *: according to Chi-square test; NS: not significant at $P \leq 0.05$

3.1.1.2 Association between child gender and Frequency rate VZV infection

According to gender, the Frequency rate of VZV infection in male children was 17.4 % and that of female gender was 18.0 %. Despite some

difference in the Frequency rate between male and female children, the difference was statistically not significant (P = 0.600), as shown in table (3-3)

Table (3.3): Association between child gender and Frequency rate VZV infection

Infection with VZV according to clinical findings	**Total** ***n*** **= 4545**	**Male** ***n*** **= 2787**	**Female** ***n*** **= 1758**	χ^2	***P****
Positive, *n* (%)	800 (17.6 %)	484 (17.4 %)	316 (18.0 %)	0.275	0.600 NS
Negative, *n* (%)	3745 (82.4 %)	2303 (82.6 %)	1442 (82.0 %)		

n: number of cases; *: according to Chi-square test; NS: not significant at $P \leq 0.05$

3.1.1.3 Mean age and distribution according to gender

The mean age of the 43 children selected for molecular study was 8.44 ±1.65 years and the age range was 6 – 11 years, as shown in Figure (3.2) The distribution of children according to one year age interval was as following: children aged 6 years accounted for 7 out of 43 (16.3 %), children aged 7 years accounted for 6 out of 43 (14.0 %), children aged 8 years accounted for 10 out of 43 (23.3 %), children aged 9 years accounted for 7 out of 43 (16.3 %), children aged 10 years accounted for 7 out of 43 (16.3 %) and children aged 11 years accounted for 6 out of 43 (14.0 %), as shown in Figure (3.2).

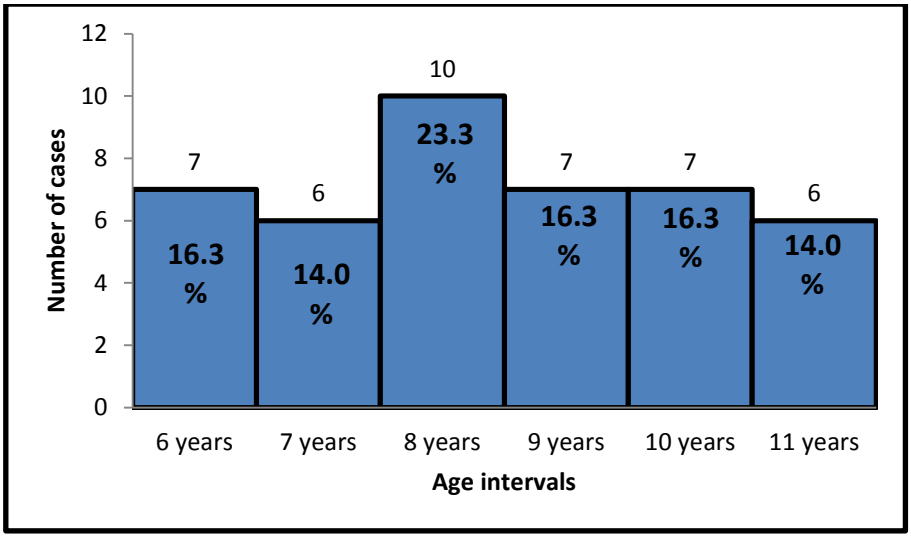

Figure 3.2: Histogram showing the distribution of children infected with VZV according to one year intervals for 43 patients

The study sample included 23 male children (53.5 %) and 20 female children (46.5 %), as shown in figure (3.3). The slight difference in male proportion from that of female proportion was statistically insignificant (*P* = 0.647).

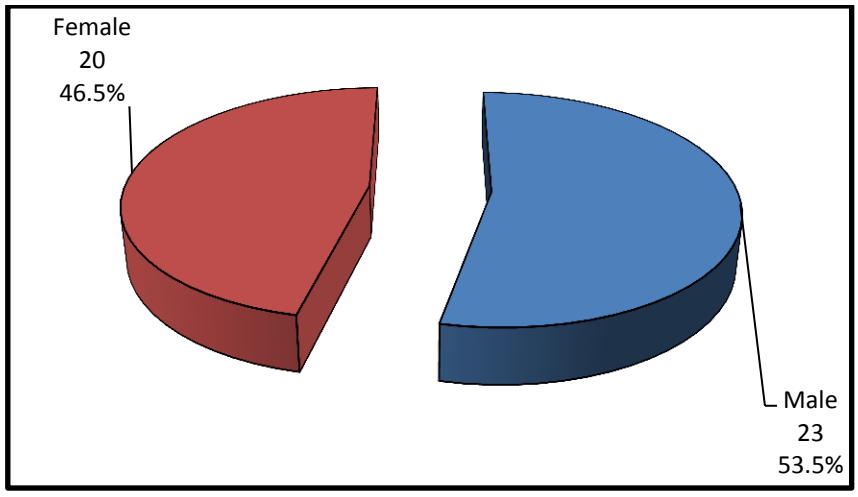

Figure 3.3: Pie chart showing the distribution of children infected with VZV according to gender

3.1.2 Laboratory diagnosis of *Varicella Zoster Virus* infection

Out of 800 children with clinical manifestation of VZV infections, whereas 43 were selected randomly for the purpose of molecular diagnosis and identifying genetic strains of VZV in laboratory.

3.1.2.1Confirmative Detection of *varicella-zoster virus* by Conventional PCR using major capsid protein *(MCP)* Gene.

The DNA from 43 samples of *Varicella-Zoster virus* was extracted and purified using genomic DNA kit. The results were detected by gel electrophoresis process using 1% agarose gel and then examined under UV Light in which the DNA appeared PCR product analysis for major capsid protein (*MCP*) gene in *varicella-zoster virus* as clear compact band in Figure (3.4). Whereas conventional PCR method showed that all the 43 children were positive for VZV.

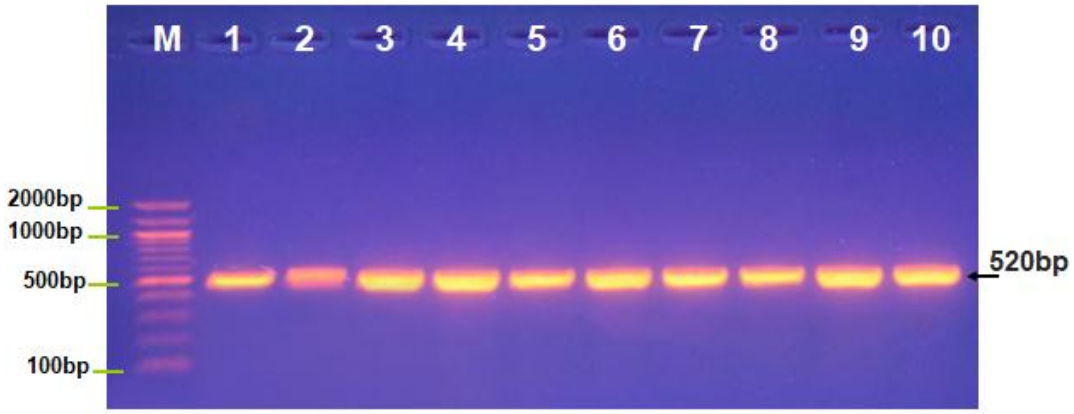

Figure 3.4: Agarose gel electrophoresis image that showed PCR product analysis. M (Marker ladder 2000-100bp). Lane (1-10) some positive *varicella-zoster virus* isolates at 520bp PCR product size.

Table (3.4) Shows concentration and purity of extracted DNA samples, where the concentration of DNA ranged from 50-150 (ng/µL) and their purities ranged from 1.7-2.1 these values are well sufficiently for amplification by PCR method.

Table 3.4: Values of extracted DNA concentration and purity of samples of extracted DNA

Sample No.	Concentration (ng/µL)	Purity (260/280)
1- 43	50-150	1.7-2.1

Based on the standard values of DNA concentration for amplification, the values of the present study are considered an efficient values and suitable for the establishment of the DNA extracted with target primers or sequences amplification.

The results of amplification of *MCP* gene with DNA extracted from 43 VZV samples showed positive PCR product with amplicon size 520 base pair. These successful bindings appeared as single bands under the U.V light using ethidium bromide as a specific DNA stain as Figure 3.4.

3.1.2.2*Varicella Zoster* gene expression

The expression of the following VZV genes was studied. These genes include VZV-MCP, VZV-ICP4 and VZV-ICP22, as shown in table 3.5. Over expression of VZV-MCP was seen in 65.1 % of cases; over expression of VZV-ICP4 was seen in 38.9 % of cases and over expression of VZV-ICP22

was seen in 44.2 % of cases. So far, gene expression of VZV-MCP was the highest, as shown in table 3.5 and figure 3.5.

Table 3.5:*Varicella Zoster* gene expression

Gene expression	VZV-MCP		VZV-ICP4		VZV-ICP22	
	n	**%**	***n***	**%**	***n***	**%**
Over-expression	28	65.1	15	38.9	19	44.2
non-expression	15	38.9	28	65.1	24	55.8

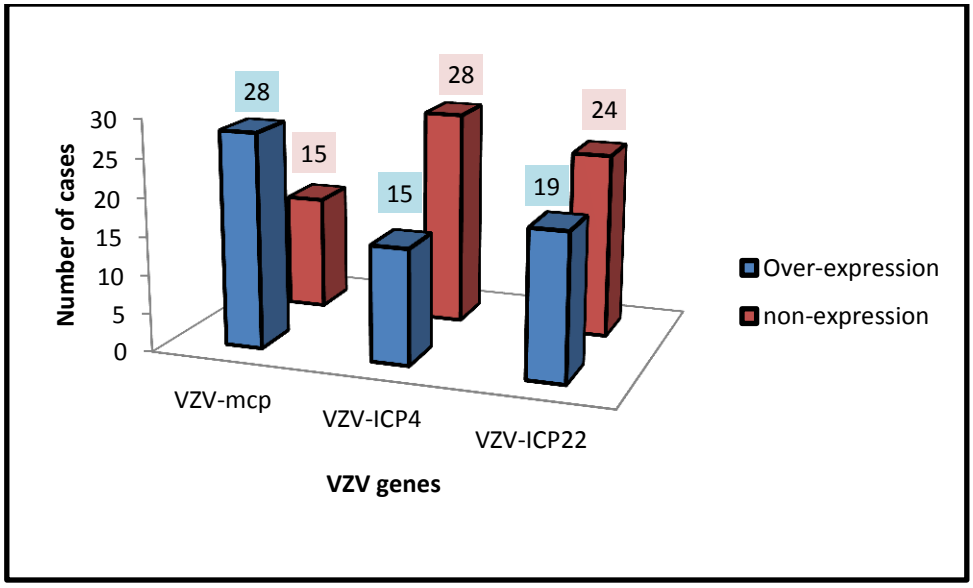

Figure 3.5 *Varicella Zoster* gene expression

We studied the correlation between VZV gene expression and age of infected children and the results are shown in table (3.6) and figures (3.6). There was no significant difference in mean age of children in whom *MCP*

was over-expressed in comparison to children in whom *MCP* was not expressed, 8.46 ±1.69 years versus 8.40 ± 1.64 years, and the level of significance (*P* = 0.905), as shown in table 3.6 and figure 3.6.

Table 3.6: Correlation between VZV gene expression and children age

VZV gene expression		**Mean age (years)**	**SD**	***P***
VZV-MCP	**Over-expression**	8.46	1.69	0.905
	non-expression	8.40	1.64	NS
VZV-ICP4	**Over-expression**	8.33	1.54	0.757
	non-expression	8.50	1.73	NS
VZV-ICP22	**Over-expression**	8.53	1.71	0.769
	non-expression	8.38	1.64	NS

Also there was no significant difference in mean age of children in whom ICP4 was over-expressed in comparison to children in whom ICP4 was not expressed, 8.33 ±1.54 years versus 8.50 ± 1.73 years, and the level of significance (*P* = 0.757), as shown in table 3.6 and figure 3.6. There was no significant difference in mean age of children in whom ICP4 was over-expressed in comparison to children in whom ICP4 was not expressed, 8.53 ± 1.71 years versus 8.38 ± 1.64 years, and the level of significance (*P* = 0.769), as shown in table 3.6 and figure 3.6.

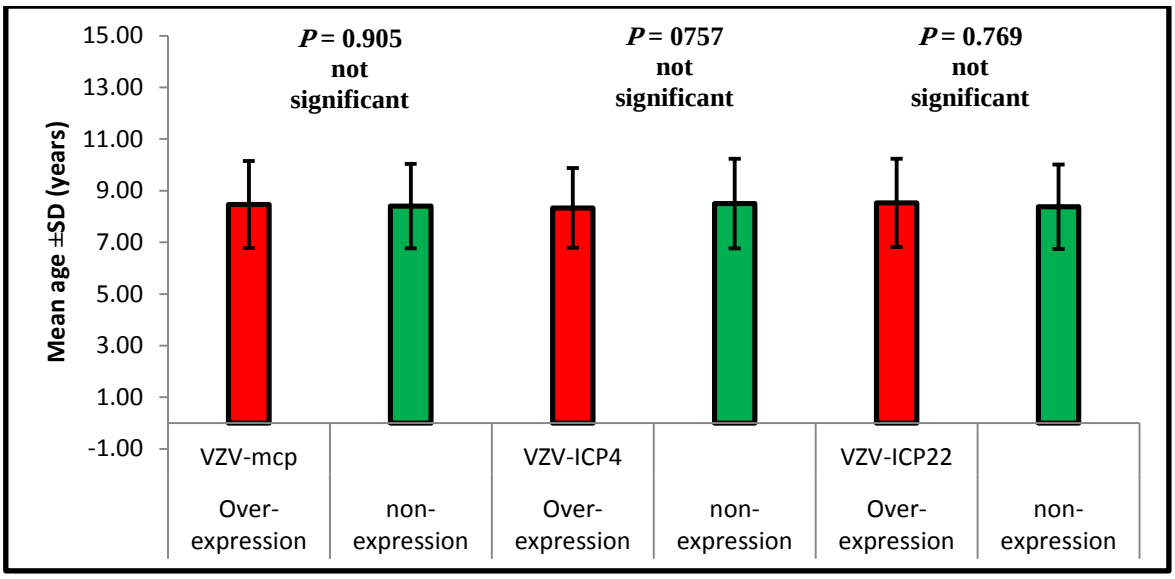

Figure 3.6 Correlation between *Varicella Zoster* gene expression and children age

In addition, we assessed the association of over expression of VZV genes, MCP, ICP4 and ICP22 to gender of infected children, and the association was demonstrated in table 3.6. Over expression of MCP was more frequently associated with male gender, 73.9 % versus 55.0 %; however, the association was statistically insignificant (P = 0.194), as shown in table 3.7. Over expression of ICP4 was less frequently associated with male gender, 26.1 % versus 45.0 %; however, the association was statistically insignificant (P = 0.194), as shown in table 3.7. Over expression of ICP4 was less frequently associated with male gender, 30.4 % versus 60.0 %; however, the association was statistically insignificant (P = 0.052), as shown in table 3.7.

Table 3.7: Association between *Varicella Zoster* gene expression and children gender

VZV genes		**Total** ***n*** **= 43**	**Male** ***n*** **= 23**	**Female** ***n*** **= 20**	χ^2	***P***
MCP	Over-expression	28 (65.1 %)	17 (73.9 %)	11 (55.0 %)	1.685	0.194 NS
	non-expression	15 (38.9 %)	6 (26.1 %)	9 (45.0 %)		
ICP4	Over-expression	15 (38.9 %)	6 (26.1 %)	9 (45.0 %)	1.685	0.194 NS
	non-expression	28 (65.1 %)	17 (73.9 %)	11 (55.0 %)		
ICP22	Over-expression	19 (44.2 %)	7 (30.4 %)	12 (60.0 %)	3.792	0.052 NS
	non-expression	24 (55.8 %)	16 (69.6 %)	8 (40.0 %)		

In the next pages, Real time PCR amplification plot results for MCP, ICP4, ICP22 and Human housekeeping GAPDH genes are demonstrated, they showed threshold cycle numbers (Ct value) in *Varicella Zoster* virus positive isolates.

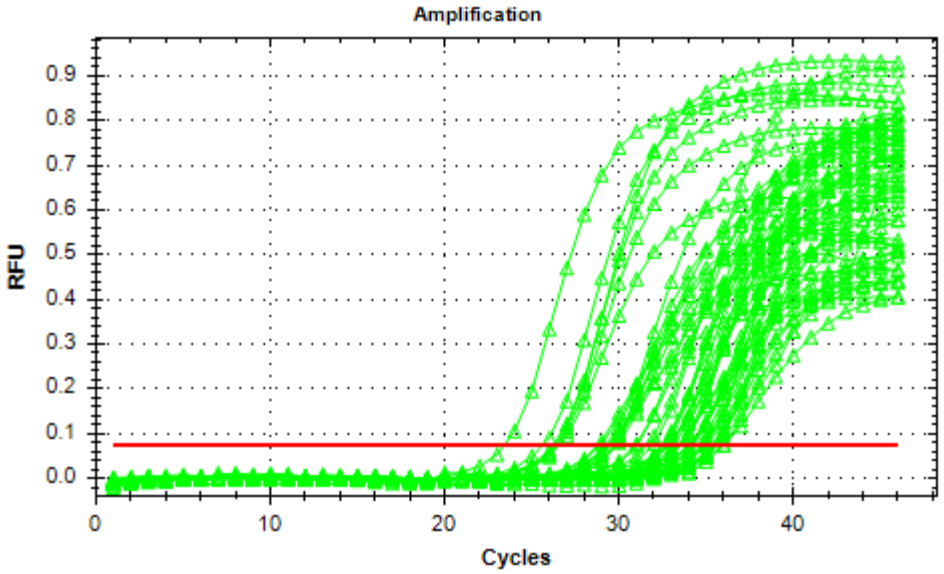

Figure 3.7 Real time PCR amplification plot for *varicella-zoster virus MCP* gene that showed threshold cycle numbers (Ct value) in *varicella-zoster virus* positive isolates.

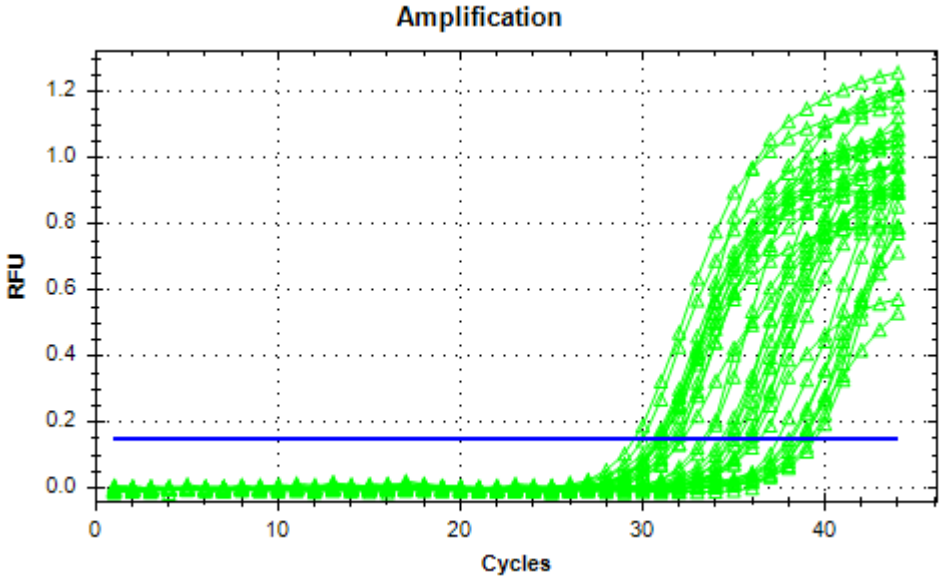

Figure 3.8 Real time PCR amplification plot for *varicella-zoster virus ICP4* gene that showed threshold cycle numbers (Ct value) in *varicella-zoster virus* positive isolates.

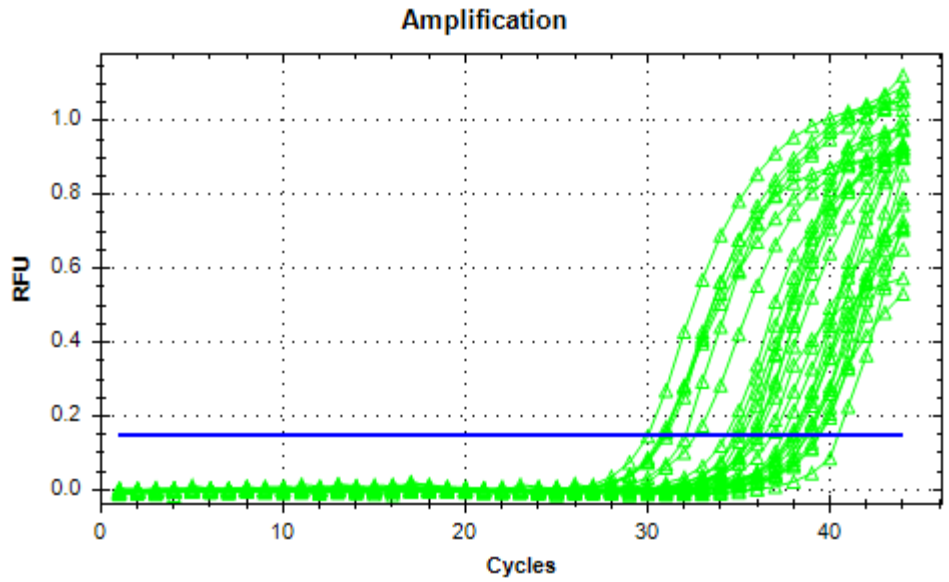

Figure 3.9 Real time PCR amplification plot for *varicella-zoster virus ICP22* gene that showed threshold cycle numbers (Ct value) in *varicella-zoster virus* positive isolates.

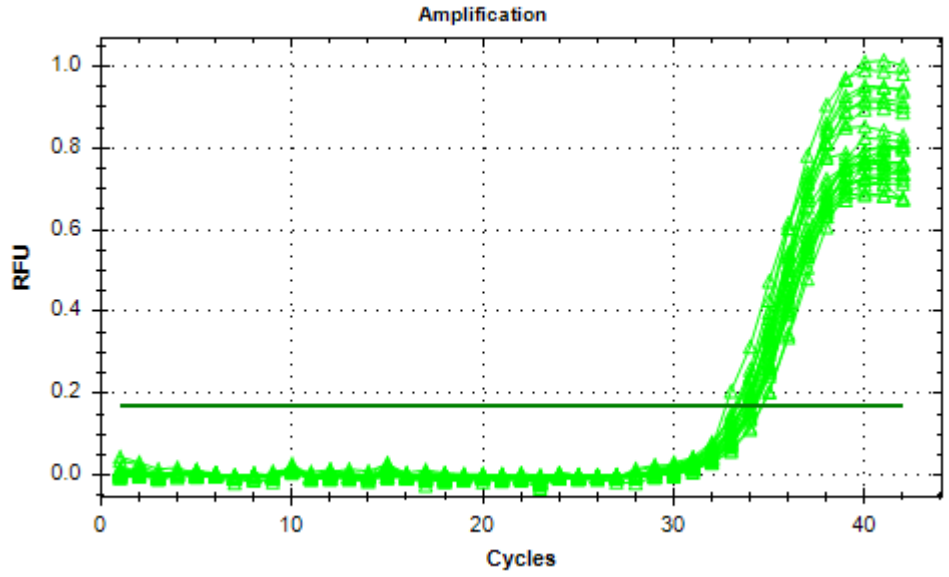

Figure 3.10 Real time PCR amplification plot for *Human housekeeping GAPDH* gene that showed threshold cycle numbers (Ct value) in *varicella-zoster virus* positive isolates.

3.1.2.3 Genotyping of *Varicella Zoster Virus* of ORF38, ORF54 and ORF62 using Restriction Fragment Length Polymorphism (RFLP) Technique

Forty-three chickenpox patients isolates taken, after the DNA extraction and PCR amplification performed by specific primers of (Open Reading Frame) ORFs 38, 54 and 62, then RFLP assay and digestion carried out by *Pst*I (for ORF38), *Bgl*I (for ORF54) and *Sma*I (for ORF62) restriction enzymes.

RFLP analyses of ORF38 by specific restriction enzymes showed that all 43 isolates were cleaved by *Pst*I enzyme (100% *Pst*I +) and there were no *Pst*I¯ isolates as fig (3.14). Digestion of ORF54 by *Bgl*I restriction enzyme so31 isolates were cleaved (72% *Bgl*I+) whereas 12 isolates were negative to *Bgl*I (28% *Bgl*I ¯) as fig (3.12). While there's no *Sma*I+ (0% *Sma*I+) that's meaning no ORF62 to cleavedas fig (3.16).

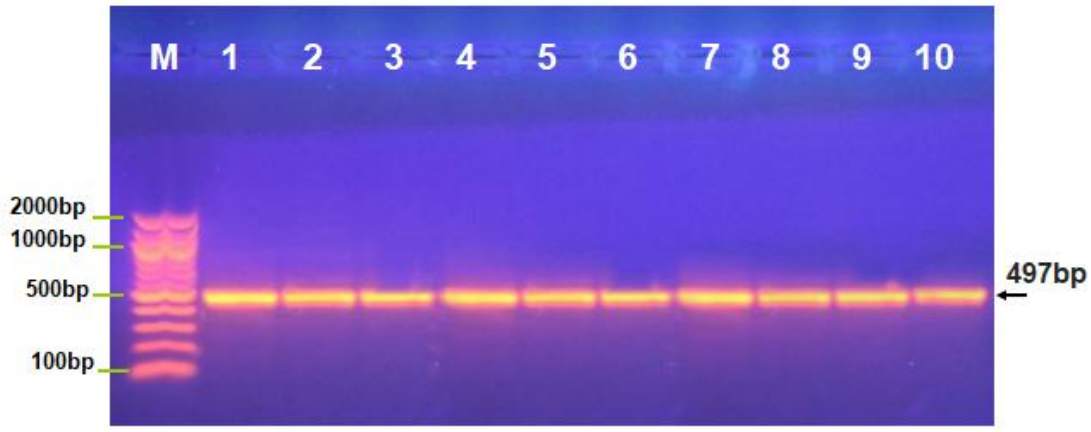

Figure 3.11 Agarose gel electrophoresis image that showed PCR product genotyping analysis for ORF54 in varicella-zoster virus. M (Marker ladder 2000-100bp). Lane (1-10) some positive ORF54 varicella-zoster virus isolates at 497bp PCR product size.

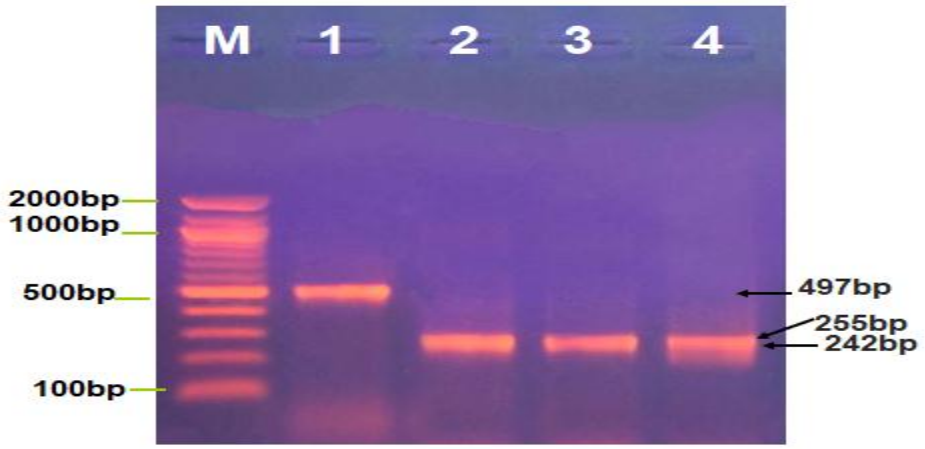

Figure 3.12 Agarose gel electrophoresis image that showed RFLP-PCR product genotyping analysis for ORF54 in varicella-zoster virus by using restriction enzyme (*BglI*). M (Marker ladder 2000-100bp). Lane (1) positive control ORF54 varicella-zoster virus isolate at 497bp PCR product size and lane (2-3) positive digestion *BglI* restriction enzyme for some varicella-zoster virus isolates into overlapping 242bp product size.

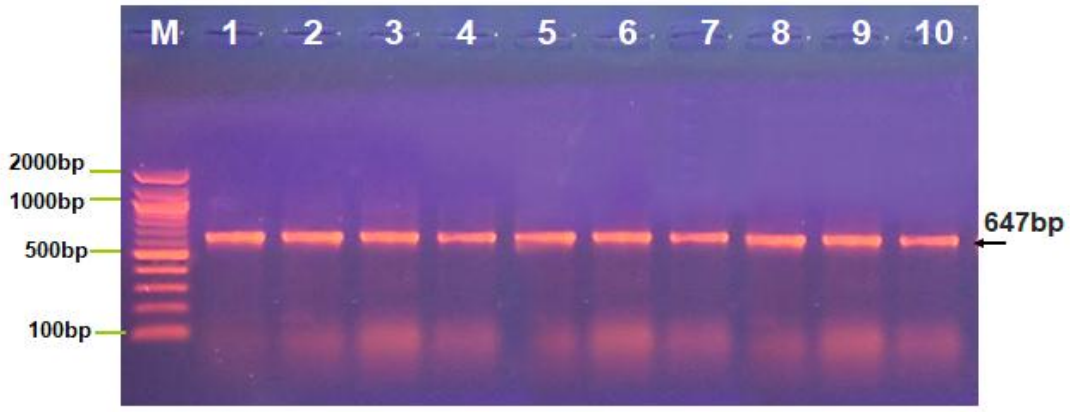

Figure 3.13 Agarose gel electrophoresis image that showed PCR product genotyping analysis for ORF38 in varicella-zoster virus. M (Marker ladder 2000-100bp). Lane (1-10) some positive ORF54 varicella-zoster virus isolates at 647bp PCR product size.

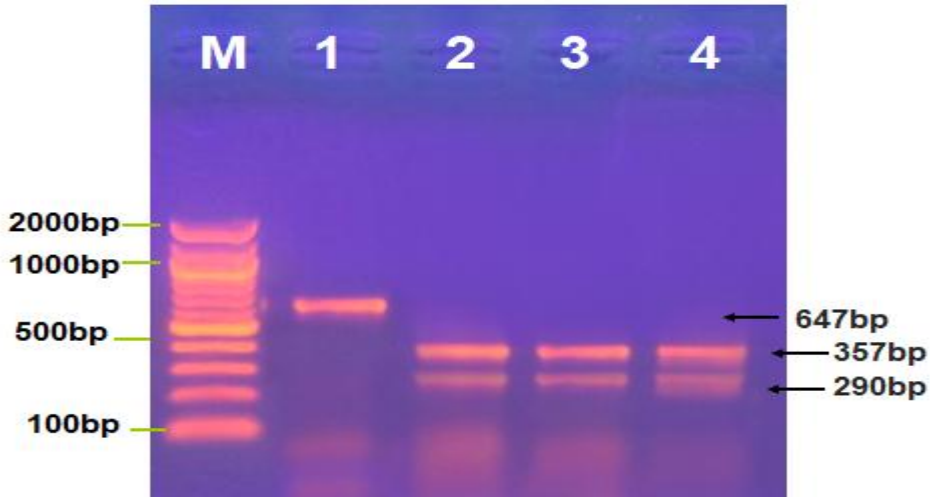

Figure 3.14 Agarose gel electrophoresis image that showed RFLP-PCR product genotyping analysis for ORF38 in varicella-zoster virus by using restriction enzyme (*PstI*). M (Marker ladder 2000-100bp). Lane (1) positive control ORF54 varicella-zoster virus isolate at 647bp PCR product size and lane (2-3) positive digestion *PstI* restriction enzyme for some varicella-zoster virus isolates into 357bp and 290bp product size.

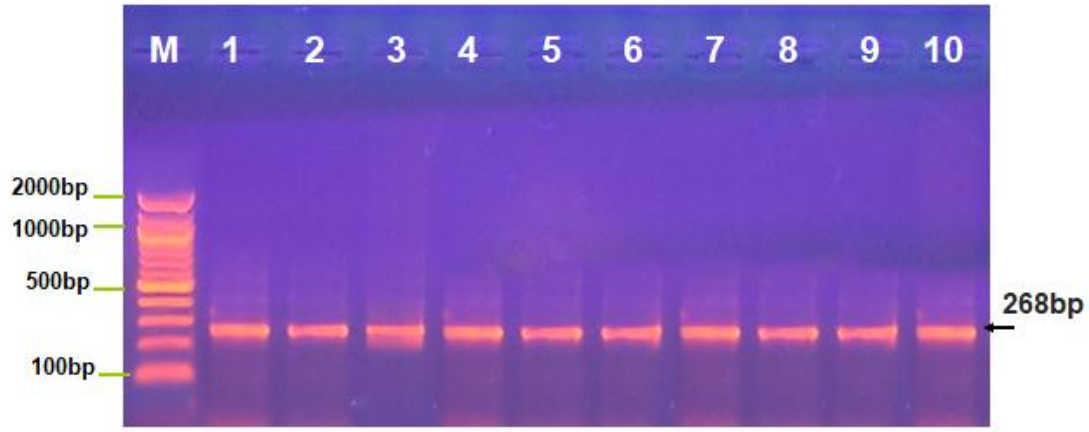

Figure 3.15 Agarose gel electrophoresis image that showed PCR product genotyping analysis for ORF62 in varicella-zoster virus. M (Marker ladder 2000-100bp). Lane (1-10) some positive ORF62 varicella-zoster virus isolates at 268bp PCR product size.

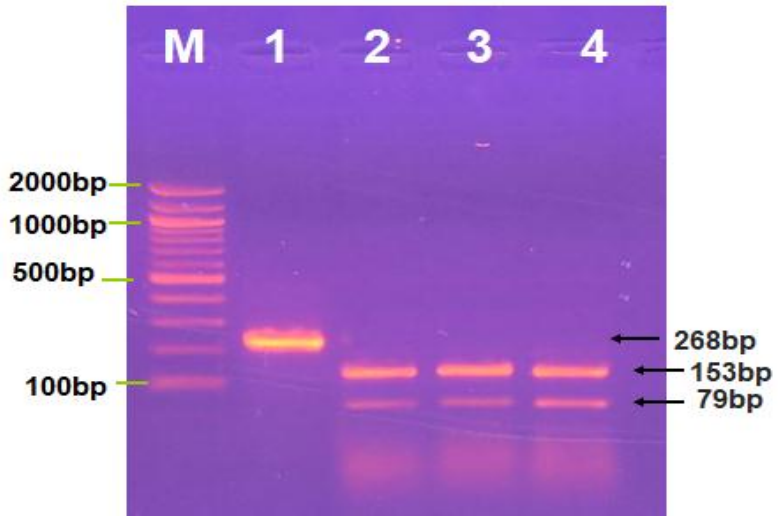

Figure 3.16 Agarose gel electrophoresis image that showed RFLP-PCR product genotyping analysis for ORF62 in varicella-zoster virus by using restriction enzyme (*SmaI*). M (Marker ladder 2000-100bp). Lane (1) positive control ORF54 varicella-zoster virus isolate at 268bp PCR product size and lane (2-3) positive digestion *SmaI* restriction enzyme for some varicella-zoster virus isolates into 153bp and 79bp product size.

The present study included three single nucleotide polymorphism (SNP) alleles were estimated and these were ORF38, ORF54 and ORF62. All the cases were positive for ORF38 (100.0 %). The number of cases that were positive for ORF54 were 31 out 43 (72.1 %) whereas, none of the cases were positive for ORF62 (0.0%) as shown in figures (3.14, 3.12 and 3.16).

Accordingly virus genotype strains were identified. Cases which were positive for ORF38 and ORF54 SNPs were considered genotype B whereas cases which were positive for ORF38 only were labeled as genotype A.

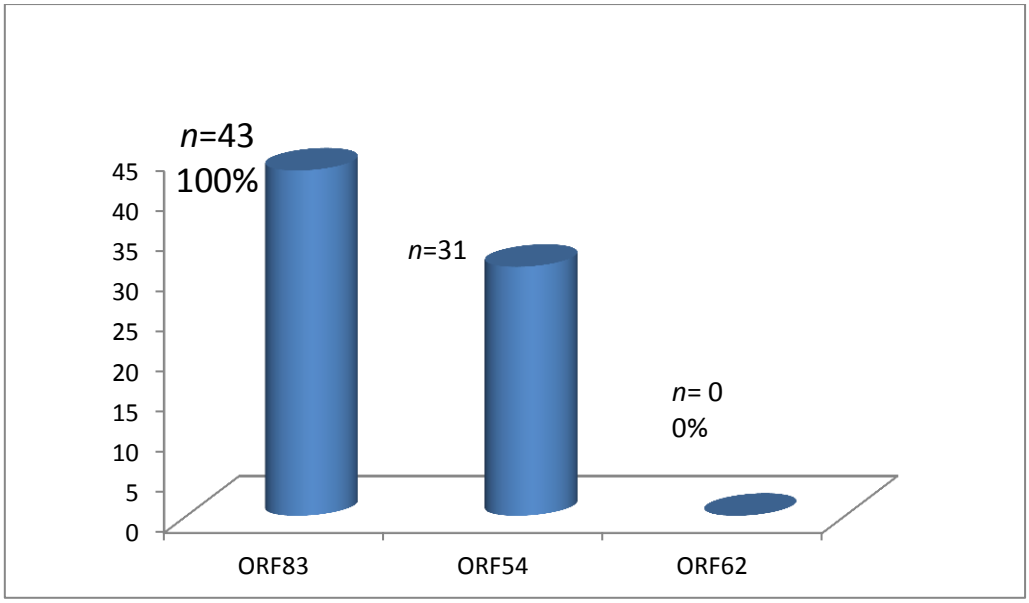

Figure 3.17 The number and percentage of cases according to single nucleotide polymorphism alleles (ORF43, ORF54 and ORF62)

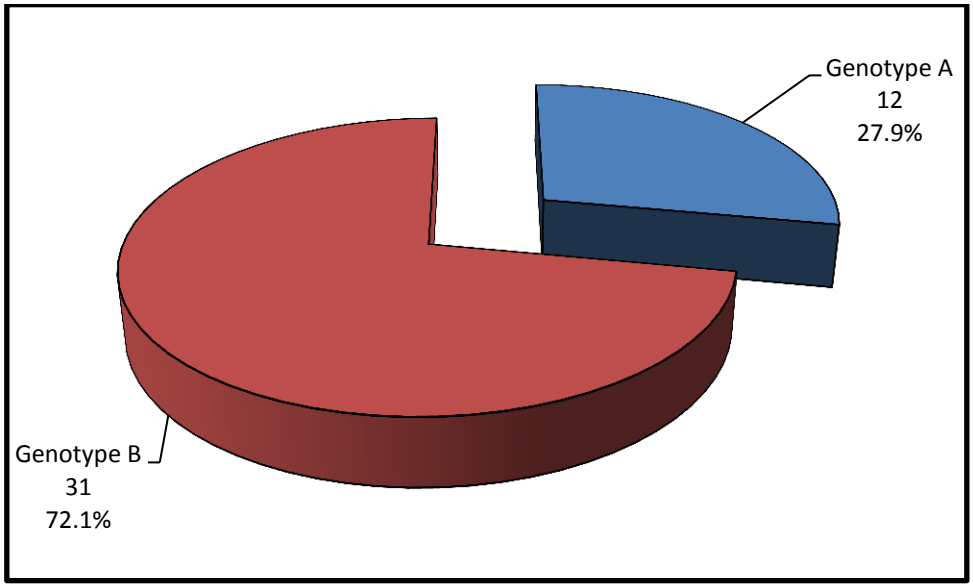

Figure 3.18 The number and percentage of cases according to genotype (A versus B)

The correlation between VZV gene SNP alleles and age and gender of children were not possible for both ORF38 and ORF62 since ORF38 is positive in all case and ORF62 is negative in all cases. Therefore, we linked ORF54 only to age and gender of infected children, there was no significant association between age of child and ORF54 SNP allele frequency (P= 0.951), as shown in table 3.8 and figure 3.19. In addition, the association between ORF54 and gender of infected children was insignificant (P = 0.078).

Genotypes A and B were also neither correlated significantly to age of children nor gender of children, as shown in Figure 3.20 and figure 3.20.

Table 3.8: Comparison of mean age between cases with positive ORF54 and cases with negative ORF54

ORF54	***n***	**Mean**	**SD**	***P*** *
Positive	31	8.45	1.83	0.951
Negative	12	8.42	1.61	NS

n: number of cases; SD: standard deviation; *: independent samples t-test; NS: not significant at $P \leq 0.05$

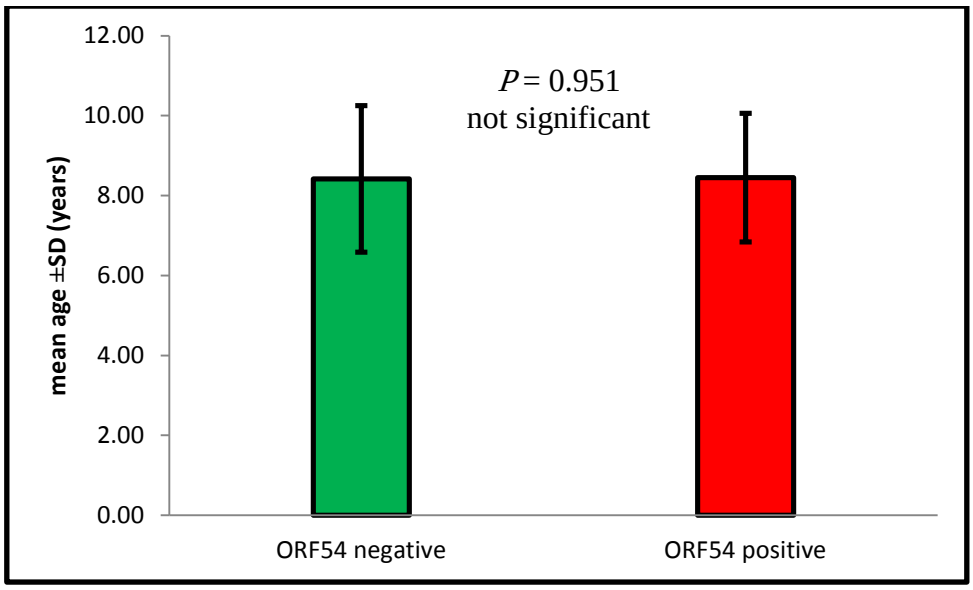

Figure 3.19 Comparison of mean age between cases with positive ORF54 and cases with negative ORF54

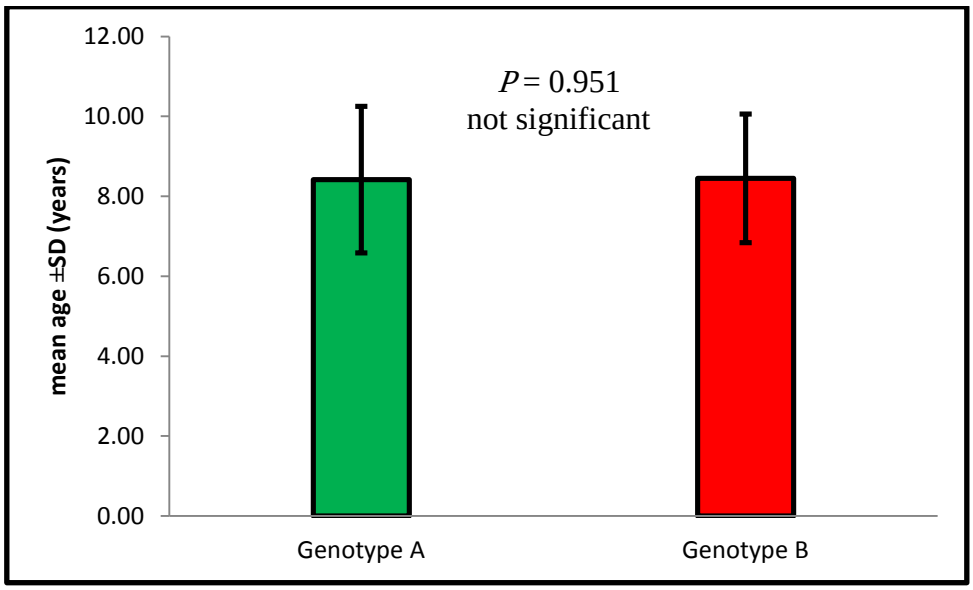

Figure 3.20 Comparison of mean age according to VZV genotype (A versus B)

3.1.2.4 Multiple sequence alignment analysis of of*MCP*Gene of VZV

Multiple sequence alignment analysis of the partial sequence in major capsid protein (*MCP*) gene for local (*Human alphaherpesvirus-3*) *varicella-zoster virus* Isolates (IQ; Iraq) IQ1,IQ2,IQ3,IQ4,IQ5,IQ6,IQ7 and IQ8 with NCBI-Blast *Human alphaherpesvirus-3* by using (MEGA 6.0, multiple alignment analysis tools).The multiple alignment analysis that showed nucleotide similarity (*) and mutation in ***MCP*** gene nucleotide sequences as fig. (3.21)

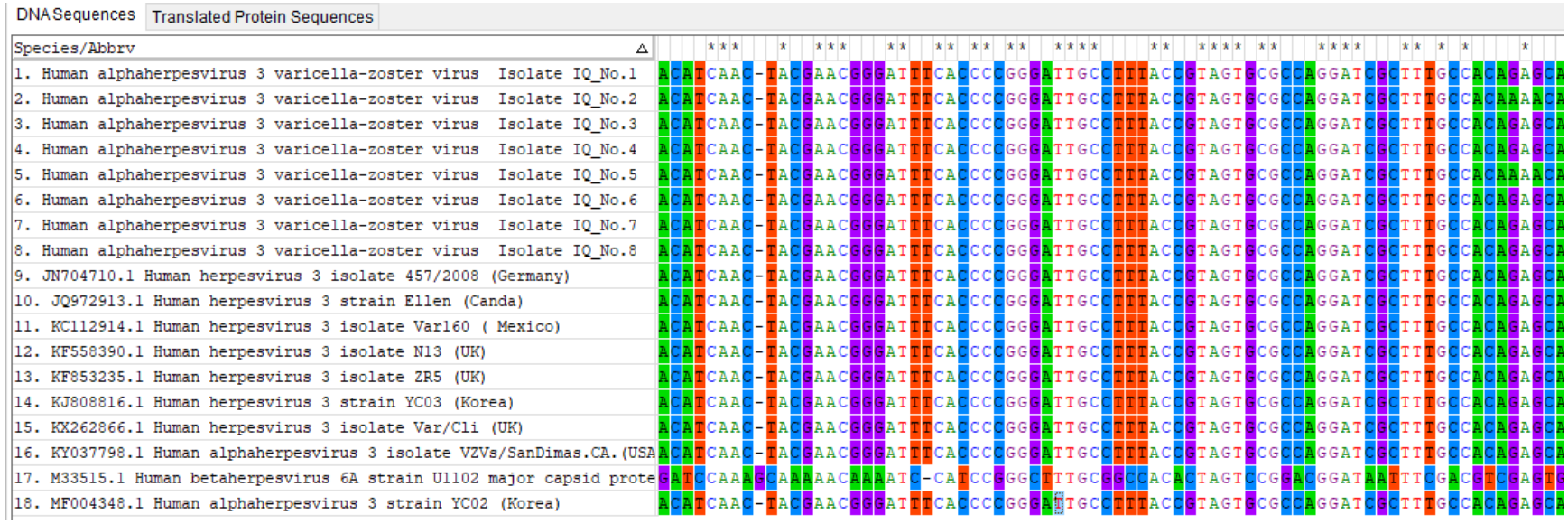

Fig. (3.21) Multiple sequence alignment analysis of the partial sequence in major capsid protein (MCP) gene for local (*Human alphaherpesvirus-3*).

3.1.2.5 Sequencing and Phylogeny Analysis of *MCP* Gene of VZV

Phylogenetic tree analysis based on major capsid protein (*MCP*) gene partial sequence that used for local (*Human alphaherpesvirus-3*) *varicella-zoster virus* IsolatesIQ1, IQ2, IQ3, IQ4, IQ5, IQ6, IQ7 and IQ8 genetic relationship analysis (Appendix-2). The phylogenetic tree was constructed using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). All local *Human alphaherpesvirus-3* Isolates were showed closed related to *Human herpesvirus-3* isolate 457/2008 Germany (JN704710.1) except local *Human alphaherpesvirus-3* Isolates IQ2, IQ3, and IQ5 were showed less genetic different than NCBI BLAST *Human alphaherpesvirus-3* Isolates at total genetic change fig (3.22).

The results of DNA sequencing should be firstly examined to confirm the nucleotide sequences and closed relationships with others world strains, test used to confirm was through NCBI-BLAST-query- nucleotide –online, it was perfect program and gave the exact results of identity percentage with other world strains and they were ranged from (98%-100%) table (3.9).

3.1.3 Submission of Iraqi *Human Herpesvirus-3* Isolates in GeneBank database of NCBI

An Eight sequences *(MCP)* gene of *Varicella-Zoster virus* which isolated from human sources in Al Diwaniyah governorate whereas each sequence have **Accession Number** (IQ1, IQ2, IQ3, IQ4, IQ5, IQ6, IQ7 and IQ8 isolates) by GeneBank database in NCBIAppendix-1

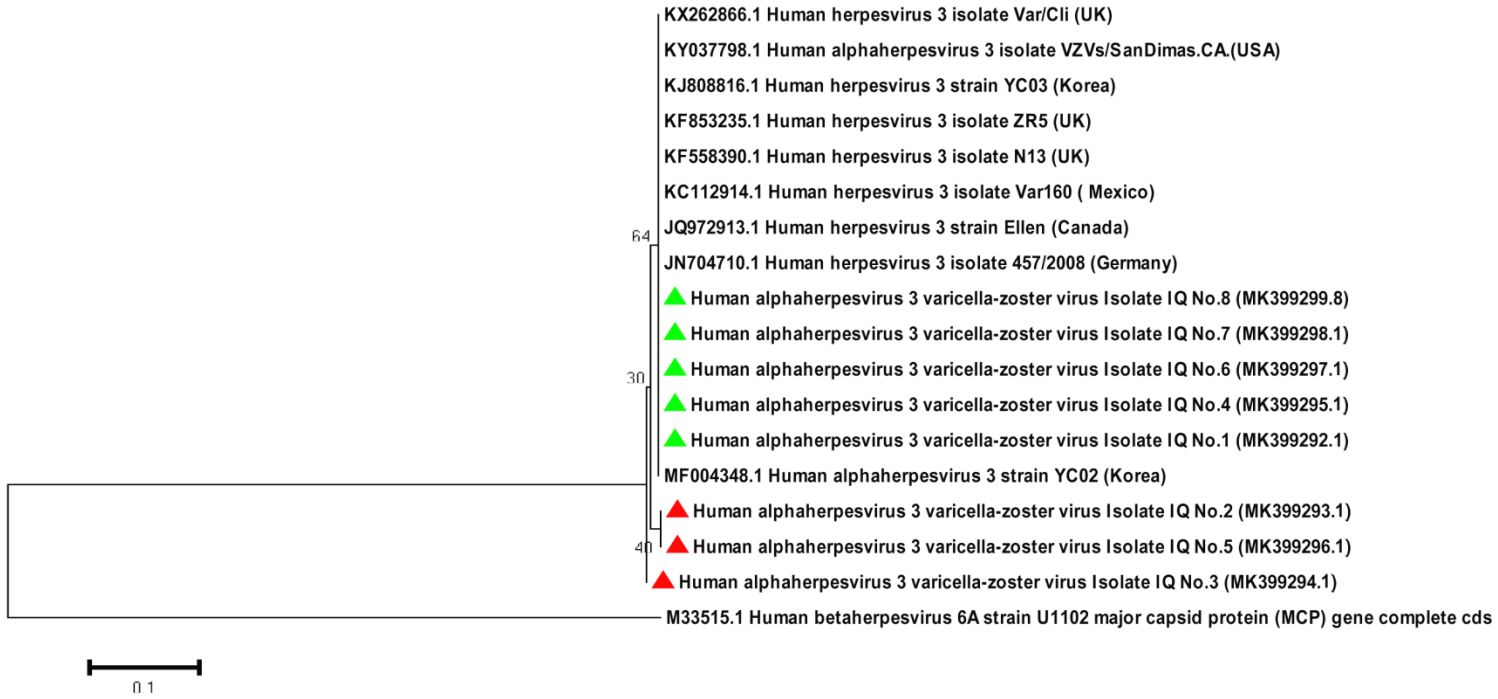

Fig. (3.22) Phylogenetic tree analysis based on *major capsid protein* (*MCP*) gene partial sequence that used for local (*Humanalphaherpesvirus-3*) *varicella-zoster virus* IsolatesIQ1, IQ2, IQ3, IQ4, IQ5, IQ6, IQ7 and IQ8 genetic relationship analysis.

Table (3.9) NCBI-Blast Homology sequence identity between local *Human herpesvirus-3* isolate and NCBI BLAST Identical *Human herpesvirus-3*isolate

Local *Human herpesvirus-3* isolate	Genbank Accession number	Identical *Human herpesvirus-3*	Country	Identity (%)
Human herpesvirus-3 Isolate IQ_No.1	MK399292.1	Human herpesvirus-3 isolate 457/2008	Germany	100%
Human herpesvirus-3 Isolate IQ_No.2	MK399293.2	Human herpesvirus-3 isolate 457/2008	Germany	98%
Human herpesvirus-3 Isolate IQ_No.3	MK399294.3	Human herpesvirus-3 isolate 457/2008	Germany	99%
Human herpesvirus-3 Isolate IQ_No.4	MK399295.4	Human herpesvirus-3 isolate 457/2008	Germany	100%
Human herpesvirus-3 Isolate IQ_No.5	MK399296.5	Human herpesvirus-3 isolate 457/2008	Germany	99%
Human herpesvirus-3 Isolate IQ_No.6	MK399297.6	Human herpesvirus-3 isolate 457/2008	Germany	100%
Human herpesvirus-3 Isolate IQ_No.7	MK399298.7	Human herpesvirus-3 isolate 457/2008	Germany	100%
Human herpesvirus-3 Isolate IQ_No.8	MK399299.8	Human herpesvirus-3 isolate 457/2008	Germany	100%

3.2. Discussion

3.2.1 Frequency rate of Varicella Zoster Virus (VZV)

In the present study infection with *Varicella Zoster Virus* (VZV) was documented clinically in 800 out of 4545 children making the Frequency rate (17.6 %) and the rate in different schools ranged from 6.2 % to 27.2 %.

In Iraq, a study on chickenpox has been performed from 2007 to 2011. It has reported a rise in the number of clinically registered chickenpox cases between 2007 and 2011, around 22% in 2007, 60% in 2008, 39% in 2009, 43% in 2010, and 74% in 2011. Moreover, most cases were 15 years of age and less (65%). The number of infected males was slightly higher than the number of females, and the highest frequencies were reported in March, April, and May (Khaleel and Abdelhussein, 2012).

Our results agree with a study conducted on the Frequency rate of VZV infection in Saudi Arabia and found that the Frequency rate was 11.3 % (Abbas *et al*., 2007).

In Qatar, according to the 2007 Qatar Annual Health Report, the number of reported cases of chickenpox among Qataris was (17.5%) (Al-Turab and Chehadeh, 2018), which is approximately similar to our reported Frequency rate among Iraqi children in Al Diwaniyah region. In addition, a study has been carried out for the detection of VZV in skin samples of 33 patients in Jordan and a total of 11 patients were positive for VZV accounting for (33 %) (Meqdam *et al*., 2001); although the Frequency rate in this Jordanian study was high, it included a small sample size.

A different study aimed to estimate the Frequency of age-specific VZV antibody in Iranian < 40 years during 2003 and 2011 and has found an increase in

the VZV seropositivity with age: 1–5 years (21.9%), 6–10 years (42.1%), 11–15 years (59.4%), and 40 years (87%) (Allami and Mohammadi, 2014). In these Iranian studies, the Frequency rate appears to be higher than that obtained in our study; however, this is not true since these Iranian studies were based on serologic Frequency rather than clinical features so that accumulation of cases along several years would build up the Frequency rate as estimated according to serologic method. Therefore we believe that the true annual incidence rate in these Iranian is much lower and is approximately similar to our results.

The varicella seroFrequency was investigated in a random sample of the Turkish population under age 30 in nine provinces of Turkey. Out of 4387, 77.8% were VZV seropositive, with no difference in the seroFrequency rate between rural (76.3%) and urban (79.0%) areas. In addition, the varicella seroFrequency was reported to increase with age, with 20% in those aged 1 year, 40% in those aged 4 years, 60% at age 6 years, 80% at age 8 years, 85% at age 10 years, and 85%–90% in those over 10 years ((Al-Turab and Chehadeh, 2018)).

In another Turkish study aimed to determine the seroFrequency of VZV-IgG antibodies among 885 children aged between 0 and 15 years in Turkey, it was found that VZV seropositivity rates were low up to the end of the first year and then showed a gradual increase. The seropositivity rates were 41.2%, 80%, and 85% for 4–5 years old group, 10–11 years old group, and 13–15 years old group, respectively (Savaş*et al*., 2004). Another study aimed to determine the varicella seroFrequency under age 30 in Eastern Turkey to design a strategy for vaccination against varicella. The varicella seropositivity was 78% increasing with age. Accordingly, authors highlighted the need of the introduction of varicella vaccine into the routine childhood vaccination program in Turkey since majority of varicella infections occur during the early childhood (Alp *et al*., 2005).

Many other studies investigated the varicella seroFrequency in different regions in Turkey (Dinleyici *et al*., 2012). One study aimed to determine the reliability of a history of varicella to detect susceptible children, adolescents, and adults in Izmir. The overall VZV seronegativity among 590 participants was 28.5%, specifically 18.8% in adolescents and 11.7% in young adults, while VZV seropositivity in children was 25.5%. Thus, it was recommended a serological testing for adolescents and adults with negative history of varicella before VZV immunization (Koturoglu *et al*., 2011). Another study in Izmir has investigated varicella seroFrequency among 2136 healthy aged 15 years and above. It has shown that VZV seropositivity was high (94.3%), and there was significant difference between VZV seroFrequency in urban and rural. Furthermore, a large proportion of the population in Izmir was naturally immunized against varicella (Kose *et al*., 2013).

3.2.2 Demographic characteristics

3.2.2.1 Age of patients with VZV

In the present study, the mean age of the 43 children selected for molecular study was 8.44 ±1.65 years and the age range was 6 – 11 years. In the United States, the highest Frequency rate of VZV infection is in the 4 to 10-year-old age group (Bakker *et al*., 2016; Schmader, 2016; Rice *et al*., 2018). This age range of predilection is similar to that found in the presents study.

In Kingdom of Saudi Arabia a study was carried out aimed to evaluate retrospectively the trend of varicella at the Preventive Medicine Department of the Armed Forces Hospital of the Southern Region from 2007 to 2012. The study showed that most cases occurred in children <15 years of age, in accordance with

our finding that children is the main population subset affected by VZV infection (Saleh and Al Moghazy, 2014). Another prospective study conducted in Riyadh, on chickenpox cases from 2001 to 2003 found that of 3802 reported chickenpox cases, 2984 (78%) occurred in children <15 years of age, confirming our finding that chickenpox is principally acquired in children (Almuneef *et al*., 2006).

In another study held in United Arab of Emirates it was found that most cases of VZV infection (89%) occurred in children aged <15 years (Uduman *et al*., 2009).

3.2.2.2 Gender of patients with VZV

According to gender, the Frequency rate of VZV infection in male children was 17.4 % and that of female gender was 18.0 %. Despite some difference in the Frequency rate between male and female children, the difference was statistically not significant (P = 0.600). In agreement with our study several other studies reported no significance difference in Frequency rate of VZV infection between male and female gender (Kanra *et al*., 2002; Savaş *et al*., 2004; Dinleyici *et al*., 2012). However, several other studies showed some male predominance but the difference was again insignificant (Uduman *et al*., 2001; Ahmed *et al*., 2007; Saleh *et al*., 2014).

In the current study the infection rate was higher in male children than in female children; however the difference in rate of infection was statistically insignificant (P = 0.647). This finding is in agreement with (Saleh and Al Moghazy, 2014) who stated that the number of varicella cases was slightly higher in males than females.

3.2.2.3 Residency of patients with VZV

In the present study, according to residency, the Frequency rate of VZV infection in urban areas was 16.5 % and that of rural areas was 18.1%. Despite some difference in the Frequency rate between rural and urban areas, the difference was statistically not significant (P = 0.207). In accordance with our study, that there is no significance difference in the Frequency rate among rural and urban areas, several other authors expressed the same findings (Kanra *et al*., 2002; Savaş *et al*., 2004; Dinleyici *et al*., 2012); however, a number of studies disagree with our results and found higher Frequency rate of VZV infection in urban areas in comparison with rural areas (Uduman *et al*., 2001; Ahmed *et al*., 2007; Saleh *et al*., 2014).

3.2.3 Molecular diagnosis of Varicella Zoster Virus infection

3.2.3.1 Conventional PCR

In this study we proved that the conventional PCR method was 100 % sensitive since it identified viral infection in all selected clinically suspected children. According to Espy *et al*., (2000) the sensitivity of conventional PCR in detection of VZV infection during phase of viremia was 88. 6 % since VZV DNA was detected in 39 of 44, which is slightly lower than the sensitivity rate of the current study. In another study, the sensitivity of nested PCR was studies in a sample of clinically infected individuals with VZV and the sensitivity rate was 96 % (Weidmann *et al*., 2003) which is nearly similar to that of the current study. In another study on 104 clinically positive samples the sensitivity of conventional PCR was shown to be 100% (Tan *et al*., 2013) in accordance with the finding of the present study. In another study on 200 clinically proven VZV infected individuals the sensitivity of PCR following DNA extraction method was 100 %

(Binkhamis *et al*., 2014), a result that is similar to that of the present study. In the review article held by Wilson *et al.*, (2012) it has been described, in support for the current study, that the sensitivity rate of PCR in detection of VZV in clinically suspected samples approaches almost 100 %.

3.2.3.2 Gene expression of VZV by Real-Time PCR

In the present study, over expression of VZV-MCP was seen in 65.1 % of cases; over expression of VZV-ICP4 was seen in 34.9 % of cases and over expression of VZV-ICP22 was seen in 44.2 % of cases. So far, gene expression of VZV-MCP was the highest.

The MCP stands for the major capsid protein which is the basic unit for the synthesis of the icosahedral structure necessary for viral assembly. Therefore over expression is detected at maximum viral replication during acute stage (Cohen, 2010). On the other the two internal capsid proteins encoded by genes ICP-4 and ICP -22 are necessary for viral replication and transition for acute into more latent phases, respectively (Reichelt *et al*., 2009). Internal capsid protein ICP-4 is mandatory for viral replication since it functions through blockage of the antigen presentation associated transporter by binding to it and by this way it prevents the cell from viral antigen presentation and gets protected against cytotoxic T cell recognition and cell elimination (Erazo *et al*., 2008); whereas, ICP-22 is necessary for viral replication since its product regulates viral genes that are necessary to transmission form early acute phase into more latent stages (Cohen, 2010).

Finding over expression of MCP in the current study supports high rate of replication during acute stage. Finding of variable rates of expression of both ICP4 and ICP22 indicates variable stages in the transition of viral infection form acute into more latent stage.

There was no significant difference in mean age of children in whom MCP was over-expressed in comparison to children in whom MCP was not expressed; there was no significant difference in mean age of children in whom ICP4 was over-expressed in comparison to children in whom ICP4 was not expressed, over expression of MCP was more frequently associated with male gender, 73.9 % versus 55.0 %; however, the association was statistically insignificant (P = 0.194). Moreover, over expression of ICP4 was less frequently associated with male gender, 26.1 % versus 45.0 %; however, the association was statistically insignificant (P = 0.194). Added to that, over expression of ICP4 was less frequently associated with male gender, 30.4 % versus 60.0 %; however, the association was statistically insignificant (P = 0.052).

To the best of our knowledge no published article has study the exact Frequency rate of MCP, ICP4 and ICP22 expression levels in a sample of human being infected recently with VZV and also no association with sociodemographic factors has been highlighted.

3.2.3.3 Genotyping of VZV according to Open Reading Frame ORF38, ORF54 and ORF62

The aim of the present study was to figure out the genetic strains of *Varicella Zoster Virus* (*VZV*) that are common in Al Diwaniyah region of Iraq. Three single nucleotide polymorphism (SNP) alleles were estimated and these were ORF38, ORF54 and ORF62. Accordingly virus genotype strains were identified. Cases which were positive for ORF38 and ORF54 SNPs were considered **genotype B** whereas cases which were positive for ORF38 and negative for ORF54 were labeled as **genotype A**, so that genotype B was more frequent accounting for (72.1 %) followed by genotype A accounting for (27.9 %),

whereas none of our cases was labeled genotype C (0.0 %). In agreement with our finding, in Kuwait, regarding VZV genotyping, a study aimed to genotype VZV in samples collected from patients using restriction fragment length polymorphism (RFLP) technique and found that the predominant genotype was B (86.6%), followed by A (11.7%) and C (1.7%) (Qasem *et al*., 2012).

In a cross sectional study conducted in Iran, 38 chickenpox patients, who referred to the hospitals of Iran University of Medical Sciences in Tehran from May 2010 to June 2015 were investigated for primers of ORFs 38, 54 and 62, then RFLP assay and digestion carried out by *Pst*I (for ORF38) and *Bgl*I (for ORF54) restriction enzymes. The finding of this Iranian study was that the predominant *VZV* genotype was genotype B with a Frequency rate of (89.5 %) in support for the finding of the current study that the major *VZV* genotype are B (Safarnezhad Tameshkel *et al*., 2016).

In a Korean study, in restriction enzyme analysis, 51 of 54 (94.3%) isolates contained a *Pst*I site in ORF38, and all isolates contained a *Bgl*I site in ORF54 (Kim *et al*., 2011). The present study agrees with finding of the Korean study that wild genotype (B) is most frequent.

In a Chinese study, analysis of the R5 variable-repeat region in those strains revealed that 9 (47.4%) were type (A), while the remaining 10 strains (52.6%) were type (B) (Liu *et al*., 2009) and this finding confirm the finding of the current study.

There was no significant association between age of child and ORF54 SNP allele frequency (P= 0.951). In addition, the association between ORF54 and gender of infected children was insignificant (P = 0.078).

Genotypes A and B were also neither correlated significantly to age of children nor gender of children. These results agreed with the Australian study of (Toi and Dwyer, 2010) who described no significant association between VZV genotype and region, age or gender.

3.2.3.4 Phylogenetic Tree Analysis

The present study revealed that the genetic stains of VZV isolated from Iraqi patients were strongly related to German strains (98 to 100 %), and also related to other strains isolated in USA, UK, Korea, Mexico and Canada (NCBI-BANK).

Conclusions & Recommendations

Conclusions & Recommendations

1. Conclusions

1. The clinical prevalence rate of VZV in Al Diwaniyah governorate was (17.6 %).
2. The clinical frequency rate was not correlated to age, gender or residency of enrolled children.
3. The most frequency VZV genotype in Al Diwaniyah governorate was the B genetic subtype (wild type) accounting for 31 out of 45 (72.1 %) whereas, the A genetic subtype accounted for 12 out of 43 (27.9 %).
4. Genotype A and B were not correlated to age, gender or residency of enrolled children.

2. **Recommendations**

1. Immunization program in Iraq should be encouraged to target the wild B genotype because is the most prevalent VZV genotype in Al Diwaniyah governorate.
2. Investigation of virus genotype after vaccination with live attenuated VZV vaccine in the further studies.
3. A larger study that is multicentric and included a number of governorates is needed in order to validate the conclusions of the present study.
4. A study aiming at culturing the virus in order to compare the sensitivity, specificity, positive predictive value, negative predicative value and accuracy of conventional PCR in detection VZV infection.
5. Assessment of correlation between serologic and molecular diagnostic methods of VZV infection.

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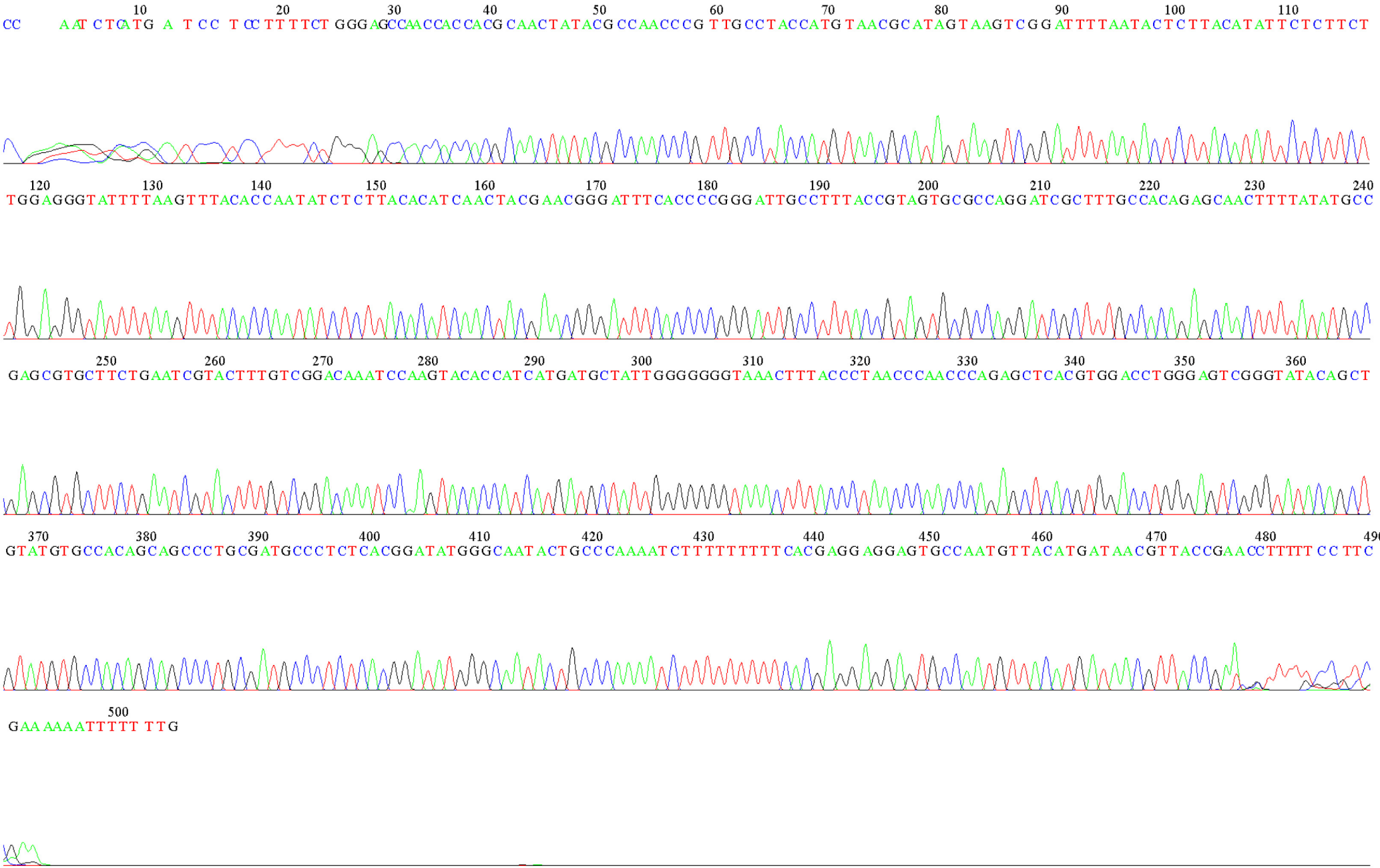
Appendices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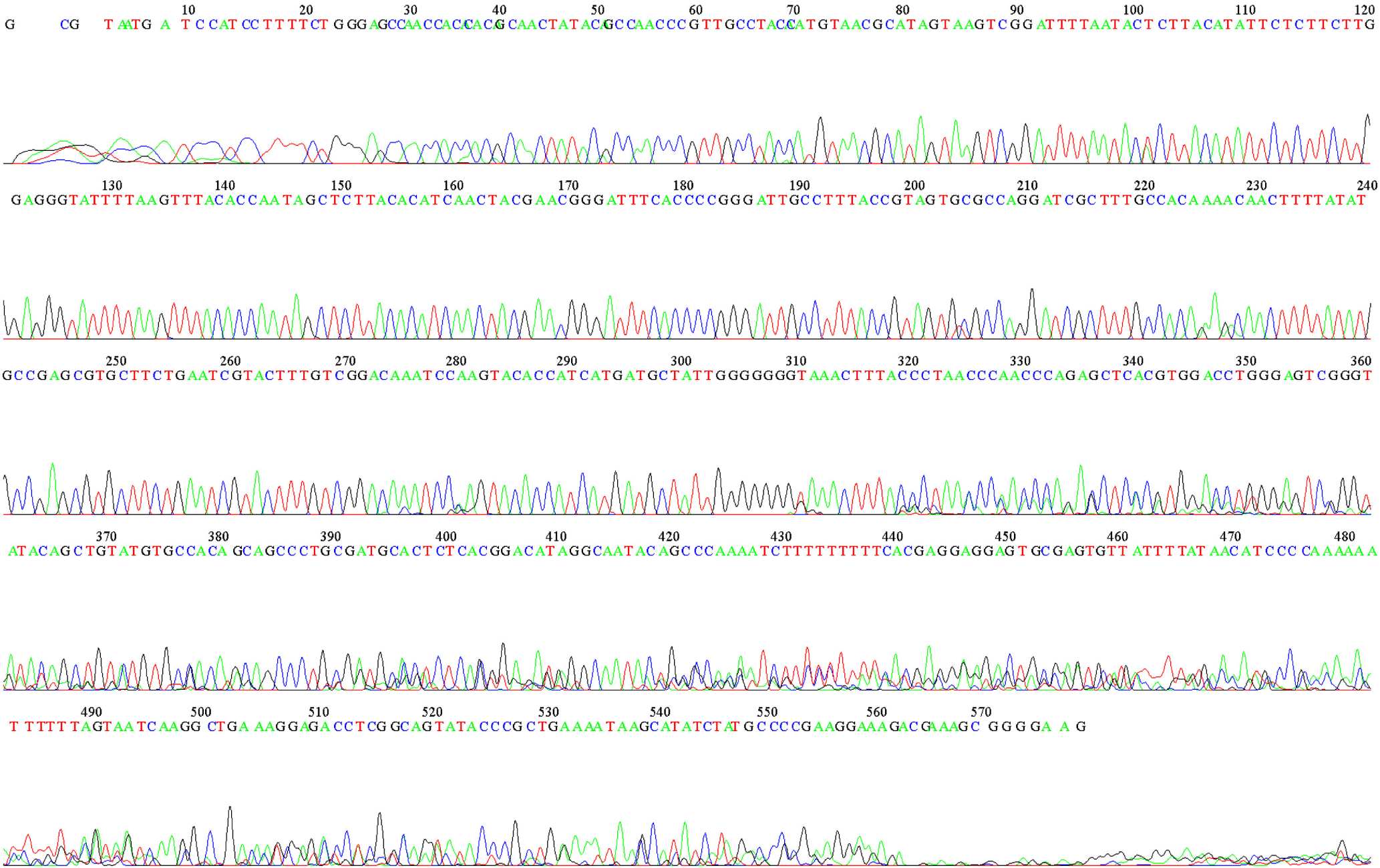

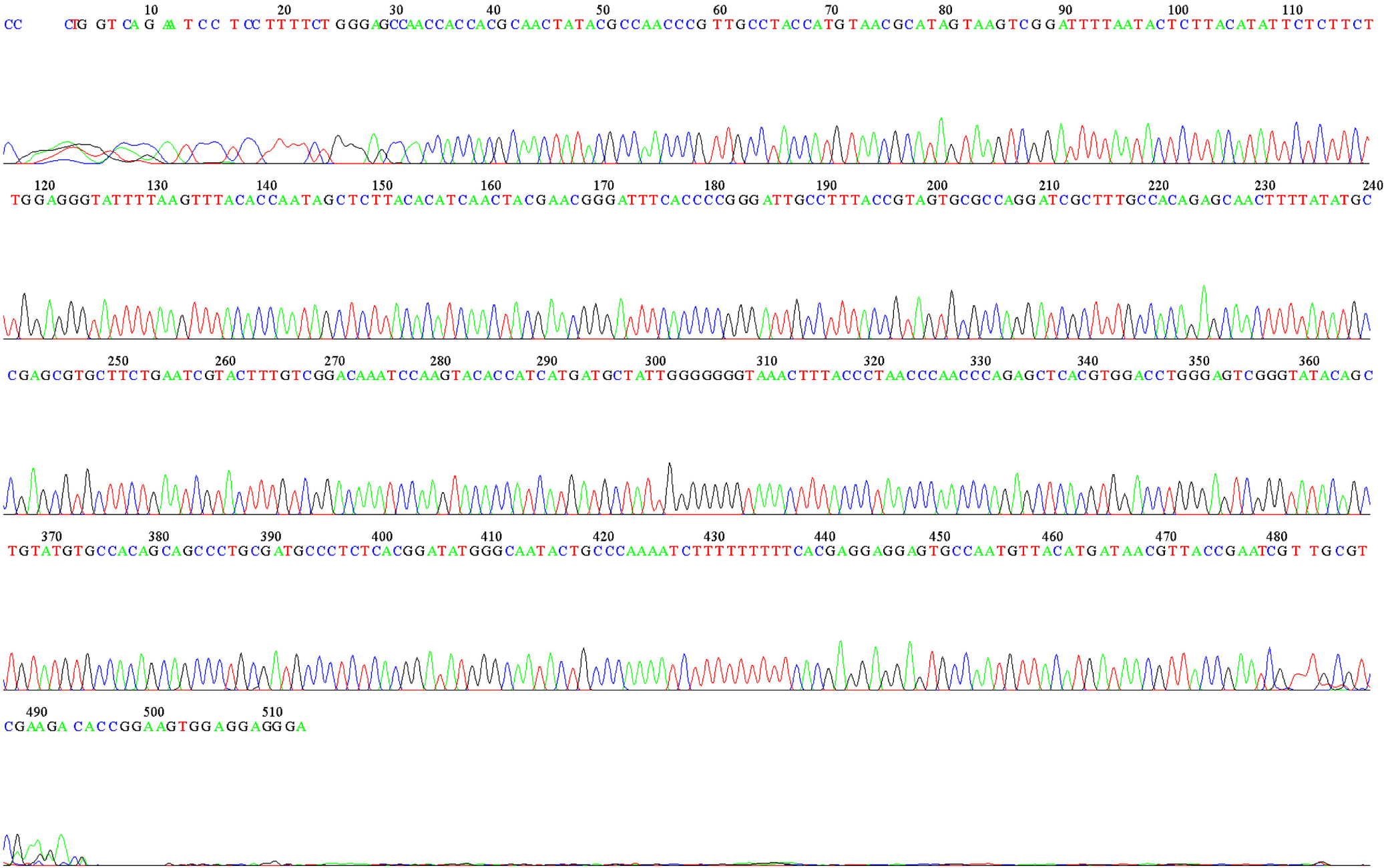
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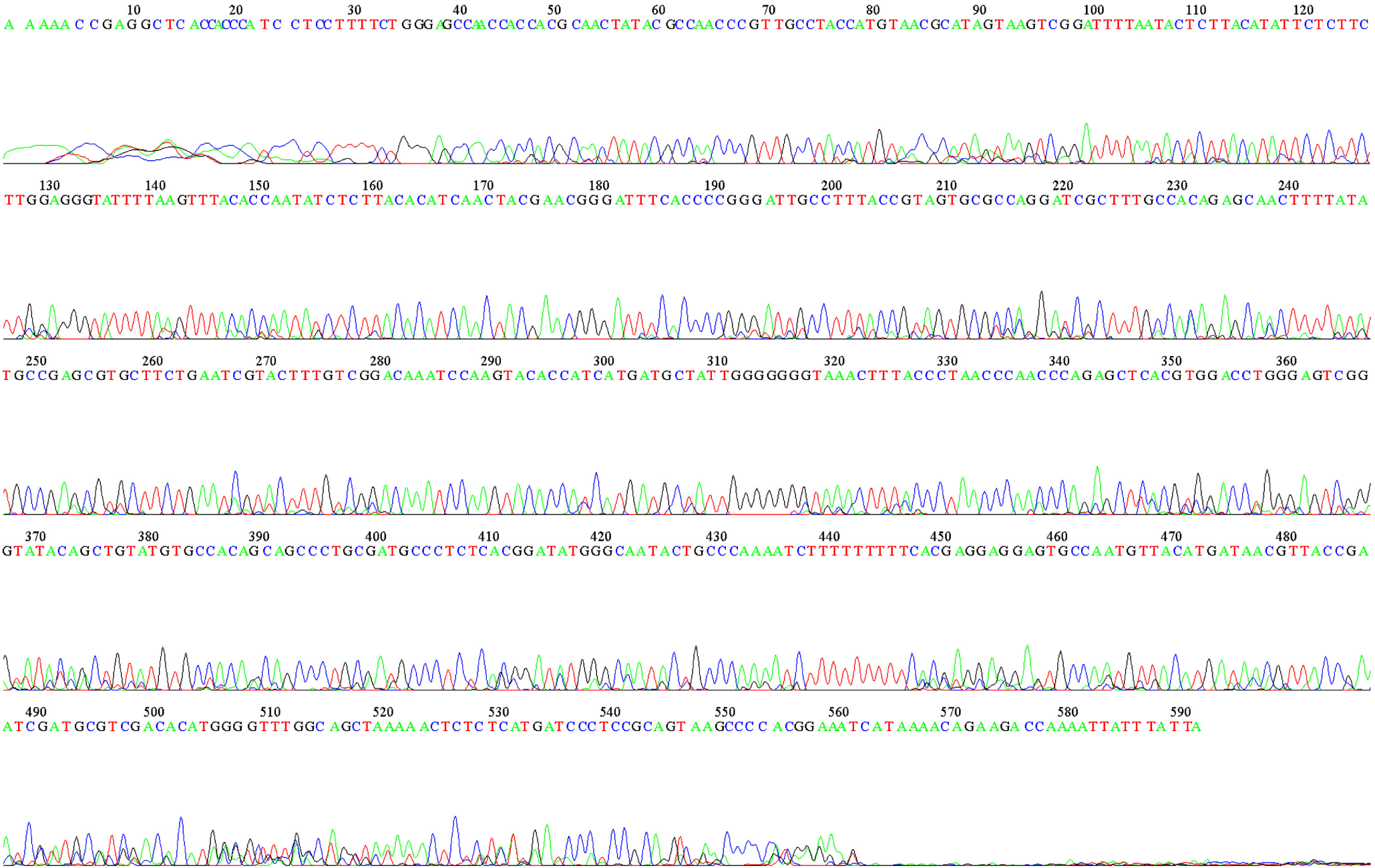
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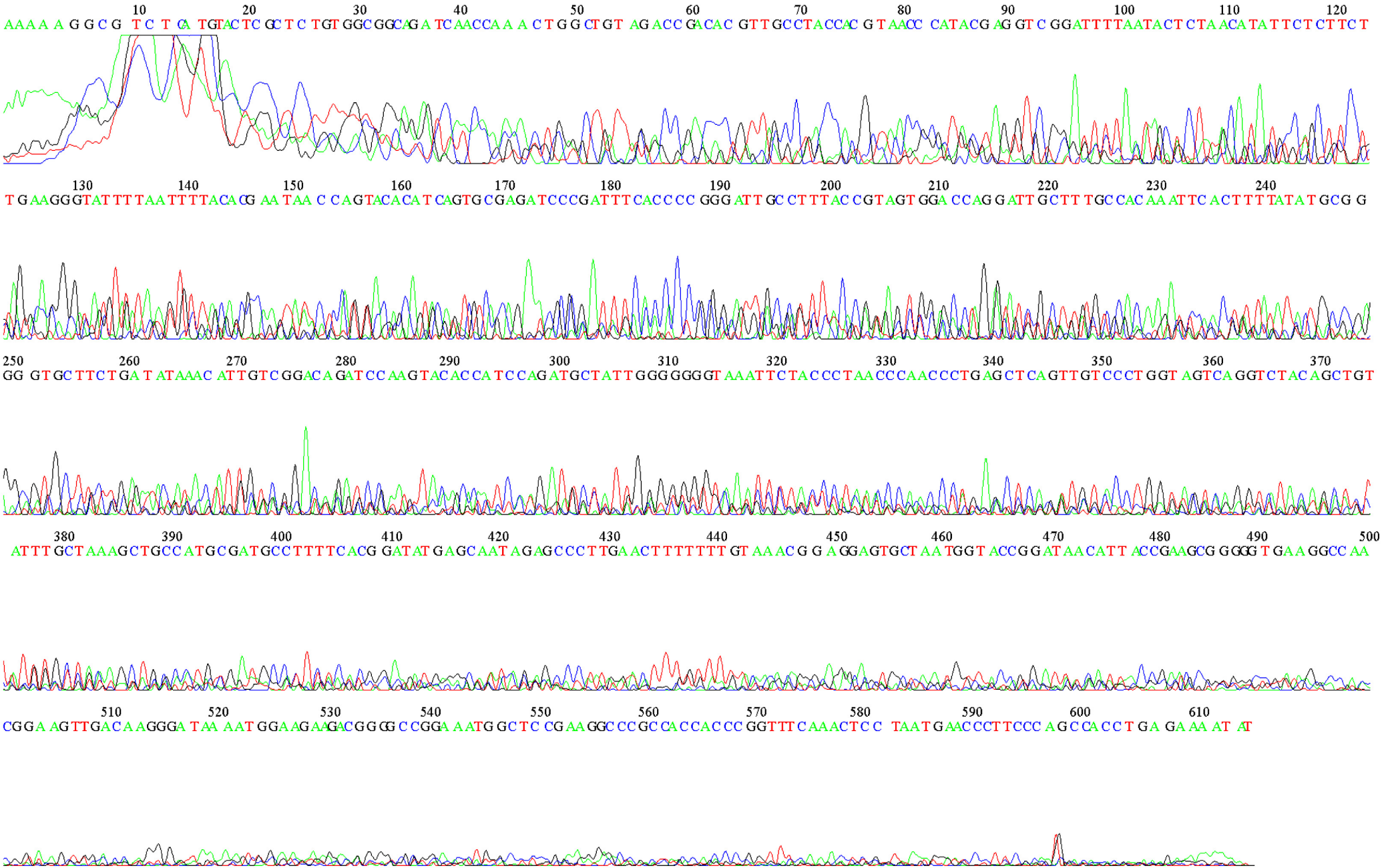
File: A5_A_F_primer.ab1 Run Ended: 2017/11/6 20:30:13 Signal G:52 A:77 C:105 T:102

Sample: A5_A_F_primer Lane: 7 Base spacing: 16.335537 615 bases in 7373 scans Page 1 of 1



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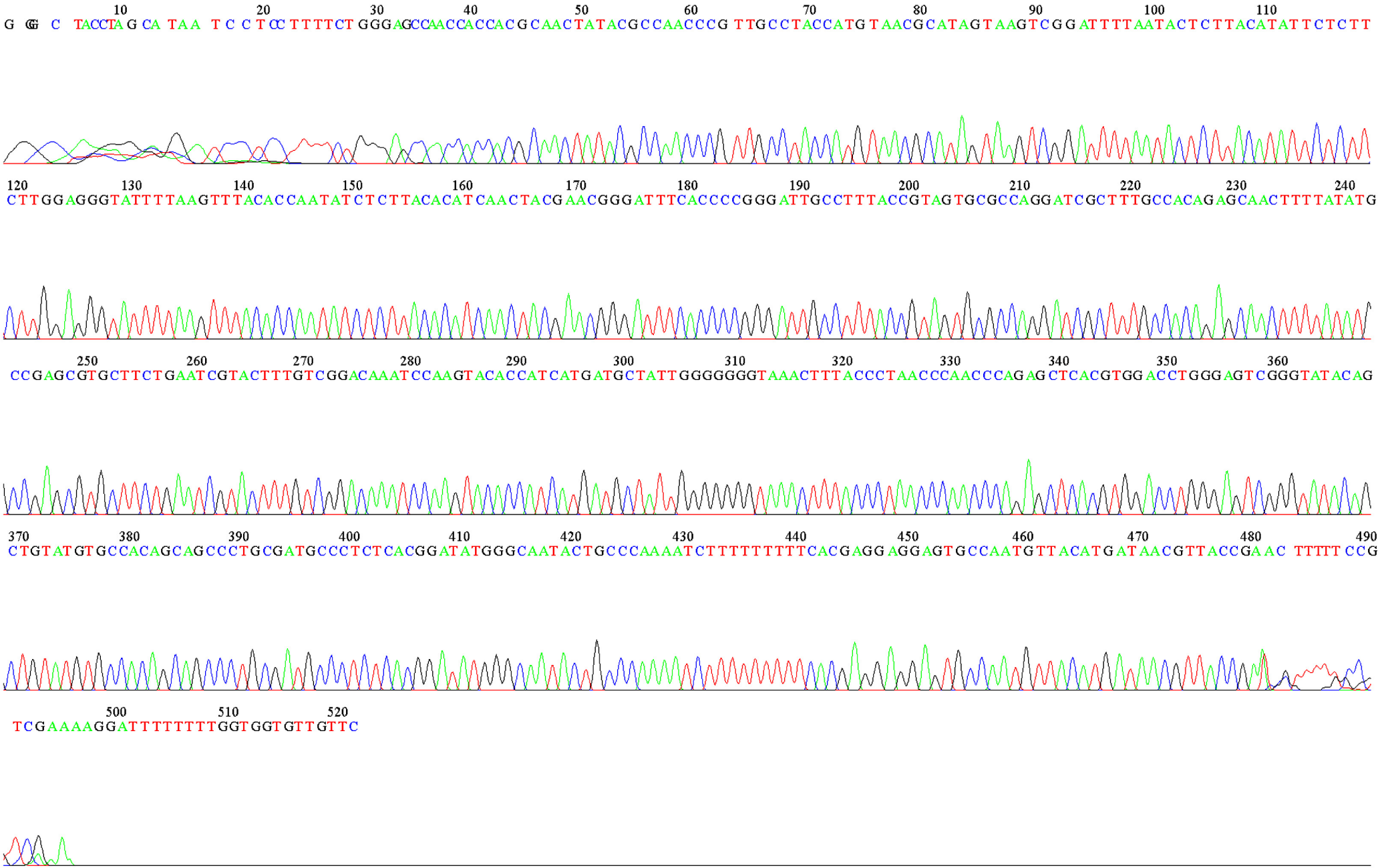
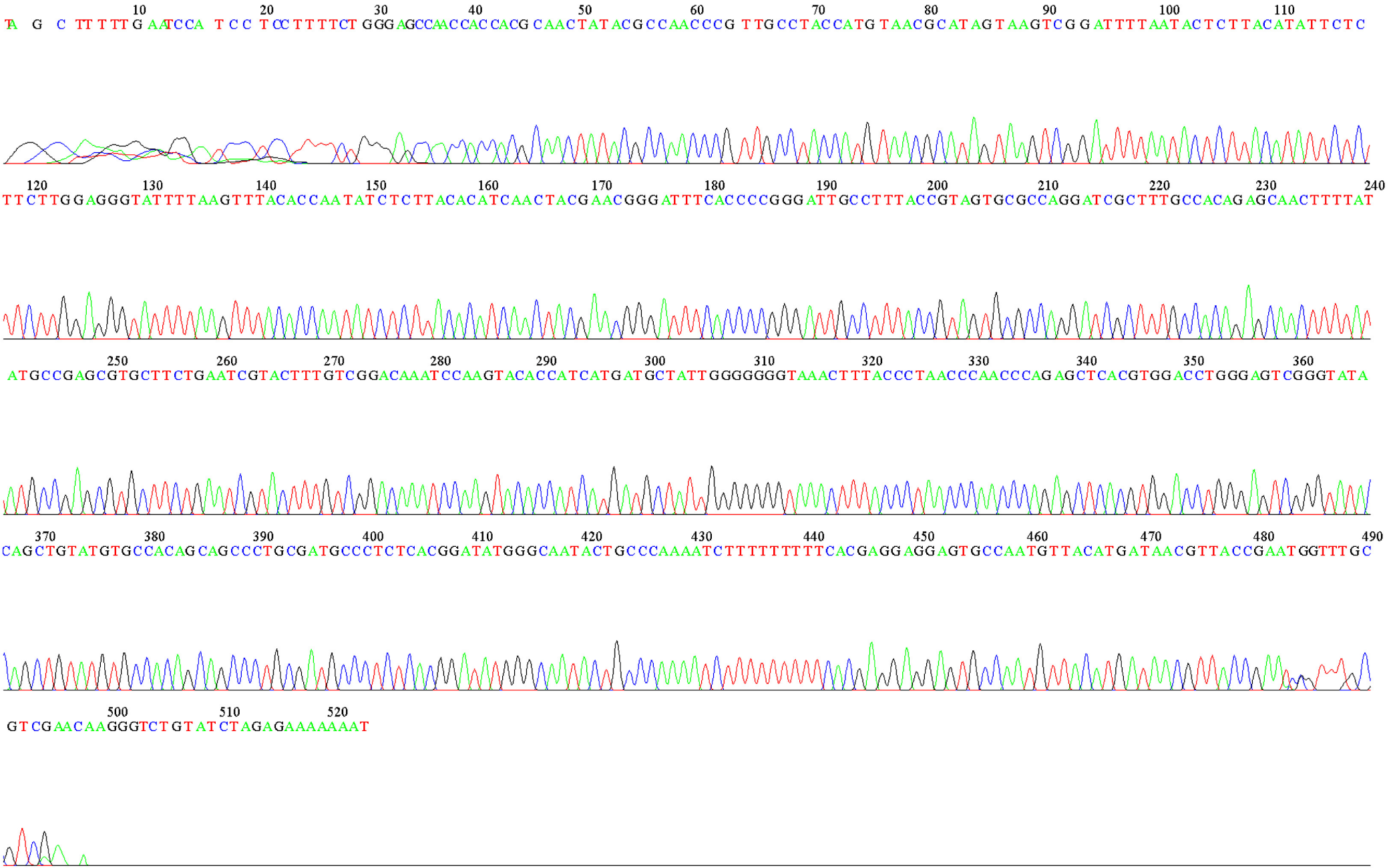
Sample: A6_A_F_primer *Lane: 5* *Base spacing: 16.300364* *522 bases in 31346 scans* *Page 1 of 2*

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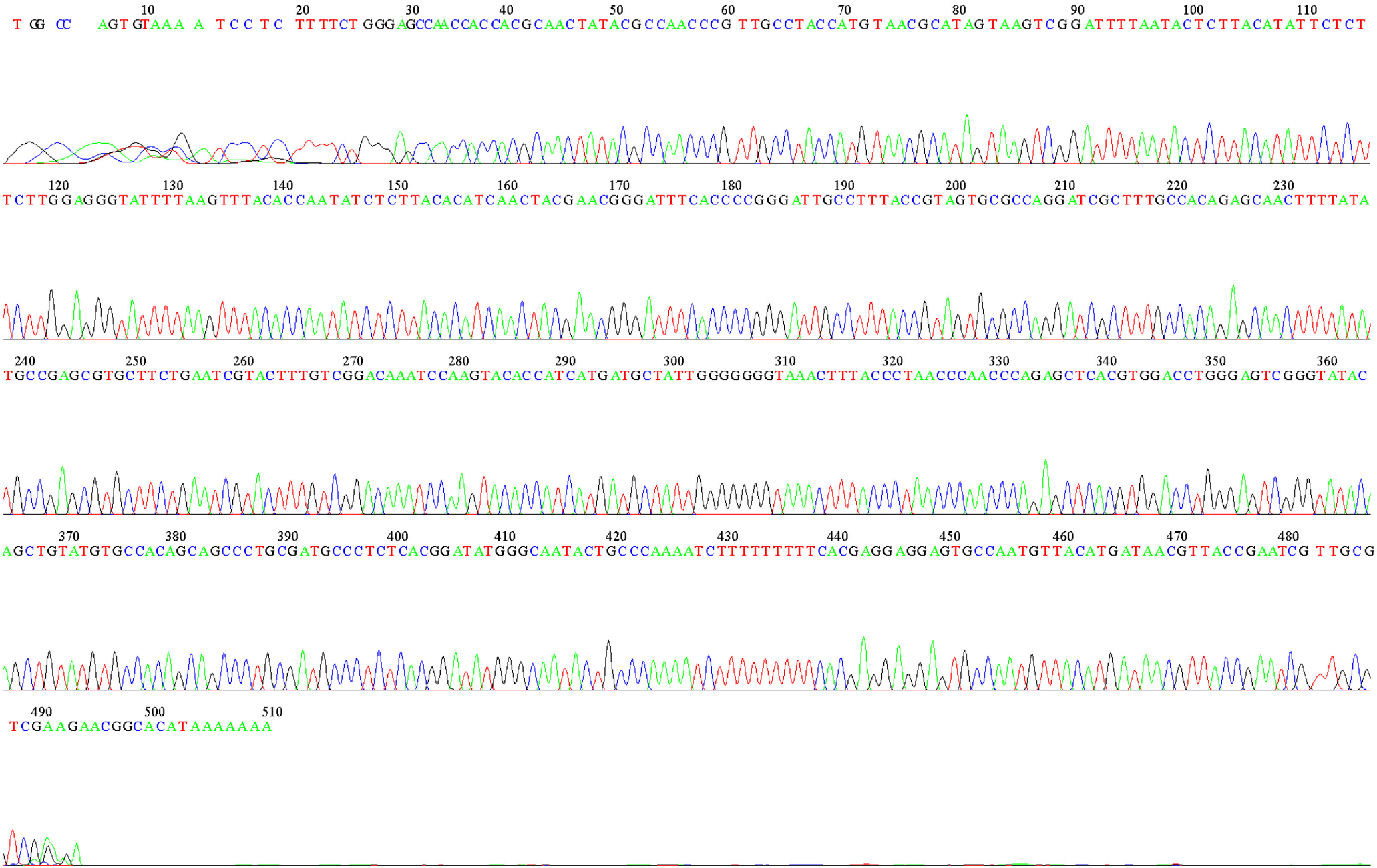
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PopSet

GenBank

Links from Nucleotide

Human alphaherpesvirus 3 major capsid protein (mcp) gene, partial cds.

PopSet: 1579876265

Sequences

Study Details

Molecular surveillance of Varicella-Zoster Virus Among School Age Children in Al-Diwaniyah province

Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.

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VERSION     MK399292.1
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REFERENCE   1  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Molecular surveillance of Varicella-Zoster Virus Among School Age
            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
            Iraq
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REFERENCE   1  (bases 1 to 350)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
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            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 350)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
            Iraq
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  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Molecular surveillance of Varicella-Zoster Virus Among School Age
            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
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  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
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  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Molecular surveillance of Varicella-Zoster Virus Among School Age
            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 350)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
            Iraq
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  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Molecular surveillance of Varicella-Zoster Virus Among School Age
            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 300)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
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REFERENCE   1  (bases 1 to 300)
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 300)
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  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
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            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
            Iraq
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      121 acgggatttc accccgggat tgcctttacc gtagtgcgcc aggatcgctt tgccacagag
      181 caacttttat atgccgagcg tgcttctgaa tcgtactttg tcggacaaat ccaagtacac
      241 catcatgatg ctattggggg ggtaaacttt accctaaccc aacccagagc tcacgtggac
      301 ctgggagtcg ggtatacagc tgtatgtgcc acagcagccc tgcgatgccc tctcacggat
      361 atgggcaata ctgcccaaaa tctttttttt tcacgaggag
//
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DEFINITION  Human alphaherpesvirus 3 isolate Varicella-zoster virus-IQ-No.8
            major capsid protein (mcp) gene, partial cds.
ACCESSION   MK399299
VERSION     MK399299.1
KEYWORDS    .
SOURCE      Human alphaherpesvirus 3 (HHV-3)
  ORGANISM  Human alphaherpesvirus 3
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            Alphaherpesvirinae; Varicellovirus.
REFERENCE   1  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Molecular surveillance of Varicella-Zoster Virus Among School Age
            Children in Al-Diwaniyah province
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 400)
  AUTHORS   Abed,A.S., Aubaid,A.H. and Al-Rodhan,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JAN-2019) Medical Microbiology, College of Medicine /
            University of Al-Qadisiyah, Jameaa, Diwanyiah, Al-Qadisiyah 00964,
            Iraq
COMMENT     ##Assembly-Data-START##
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                     /country="Iraq"
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      121 ggatttcacc ccgggattgc ctttaccgta gtgcgccagg atcgctttgc cacagagcaa
      181 cttttatatg ccgagcgtgc ttctgaatcg tactttgtcg gacaaatcca agtacaccat
      241 catgatgcta ttgggggggt aaactttacc ctaacccaac ccagagctca cgtggacctg
      301 ggagtcgggt atacagctgt atgtgccaca gcagccctgc gatgccctct cacggatatg
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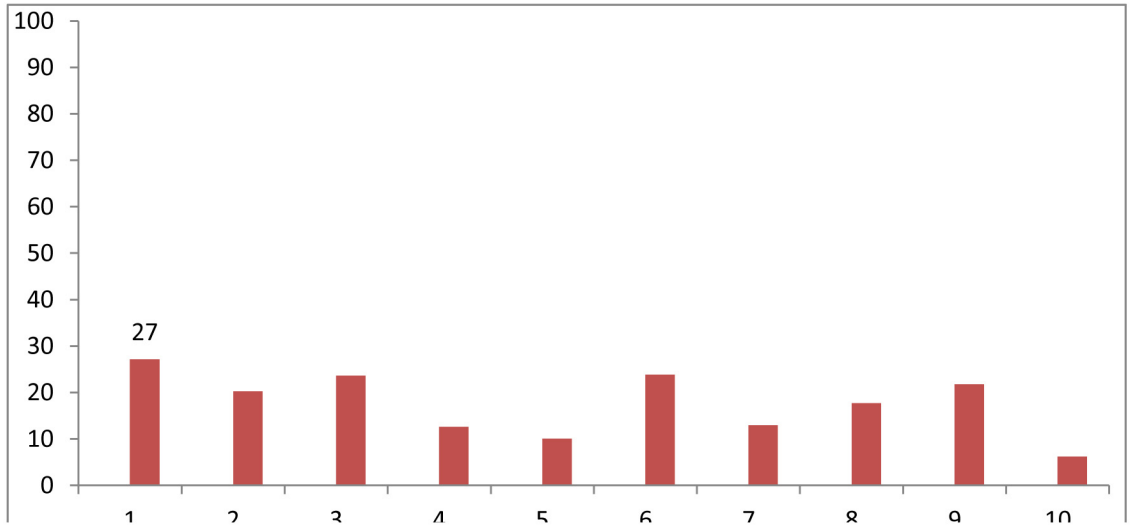
Appendix;2 Resuls of Gene Expression of MCP, ICP4 and ICP22 of VZV

VZV skin lesion samples				RFLP-SNP				Gene exprression		
Case No.	Age	Sex	PCR	ORF34	ORF54	ORF62	Genotype	VZV-mcp	VZV-ICP4	VZV-ICP22
1	6	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
2	7	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
3	8	M	Positive	Positive			A	Over expression	Non expression	Non expression
4	6	M	Positive	Positive	Positive		B	Over expression	Non expression	Over expression
5	7	M	Positive	Positive			A	Over expression	Non expression	Non expression
6	8	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
7	9	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
8	10	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
9	11	M	Positive	Positive			A	Non expression	Over expression	Over expression
10	8	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
11	9	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
12	10	F	Positive	Positive	Positive		B	Over expression	Over expression	Non expression
13	11	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
14	6	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
15	7	M	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
16	8	M	Positive	Positive			A	Non expression	Over expression	Over expression
17	9	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
18	10	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
19	11	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
20	8	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
21	9	M	Positive	Positive			A	Over expression	Non expression	Non expression
22	8	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
23	9	F	Positive	Positive			A	Over expression	Non expression	Over expression
24	10	F	Positive	Positive	Positive		B	Over expression	Non expression	Over expression
25	11	F	Positive	Positive			A	Over expression	Non expression	Over expression
26	6	F	Positive	Positive			A	Over expression	Non expression	Non expression
27	7	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
28	8	M	Positive	Positive			A	Over expression	Non expression	Non expression
29	9	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
30	10	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
31	11	M	Positive	Positive			A	Over expression	Non expression	Non expression
32	8	M	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
33	6	M	Positive	Positive			B	Non expression	Over expression	Over expression
34	7	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
35	8	M	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
36	6	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
37	7	M	Positive	Positive			A	Over expression	Non expression	Non expression
38	8	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression
39	9	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
40	10	M	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
41	10	F	Positive	Positive	Positive		B	Non expression	Over expression	Over expression
42	11	F	Positive	Positive	Positive		B	Non expression	Non expression	Over expression
43	6	F	Positive	Positive	Positive		B	Over expression	Non expression	Non expression

Appendix;1 All Study Analysis

VZV Clinical observation					
School No.	Area	Sex	Total	Clinical observation positive	Total prevlence %
1	R	M	541	147	27
2	R	M	409	83	20
3	R	F	461	109	24
4	R	F	420	53	13
5	U	M	208	21	10
6	R	F	369	88	24
7	U	F	508	66	13
8	R	M	356	63	18
9	U	M	583	127	22
10	R	M	690	43	6
Total			**4545**	**800**	**18**

School No.	Total prevlence %
S1	27
S2	20
S3	24
S4	13
S5	10
S6	24
S7	13
S8	18
S9	22
S10	6



	Gene expression		
Patient No.	VZV-mcp	VZV-ICP4	VZV-ICP22
1	Over expression	Non expression	Non expression
2	Over expression	Non expression	Non expression
3	Over expression	Non expression	Non expression
4	Over expression	Non expression	Over expression
5	Over expression	Non expression	Non expression
6	Over expression	Non expression	Non expression
7	Over expression	Non expression	Non expression
8	Over expression	Non expression	Non expression
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25	Over expression	Non expression	Over expression
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27	Non expression	Over expression	Over expression
28	Over expression	Non expression	Non expression
29	Over expression	Non expression	Non expression
30	Over expression	Non expression	Non expression
31	Over expression	Non expression	Non expression
32	Over expression	Non expression	Non expression
33	Non expression	Over expression	Over expression
34	Non expression	Over expression	Over expression
35	Non expression	Over expression	Over expression
36	Non expression	Over expression	Over expression
37	Over expression	Non expression	Non expression
38	Over expression	Non expression	Non expression
39	Non expression	Over expression	Over expression
40	Non expression	Over expression	Over expression
41	Non expression	Over expression	Over expression
42	Non expression	Non expression	Over expression
43	Over expression	Non expression	Non expression

RT-qPCR for VZV-ICP22 gene expression					
No.	CT (ICP22)	CT (GAPDH)	ΔCT	Fold change expression	Expression
1	35.15	34.71	-0.44	**0.74**	**Non expression**
2	35.36	34.15	-1.21	**0.43**	**Non expression**
3	35.81	33.41	-2.40	**0.19**	**Non expression**
4	31.00	33.53	2.53	**5.78**	Over expression
5	36.04	34.21	-1.83	**0.28**	**Non expression**
6	36.22	33.89	-2.33	**0.20**	**Non expression**
7	36.63	34.01	-2.62	**0.16**	**Non expression**
8	37.07	33.55	-3.53	**0.09**	**Non expression**
9	31.56	34.71	3.15	**8.89**	Over expression
10	31.19	34.15	2.96	**7.79**	Over expression
11	31.03	33.41	2.38	**5.22**	Over expression
12	38.12	33.53	-4.59	**0.04**	**Non expression**
13	38.18	34.21	-3.97	**0.06**	**Non expression**
14	38.34	33.89	-4.45	**0.05**	**Non expression**
15	30.21	33.01	2.80	**6.98**	Over expression
16	29.83	33.15	3.32	**9.95**	Over expression
17	30.13	33.02	2.89	**7.41**	Over expression
18	39.06	33.02	-6.04	**0.02**	**Non expression**
19	39.56	33.04	-6.52	**0.01**	**Non expression**
20	39.64	33.04	-6.60	**0.01**	**Non expression**
21	39.82	33.08	-6.74	**0.01**	**Non expression**
22	40.32	33.08	-7.24	**0.01**	**Non expression**
23	31.57	34.26	2.69	**6.45**	Over expression
24	32.39	34.26	1.87	**3.66**	Over expression
25	32.48	33.48	1.00	**2.00**	Over expression
26	34.78	33.63	-1.15	**0.45**	**Non expression**
27	29.90	33.79	3.89	**14.83**	Over expression
28	34.15	33.48	-0.67	**0.63**	**Non expression**
29	33.36	33.77	0.41	**1.33**	**Non expression**
30	33.81	33.77	-0.04	**0.97**	**Non expression**
31	33.00	33.90	0.90	**1.87**	**Non expression**
32	36.04	33.90	-2.14	**0.23**	**Non expression**
33	30.63	33.90	3.27	**9.65**	Over expression
34	30.79	33.90	3.11	**8.63**	Over expression
35	30.03	34.12	4.09	**17.03**	Over expression
36	30.40	34.12	3.72	**13.18**	Over expression
37	33.50	34.37	0.87	**1.83**	**Non expression**
38	34.48	34.37	-0.11	**0.93**	**Non expression**
39	30.78	33.48	2.70	**6.50**	Over expression
40	30.54	33.63	3.09	**8.51**	Over expression
41	30.33	33.79	3.46	**11.00**	Over expression
42	30.87	33.48	2.61	**6.11**	Over expression
43	35.91	33.63	-2.28	**0.21**	**Non expression**

RT-qPCR for VZV-ICP4 gene expression					
No.	CT (ICP4)	CT (GAPDH)	ΔCT	Fold change expression	Expression
1	35.48	34.71	-0.77	**0.59**	**Non expression**
2	36.89	34.15	-2.74	**0.15**	**Non expression**
3	34.88	33.41	-1.47	**0.36**	**Non expression**
4	33.77	33.53	-0.24	**0.85**	**Non expression**
5	36.12	34.21	-1.91	**0.27**	**Non expression**
6	33.46	33.89	0.43	**1.34**	**Non expression**
7	33.89	33.01	-0.88	**0.54**	**Non expression**
8	33.50	33.15	-0.35	**0.78**	**Non expression**
9	30.12	33.02	2.90	**7.46**	Over expression
10	30.53	33.02	2.49	**5.62**	Over expression
11	30.04	33.04	3.00	**8.00**	Over expression
12	31.19	33.04	1.85	**3.61**	Over expression
13	33.50	33.08	-0.42	**0.75**	**Non expression**
14	34.48	33.08	-1.40	**0.38**	**Non expression**
15	31.78	34.26	2.48	**5.58**	Over expression
16	31.12	34.26	3.14	**8.82**	Over expression
17	30.95	33.48	2.53	**5.78**	Over expression
18	35.36	33.63	-1.73	**0.30**	**Non expression**
19	35.51	33.79	-1.72	**0.30**	**Non expression**
20	36.20	33.48	-2.72	**0.15**	**Non expression**
21	36.26	33.77	-2.49	**0.18**	**Non expression**
22	36.31	33.77	-2.54	**0.17**	**Non expression**
23	36.77	33.90	-2.87	**0.14**	**Non expression**
24	36.85	33.90	-2.95	**0.13**	**Non expression**
25	36.98	33.90	-3.08	**0.12**	**Non expression**
26	37.29	33.90	-3.39	**0.10**	**Non expression**
27	31.21	34.12	2.91	**7.52**	Over expression
28	38.33	34.12	-4.21	**0.05**	**Non expression**
29	38.40	34.37	-4.03	**0.06**	**Non expression**
30	39.24	34.37	-4.87	**0.03**	**Non expression**
31	39.66	33.48	-6.18	**0.01**	**Non expression**
32	39.84	33.63	-6.21	**0.01**	**Non expression**
33	31.24	33.79	2.55	**5.86**	Over expression
34	30.59	33.48	2.89	**7.41**	Over expression
35	31.62	33.63	2.01	**4.03**	Over expression
36	30.23	33.79	3.56	**11.79**	Over expression
37	39.62	33.41	-6.21	**0.01**	**Non expression**
38	36.53	33.11	-3.42	**0.09**	**Non expression**
39	30.51	33.45	2.94	**7.67**	Over expression
40	31.56	34.01	2.45	**5.48**	Over expression
41	30.31	33.55	3.24	**9.42**	Over expression
42	30.45	33.02	2.57	**5.94**	**Non expression**
43	36.20	33.02	-3.18	**0.11**	**Non expression**

Appendix;3 RT-qPCR for VZV-mcp gene expression

No.	CT (mcp)	CT (GAPDH)	ΔCT	Fold change expression	Expression
1	30.54	34.71	4.18	**18.06**	Over expression
2	29.73	34.15	4.42	**21.36**	Over expression
3	30.18	33.41	3.24	**9.42**	Over expression
4	30.93	33.53	2.60	**6.06**	Over expression
5	29.73	34.21	4.48	**22.35**	Over expression
6	29.25	33.89	4.64	**24.93**	Over expression
7	30.14	33.01	2.88	**7.34**	Over expression
8	30.59	33.15	2.55	**5.87**	Over expression
9	32.11	33.02	0.91	**1.88**	**Non expression**
10	32.24	33.02	0.78	**1.72**	**Non expression**
11	32.47	33.04	0.57	**1.48**	**Non expression**
12	30.76	33.04	2.28	**4.86**	Over expression
13	30.84	33.08	2.24	**4.72**	Over expression
14	30.92	33.08	2.16	**4.47**	Over expression
15	34.17	34.26	0.09	**1.06**	**Non expression**
16	34.27	34.26	-0.01	**0.99**	**Non expression**
17	34.51	33.48	-1.03	**0.49**	**Non expression**
18	30.28	33.63	3.35	**10.20**	Over expression
19	30.29	33.79	3.50	**11.31**	Over expression
20	29.32	33.48	4.16	**17.88**	Over expression
21	32.24	33.77	1.53	**2.89**	Over expression
22	29.81	33.77	3.96	**15.56**	Over expression
23	31.60	33.90	2.30	**4.92**	Over expression
24	31.58	33.90	2.32	**4.99**	Over expression
25	31.93	33.90	1.97	**3.92**	Over expression
26	31.29	33.90	2.61	**6.11**	Over expression
27	33.61	34.12	0.51	**1.42**	**Non expression**
28	32.47	34.12	1.65	**3.14**	Over expression
29	30.93	34.37	3.44	**10.85**	Over expression
30	32.11	34.37	2.26	**4.79**	Over expression
31	31.28	33.48	2.20	**4.59**	Over expression
32	31.32	33.63	2.31	**4.96**	Over expression
33	35.16	33.79	-1.37	**0.39**	**Non expression**
34	35.28	33.48	-1.80	**0.29**	**Non expression**
35	35.52	33.63	-1.89	**0.27**	**Non expression**
36	34.44	33.79	-0.65	**0.64**	**Non expression**
37	30.40	33.41	3.01	**8.06**	Over expression
38	29.48	33.11	3.63	**12.38**	Over expression
39	34.73	33.45	-1.28	**0.41**	**Non expression**
40	35.70	34.01	-1.69	**0.31**	**Non expression**
41	33.80	33.55	-0.25	**0.84**	**Non expression**
42	34.36	33.02	-1.34	**0.40**	**Non expression**
43	30.37	33.02	2.65	**6.28**	Over expression

الخلاصة

الحماق (جدري الماء) هي الاصابة الاولية لفيروس الحماق (VZV) ، وهي عدوى خفيفة ذاتية الحد، ولكنها أيضًا معدية و يمكن أن تسبب مضاعفات شديدة بين مجموعة الأفراد ضعيفو المناعة. عادة ما تكون الاصابة في مرحلة الطفولة تعطينا مناعة ضد المرض مدى الحياة ، ولكن البالغين الذين ليس لديهم اصابة سابقا معرضون أيضًا للإصابة باي وقت. و من المرجح أن تتطور اصابة الأشخاص ضعيفو المناعة الى مضاعفات خطيرة.

الهدف من هذه الدراسة هو معرفة السلالات الوراثية لفيروس الحماق (VZV) الشائعة في محافظة الديوانية. حيث تم تسجيل ٤٥٤٥ طفلاً، وجمعت البيانات المتعلقة بهؤلاء الأطفال من ١٠ مدارس ابتدائية في محافظة الديوانية في منطقة الفرات الوسطى في العراق ، في حين وثقت العدوى في ٨٠٠ من بين ٤٥٤٥ طفلاً، حيث كان معدل الانتشار (١٧.٦٪) و لم يتم الحصول على فرق كبير من عدوى VZV فيما يتعلق بالعمر والجنس.

ثلاثة واربعون عينة تم اختيارها عشوائيا للتشخيص الجزيئي وتحديد السلالات الوراثية في المختبر ، حيث كانت النتائج بواسطة PCR التقليدية أن جميع الأطفال ٤٣ كان إيجابيا ل VZV. و كان متوسط عمر ٤٣ طفلاً الذين تم اختيارهم للدراسة الجزيئية ٨.٤٤ ± ١.٦٥ سنة و معدل اعمارهم يتراوح من ٦ إلى ١٠ سنوات. شملت عينات الدراسة ٢٣ طفلاً (ذكراً) (٥٣.٥٪) و ٢٠ طفلة (انثى) (٤٦.٥٪). حيث وجد ان الفارق طفيف في نسبة الذكور عن نسبة الإناث، إحصائياً (P = 0.647).

تم العمل على ثلاثة من النيوكليوتيدات أحادية النواة متعددة الاشكال (SNP) وكانت هذه ORF38، ORF54 و ORF62. كانت جميع الحالات إيجابية بالنسبة لـ ORF38 (١٠٠%) و كان عدد الحالات الإيجابية لـ ORF54 ٣١ من أصل ٤٣ (٧٢.١٪) بينما لم تكن أي من الحالات إيجابية لـ ORF62(٠ %). وفقا لذلك تم تحديد سلالات الفيروس الوراثي. تم اعتبار الحالات التي كانت إيجابية لـ ORF38 و ORF54 SNPs من النمط الوراثي (B) في حين تم تصنيف الحالات التي كانت إيجابية لـ ORF38 فقط على النمط الوراثي (A). ومع ذلك ، لم يتم تصنيف أي حالة على أنها النمط الوراثي (C) (ORF62).

لذلك ، كان النمط الوراثي الأكثر انتشارًا في VZV في محافظة الديوانية هو النوع الفرعي الوراثي ORF54 & ORF38 (B) (النوع الضاري) والذي يمثل ٣١ من أصل ٤٣ (٧٢.١٪) ، في

حين أن النوع A الوراثي يمثل ١٢ من أصل ٤٣ (٢٧.٩٪)). لم تكن الأنماط الجينية A و B مرتبطة بشكل كبير بسن ولا بجنس الأطفال.

التعبير الجيني كان عن VZV-MCP في (٦٥.١ ٪) من الحالات؛ التعبير الجيني عن VZV-ICP4 في (٣٤.٩٪) من الحالات ، وشوهد التعبير الجيني عن VZV-ICP22 في (٤٤.٢٪) من الحالات، حيث كان التعبير الجيني لـ VZV-MCP هو الأعلى. استنتجت الدراسة الحالية ان النمط الوراثي الأكثر انتشارًا ل VZV في محافظة الديوانية هو النوع الفرعي B الوراثي (النوع الضاري) أكثر من غيره.