MEASLES VIRUS RNA INVESTIGATION IN PERIPHERAL WHOLE BLOOD SPECIMENS COLLECTED FROM SUBACUTE SCLEROSING PANENCEPHALITIS PATIENTS BY REAL-TIME PCR METHOD

by

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This thesis is dedicated to my family…

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ABSTRACT

MEASLES VIRUS RNA INVESTIGATION IN PERIPHERAL WHOLE BLOOD SPECIMENS COLLECTED FROM SUBACUTE SCLEROSING PANENCEPHALITIS PATIENTS BY REAL-TIME PCR METHOD

Subacute Sclerosing Panencephalitis (SSPE) disease is an infection resulted from a virus which undergoes mutation during measles infection and is transferred to the brain in its dormant form. It can turn into its active form after many years and leads to irreversible damages in CNS and followed by death. Studies performed with real-time PCR method in diagnosis of SSPE is generally based on using brain biopsy materials for investigation measles virus (MV) RNA considering its being most reliable and frequently positive material. However, that positive results were found in a study carried out with blood samples of SSPE patient, shed a light on using blood material, which is easier to obtain, as a target material instead of brain material. In this study, measles vaccine and cell cultured measles strain from a patient were used as positive controls and whole blood specimens of six healthy donors provided from Yeditepe University Hospital were used as negative controls. Since it is determined that RNA encoded by *N* (nucleoprotein) gene belonging to the genome of the measles virus causing SSPE is the most abundant transcript, it was aimed to amplify *N* gene of MV-RNA. In this study we succeeded to obtain a positive result in a SSPE patient blood sample. However, no N RNA detection could be achieved in rest of the other patient blood samples. As conclusion, real-time PCR method validation we performed was confirmed with one positive result. Nevertheless, development of more comprehensive multiplex real-time PCR methods using internal control and additionally further detailed molecular analysis by sequencing will be required to obtain more accurate and conclusive results in diagnosis of SSPE.

ÖZET

SUBAKUT SKLEROZAN PANENSEFALİT HASTALARINDAN TOPLANAN PERİFERİK TAM KAN ÖRNEKLERİNDE EŞ-ZAMANLI PZR YÖNTEMİYLE KIZAMIK RNA' SININ ARAŞTIRILMASI

Subakut Sklerozan Panensefalit (SSPE) SSPE hastalığı, kızamık enfeksiyonu sırasında mutasyona uğramış kızamık virüsünün dormant haldeyken beyine yerleşmesiyle hastalık geçirildikten yıllar sonra aktif hale geçerek beyinde geri dönüşü olmayan tahribatlara yol açan ölümcül bir hastalıktır. SSPE'nin teşhisinde kızamık virüsünün araştırılması için eş-zamanlı PZR tekniği kullanılarak yapılan çalışmalar en güvenilir pozitif sonuçları vermesi bakımından genellikle beyin biyopsi materyallerinin kullanımına dayanmaktadır. Ancak kan örnekleriyle yapılan bir çalışmada pozitif sonuçlara rastlanması, hedef materyal olarak beyin yerine daha kolay elde edilen kan örneklerinin kullanımının önünü açmıştır. Bu çalışmada kızamık aşısı ve bir hastadan hücre kültürü yöntemiyle elde edilmiş kızamık suşu pozitif kontrol, Yeditepe Üniversitesi Hastanesi'nin sağladığı altı sağlıklı donörden elde edilen tam kan örnekleri ise negatif kontrol olarak kullanılmıştır. SSPE'ye sebep olan kızamık virüsünde *N* (nükleoprotein) genince kodlanan RNA'nın en fazla rastlanılan trankript olması nedeniyle *N* geninin çoğaltılmasına karar verilmiştir.Biz bu çalışmada bir SSPE hastasının kan örneğinden pozitif sonuç almayı başardık. Ancak diğer hastalarda N RNA varlığını tespit edemedik. Sonuç olarak eş-zamanlı PZR validasyonu bir pozitif sonuçla doğrulanmış oldu. Ancak internal kontrol kullanılarak uygulanan daha kapsamplı multipleks eş-zamanlı PZR yöntemleri ve sekanslama ile birlikte yapılan daha detaylı moleküler analizler SSPE teşhisinde daha tutarlı ve güvenilir sonuçların elde edilmesi için gereklidir.

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LIST OF SYMBOLS / ABBREVIATIONS

AT	Adenine, Guanine
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dH_2O	Distilled water
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraactic acid
CNS	Central Nervous System
CSF	Cerebrospinal fluid
EEG	Electroencephalography
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INF-α	Interferon alpha
IL-2	Interleukin 2
IL-4	Interleukin 4
$MgCl_2$	Magnesium chloride
µl	Microliters
µM	Micromolar
ml	Milliliters
mM	Millimolar
NCBI	National Center for Biotechnology Information
PHA	Phytohaemagglutinin
Poly (I-C)	polyinosinic: polycytidilic acid).
RNA	Ribonucleic acid
SNP	Single-nucleotide polymorphism

TBE	Tris/Borate/EDTA
TRIS	Tris(hydroxtmethyl)aminomethane
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
UV	Ultraviolet
WHO	World Health Organization

1. INTRODUCTION

Subacute sclerosing panencephalitis (SSPE), is a devastating, progressive **"**slow virus**"** brain disease most likely resulted from measles virus which undergoes mutation during measles infection and is transferred to the brain in its dormant form. It can turn into its active form after many years and leads to irrevesible damages followed by death within approximately 1-3 years. Despite the fact that there are various considerable theories , the subject of which mechanism provides the persistence of the virus remains unclear.

Disease generally affects young children who had measles some 6–7 years earlier. Although it is known as a childhood disease, information about SSPE cases diagnosed in nursing infancy and adulthood is gradually increasing. Latest studies suggest that this period may vary between 9 months to 30 years.

Brain biopsies or postmortem histopathological examinations reveal evidence of astrogliosis, neuronal loss, degeneration of dendrites, demyelination, neurofibrillary tangles, and infiltration of inflammatory cells. Patients usually suffer from myoclonus, dementia, visual disturbances, pyramidal and extrapyramidal signs and show behavioral changes. It is also known from the stated cases that uncommon neurological pictures can be observed during the disease. The conventional methods for diagnosis is based upon characteristic clinical manifestations, the presence of characteristic periodic EEG discharges, and confirmation of increased antibody titer against measles in the sera and CSF. Detecting measles virus RNA (MV-RNA) with Polymerase Chain Reaction (PCR) methods is an alternative technique which has many advantages and realibility.

The aim of this study was the development and validation of the most convenient real-time Polymerase Chain Reaction (real-time PCR) protocol for detecting measles virus RNA (MV-RNA) targeting *N* (Nucleoprotein) gene in the peripheral whole blood samples collected from the patients who were diagnosed with SSPE clinically and serologically.

2. THEORETICAL BACKGROUND

2.1. HISTORY

Two children from Tennessee were the first reported cases who were seen by Dawson in 1933 and 1934 respectively. A progressive fatal disorder consisting of limb jerking and mental deterioration were observed in both cases. Dawson considered the pathological transforms in their brains as an indication of encephalitis caused by viral infection [1,2]. Pette & DoÅNring [3], and Von Bogaert[4] reported two other cases in 1939 and 1945 respectively. The four aforementioned cases showed a similar clinical course but revealed diverse brain pathology. The combined findings in those cases showed the presence of inflammation with intraneuronal eosinophilic inclusions and nodular sclerotic changes in the white and gray matter. Poser & Radermecker have studied seven cases in 1957, three with postmortem, and they came to the conclusion that all of the cases have been affected by the same syndrome called **"**Subacute Sclerosing Leucoencephalitis" by them [5]. The term `SSPE` (Subacute Sclerosing Panencephalitis) was coined by Greenfield in 1950[6]. In the understanding of SSPE, a huge progress was made by Boutellie who with the help of the electron microscope identified in the brain of patients with SSPE particles which resembled nucleocapsids of paramyxovirus [7]. The learning that all patients with SSPE have increased titers of measles antibodies in blood and CSF [8] is led by this very important finding pointing to the role of measles virus (MV) in the pathology of SSPE. Following this crucial observation, worldwide epidemiological studies identified the relationship between the incidence of clinical measles morbidity and SSPE. The abrupt and distinct reduction in the frequency of measles and SSPE has been result of a worldwide measles immunization program led by this pivotal observation.

2.2. EPIDEMIOLOGY

Despite the fact that the pandemic of SSPE was almost eradicated due to mass immunization during 1960–1980's, SSPE is still a fatal disease for the young children, even adults in countries where measles immunization is incomplete and in world regions where genetic polymorphism to this particular infection is present.

It is revealed that the disease occurs in male children 2.5 times more than female children [9,10]. According to some other publication this ratio is 4/1 [11]. In İstanbul occurance ratio between genders was determined as 2,3/1 [12].

According to epidemiological data from the USA, only 2–3 cases of SSPE were recorded during the early 90's of the previous century. However, case number increased to 13 during 2000 [13,25].

2.2.1. Measles Case Prevalence in Foreign Countries

In Israel, 992 cases of measles younger than 15 years were registered in the Jerusalem district between 2007 and 2008 [14]. According to the data released by Health Protection Agency (HPA), 485 laboratory confirmed cases of measles have been reported in England and Wales up to the end of May 2011, surpassing the annual 2010 total of 374 cases. In the US, 198 confirmed cases of measles and 15 outbreaks have been documented by the Centers for Disease Control (CDC) as for August 26, 2011. Out of the 198 cases,170 (90%), occurred in US [13, 25].

2.2.2. Measles Case Prevalence in Turkey

In 2013, an increase in measles cases during 2012-2013 were reported by Ministery of Health in accordance with the disease development worldwide. It was also added that 349 cases in 2012 and almost 7000 cases in 2013 were recorded. It was revealed that İstanbul, Şanlıurfa, Adana, Diyarbakır, Van, Siirt, Ankara and Ağrı were the cities which have the highest prevalence of measles and in addition to that measles cases were recorded in 73 cities [15].

This increase in number of measles cases may also contribute to increase in SSPE cases.

2.2.3. SSPE Prevalence in Foreign Countries

There are publications suggesting that SSPE prevalence is 1/10.000, 1/300.000 or 1/1.000.000 (9) worldwide [16].

The frequency of the SSPE is higher in eastern countries. The rate of SSPE in India is $21/10^6$ and in Japan $11/10^6$ [17]. The highest frequency of SSPE ever reported is from Papua-New Guinea (PNG)**.** In a recent study from Madang Province in PNG, a rate of $54/10^6$ was found among individuals younger than 20 years during the years 2007–2009 [18]**.** According to a publication released in September 2013, SSPE prevalence is recently recorded as 0.83/100.000 in Australia. [19]**.**

2.2.4. SSPE Prevalence in Turkey

In Turkey frequency rate was reported as 2/1.000.000 in between 2002-2004 [12]**,** 0,83/1.000.000 between 1975-1987 [20] and 0,46/1.000.000 between 1997-1999 [21].

Ministery of Health has recently stated that 200 patients in Diyarbakır, 100 patients in Batman, 150 patients in Gaziantep are currently registered**.** According to Ministery of Health SSPE Scientific Investigation Comitte Report there were 1131 SSPE patient between 1995-2005 [22].

2.3.5. Lessons Learned From SSPE

The data obtained thus far asseverate that SSPE disease occurs in children who are not vaccinated or vaccinated however could not reach the adequate level of immunity or catch the disease before vaccination. Administered vaccination in the light of these knowledge led to mass immunization against measles and almost total eradication of SSPE in many countries. Setting the age of immunization to the first two years of life also played a significant role in this dramatic achievement.

In 2005, a SSPE case occurred in Turkey and the topic moved to parliament. This case highlighted the importance of complete measles immunization again and leaded to taking

serious precautions and improvement of vaccination schedule. According to latest information released by Hekimlerbirliği Vakfı ; measles vaccination should be initiated for infants aged 9 months. After 6 months, infants should be vaccinated against Measles, Mumps and Rubella (MMR) with single mixed vaccine again. This vaccination should be repeated after five years [23].

In a study among children born between 1966 and 1971 performed in İsrail, the rate of SSPE in unvaccinated children born during this period was 16 times higher than that found for vaccinated children ($171/10^6$ live births compared to $11/10^6$). This time period was deliberately chosen due to mass measles vaccination at nine months of age started in Israel during April 1967. Hence, the size of the unvaccinated group was still important for analysis of the role of vaccination in decrease of incidence of SSPE. This dramatic reduce in the annual rate of SSPE continued until no new cases were reported after 1986 (Figure 2.1.) [24, 25].

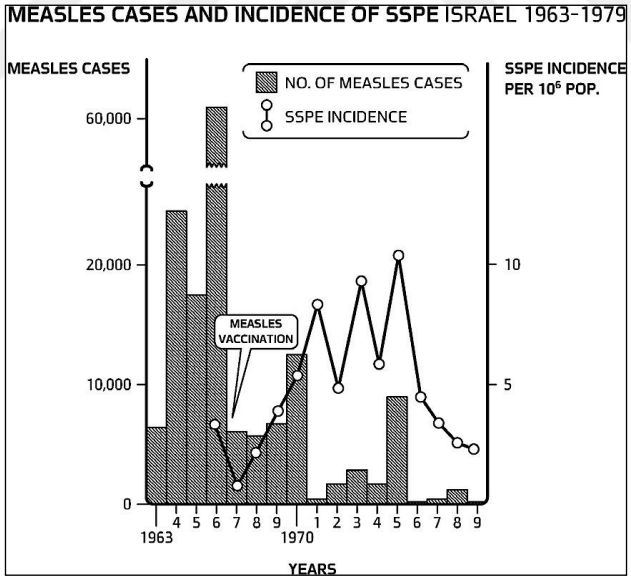


Figure 2.1. The progressive increase in the age of onset of SSPE in Israeli Jewish patients [24]

The age of onset of the disease increased concomitantly with the decrease in the rate of SSPE, and reached an average age of 18 years during 1983–1985 [24, 25]

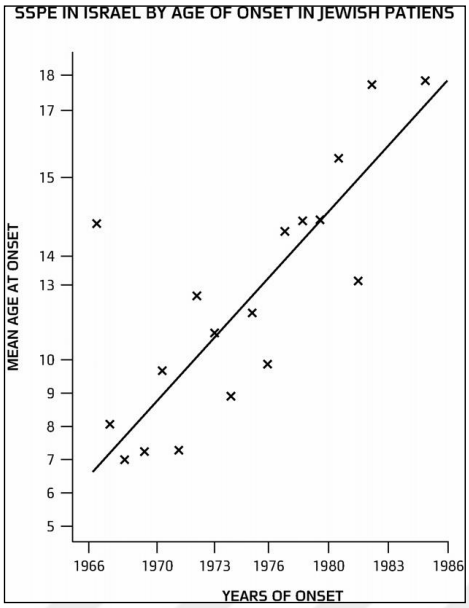


Figure 2.2. Progressive increase in age of onset of SSPE in Israeli Jews [24]

2.3. ETIOLOGY

2.3.1. Measles Virus

Measles virus can be described as a single-stranded, negative-sense, enveloped RNA virus of the genus *Morbillivirus* within the family *Paramyxoviridae*. It is 100-200 nm in diameter and has single-stranded RNA [26]. Only natural host of MV is human [27] and there is no animal reservoirs known to exist. MV is an enveloped virus; lipid-containing envelopes with a double layer structure, develops from the plasma membrane of the host cell [10, 28].

The measles, an infection of the respiratory system, is caused by the measles virus. Symptoms consist of fever, cough, runny nose, red eyes and erythematous maculopapular rash. Being very contagious, the virus is spread by direct contact with secretions or it may go around by coughing and sneezing by way of close personal contact [29].

2.3.2. Genotypes and Genome of Measles Virus

WHO identified the measles genotypes and the reference strains of each genotype have

been shown in Figure 2.3**.** [30].

Genotype – Génotype	Status[a] – Activité[a]	Reference strains (MVi)[b] – Souche de référence (MVi)[b]	H gene accession[c] – Accession au gène H[c]	N gene accession – Accession au gène N
A	Active	Edmonston-wt.USA/54	U03669	U01987
B1	Inactive	Yaounde.CAE/12.83 «Y-14»	AF079552	U01998
B2	Active	Libreville.GAB/84 «R-96»	AF079551	U01994
B3	Active	New York.USA/94 Ibadan.NIE/97/1	L46752AJ239133	L46753AJ232203
C1	Active	Tokyo.JPN/84/K	AY047365	AY043459
C2	Active	Maryland.USA/77 «JM»Erlangen.DEU/90 «WTF»	M81898Z80808	M89921X84872
D1	Inactive	Bristol.UNK/74 (MVP)	Z80805	D01005
D2	Active	Johannesburg.SOA/88/1	AF085198	U64582
D3	Active	Illinois.USA/89/1 «Chicago-1»	M81895	U01977
D4	Active	Montreal.CAN/89	AF079554	U01976
D5	Active	Palau.BLA/93Bangkok.THA/93/1	L46757AF009575	L46758AF079555
D6	Active	New Jersey.USA/94/1	L46749	L46750
D7	Active	Victoria.AUS/16.85Illinois.USA/50.99	AF247202AY043461	AF243450AY037020
D8	Active	Manchester.UNK/30.94	U29285	AF280803
D9	Active	Victoria.AUS/12.99	AY127853	AF481485
D10	Active	Kampala.UGA/51.00/1	AY923213	AY923185
E	Inactive	Goettingen.DEU/71 «Braxator»	Z80797	X84879
F	Inactive	MVs/Madrid.SPA/94 SSPE	Z80830	X84865
G1	Inactive	Berkeley.USA/83	AF079553	U01974
G2	Active	Amsterdam.NET/49.97	AF171231	AF171232
G3	Active	Gresik.INO/17.02	AY184218	AY184217
H1	Active	Hunan.CHN/93/7	AF045201	AF045212
H2	Active	Beijing.CHN/94/1	AF045203	AF045217

Figure 2.3. Reference strains to be used for genetic analysis of wild-type measles viruses: 2005 [30]

C1	MF	SSPE case/Europe/early 70s	This work	X84882
	SIP3A	SSPE case IP3/USA/early 70s	Cattaneo *et al.* (1989*b*)	X16566
	S(A)	SSPE case A/Germany/mid 80s	Cattaneo *et al.* (1989*b*)	X16567
	YA	SSPE case Yamagata-I/Japan/late 80s	Yoshikawa *et al.* (1990)	NA
	SMa81	SSPE case/Madrid/1981/1970	This work	X84866
	S(K)	SSPE case K/Germany/mid 80s	This work	X84883
	Mad78	Wild-type/Madrid/1978	This work	X84867
	Mad79	Wild-type/Madrid/1979	This work	X84868
C2	JM	Wild-type/Bethesda USA/1977	Taylor *et al.* (1991)	D01002
	S(B)	SSPE case B/Austria/mid 80s	Cattaneo *et al.* (1989*b*)	X16568
	WTF	Wild-type/Germany/1990	This work	X84872
	Bil	Wild-type/The Netherlands/1991	This work	X84878
	DL	Wild-type/Germany/1992	This work	X84873
	LB	Wild-type/Germany/1993	This work	X84880
	Ma92A	Wild-type/Madrid/1992	This work	X84869
	Ma92R	Wild-type/Madrid/1992	This work	X84870
	Ma93F	Wild-type/Madrid/1993	This work	X84871
C3	SBI	Wild-type/Bonn Germany/1992	This work	X84874
D1	MVO	Wild-type/Bristol UK/1974	Taylor *et al.* (1991)	D01004
	MVP	Wild-type/Bristol UK/1974	Taylor *et al.* (1991)	D01005
	S33	SSPE/N.Ireland/1983	Taylor *et al.* (1991)	D01008
	S81	SSPE/N.Ireland/1986	Taylor *et al.* (1991)	D01007
D2	CL	Wild-type/Birmingham UK/1988	Schulz *et al.* (1992)	NA
	SE	Wild-type/Birmingham UK/1988	Schulz *et al.* (1992)	NA
	TT	Wild-type Kawasaki case/London/1991	Schulz *et al.* (1992)	NA
	Can	Wild-type/Canada/1989	Rota *et al.* (1994*b*)	U01976
	Chil	Wild-type/Chicago USA/1989	Rota *et al.* (1994*b*)	U01977
	Chi2	Wild-type/Chicago USA/1989	Rota *et al.* (1994*b*)	U01978
	SD	Wild-type/San Diego USA/1989	Rota *et al.* (1994*b*)	U01995
	JK	MIBE/USA/1990	Rota *et al.* (1994*b*)	U01988
D3	Ma94B	Wild-type/Madrid/1994	This work	X84863
E	Brx	Encephalitis case/Germany/1971	This work	X84879
	WFK	Wild-type/Woodfolk USA/early 70s	This work	X84877
	CM	Wild-type/USA/late 70s	Taylor *et al.* (1991)	D01003
	S(C)	MIBE/USA/late 70s	Cattaneo *et al.* (1989*b*)	X16569
F	SMa79	SSPE case/Madrid/1979/1967	This work	X84864
	SMa94	SSPE case/Madrid/1994/1968	This work	X84865
G	Be83	Wild-type/Berkeley USA/1983	Rota *et al.* (1994*b*)	U01974
	B083	Wild-type/Boston USA/1983	Rota *et al.* (1994*b*)	U01990

Figure 2.4. Some certain measles strains released in 1995 (SSPE caused strains are marked with yellow) [31]

In a publication released in Spain, in 1995, measles genotypes with the ones which had caused SSPE until then were displayed [31]. In addition to that, in a study comprising the molecular investigation of two cases of SSPE in Croatia in 2002, it was revealed that the measles virus caused SSPE in two cases belonged to D6 genotype [32].

2.3.3. Genome of Measles Virus

Measles virus genome codes six structural; fusion, hemagglutinin, matrix proteins, phosphoprotein, nucleoprotein, large protein, and three accessory proteins; V, C and R proteins.

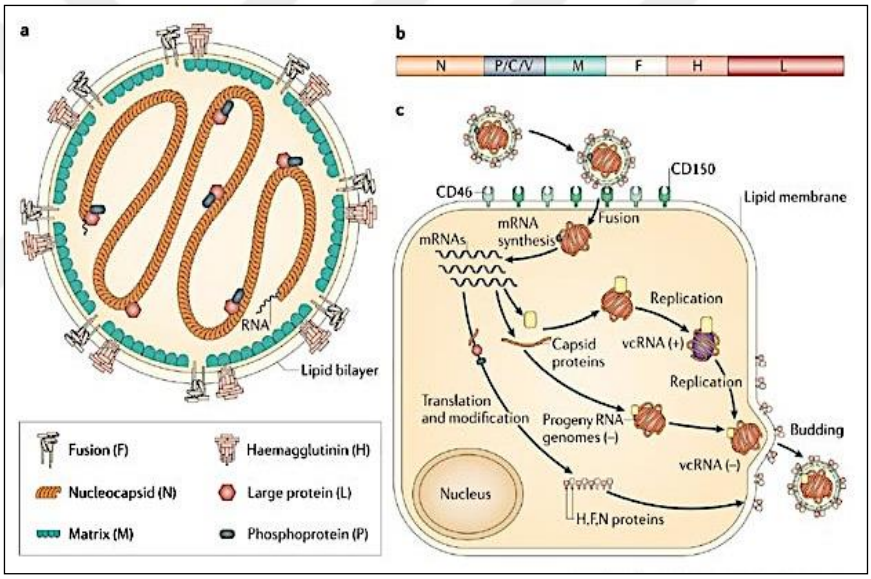


Figure 2.5. Measles virus structure indicating viral proteins and viral body forming [33]

To describe in general; **Phosphoprotein (P-protein), large (L)** protein and **nucleoprotein (N)** protein are **nucleocapsid** proteins, in other words they form transcriptionally active **ribonucleoprotein (RNP)** [27]**.** Other three proteins are **matrix protein (M)** forming conjugate with viral envelope and **hemagglutinin (H)** and **fusion (F) proteins** as surface proteins [26**,** 34, 35].

F protein lies within virus envelope. It is responsible for fusion of virus membrane with host cell membranes, virus entry into the cell and hemolysis [36, 37]**.** Binding of the host cellular receptors is the main function of the H protein. CD46 and CD150 (also known as

SLAM) are the two identified receptors. CD46 constitutes a complement regulatory molecule expressed on all nucleated cells of humans. Signalling Lymphocyte Activation Molecule (SLAM) is expressed on activated T and B lymphocytes and antigen-presenting cells. The binding sites on H for these receptors overlap and strains of MV differ in the efficiency with which each receptor is used. Most vaccine strains bind to CD46 and SLAM while wild-type MV binds to cells mainly through the cellular receptor SLAM. Other unknown receptors for MV probably exist on human endothelial and epithelial cells [33]**.** A conformational change happens in H protein when it binds to cell surface receptors and fusion process is activated [10]. M protein is located on the inside of the cell membrane. It is associated with viral nucleocapsid with its one end, cytoplasmic plica of the F and H proteins with its other end [28, 37]**.** It is associated with inner side of lipid layer as well. It plays a substantial role in budding of virus particles in the plasma membrane [10, 36]. It is associated strictly with Nucleoprotein (N) genome [36]. Possible role of N protein is to package the viral genomic nucleic acid, to protect it, and to participate in the formation of replication complex [38].

Transcription, replication and the efficiency with which the nucleoprotein assembles into nucleocapsids are regulated by the P protein. Defined as non-structural proteins, (V and C) are alternatively translated from the RNA, or an edited RNA, coding for the phosphoprotein (P). Detailed functions of proteins have not been identified, but each of the proteins seems to contribute to the virulence of MV by regulating transcription and sensitivity to the antiviral effects of IFN α/β [33].

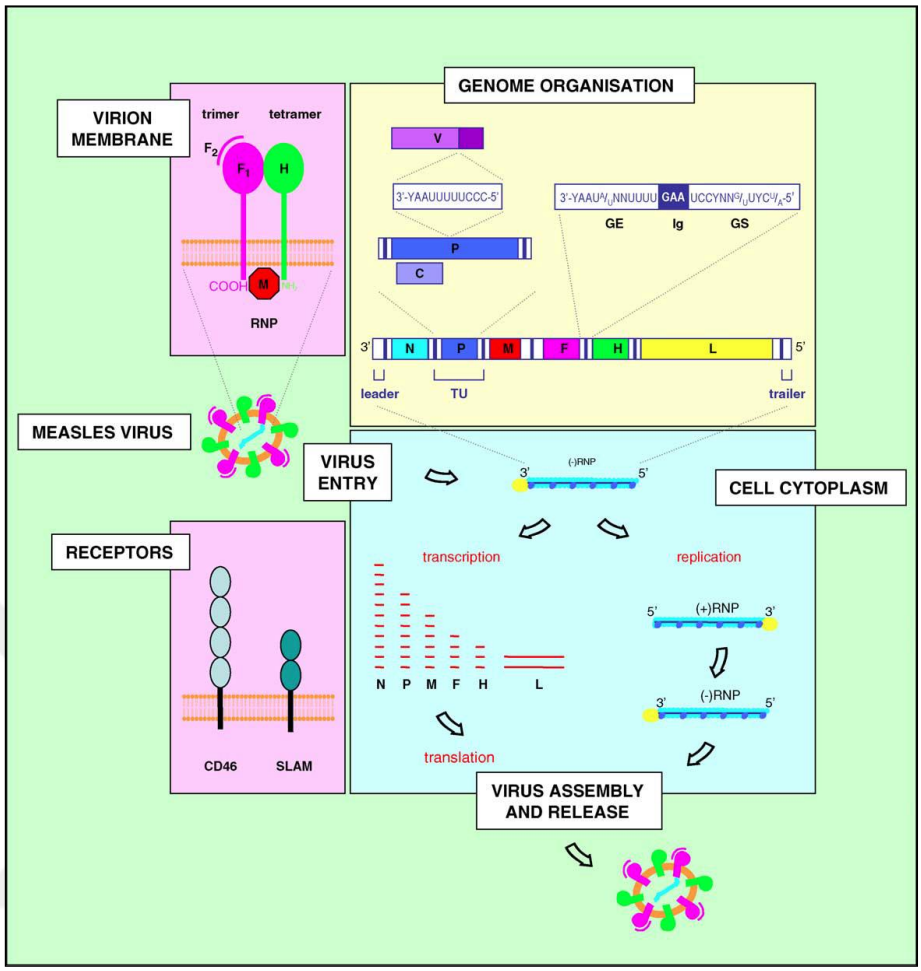


Figure 2.6. Schematic representation of the virion membrane, genome organization and the life-cycle of measles virus including the receptors used by the wild-type and vaccine viruses [33]

The functions undertaken by envelope proteins such as budding (M), absorption (H) dissolving membranes and fusion (F) to form virulent organism are of importance. Other functions such as directing and sorting matured virion into inner membrane of cytoplasm are associated with F and H proteins settled in outer side.

Without budding, the virus enters the sleep phase, i.e. wanders aimlessly in the host. Recently it has been reported that, especially in SSPE cases, virus uses trans-synaptic transmission to spread without budding [34].

2.3.4. Measles Complication

The complications of acute measles may develop in the early period right after infection. Generally, respiratory and intestinal tract are affected developing otitis media, pneumonia, diarrhea and encephalitis most commonly. Neurologic complications developing acute and late are shown in Table 2.1..

Complications developing late are SSPE and MIBE (Measles Inclusion Body encephalitis). Both diseases develop as a result of persistence that MV gains in MSS cells after acute infection. Both the SSPE and MIBE are diseases with fatal course [37]. MIBE is subacute measles encephalitis. MIBE patients are often children who repressed their immunized systems due to chemotherapy for leukemia or lymphoma. The symptoms of MIBE start within 6 months after measles. However, this period takes up years in SSPE. Adult cases without measles story have also been reported in MIBE. MIBE can be described as acute encephalitis of the delayed type [40, 41].

Various neurological complications of measles	
• Post-measles encephalitis	Develops soon after infection, reflect an autoimmune reaction
• Measles inclusion body encephalitis (MIBE)	Develops weeks or months after infection, in patients with defective cell mediated immunities like HIV infection
• Subacute sclerosing panencephalitis	Persistent defective measles virus infection
• Postinfectious	Acute immune reaction
• Transverse myelitis	Rare, acute immune reaction

Table 2.1. Various neurological complications of measles [42]

2.3.4.1. SSPE Development

It is considered that MV have settled in brain and gained persistence there before appearance of disease symptoms in SSPE.

In entry of measles virus into the brain, 3 potential routes exist. It has been suggested that access to the brain via nerve bundles in the olfactory bulb may occur, as the virus can infect neurons. On the other hand, the virus can replicate in capillary endothelial cells and so may infect brain capillaries and release viral particles directly into the brain parenchyma. It seems that, vascular endothelial cells play an important role both in propagation of the virus into the central nervous system and in viral persistence. As a 3rd route, transport of virus directly across the blood-brain barrier may be led by infection of monocytes in peripheral blood as monocytes periodically transmigrate into the brain and differentiate into resident perivascular macrophages or microglial cells [43].

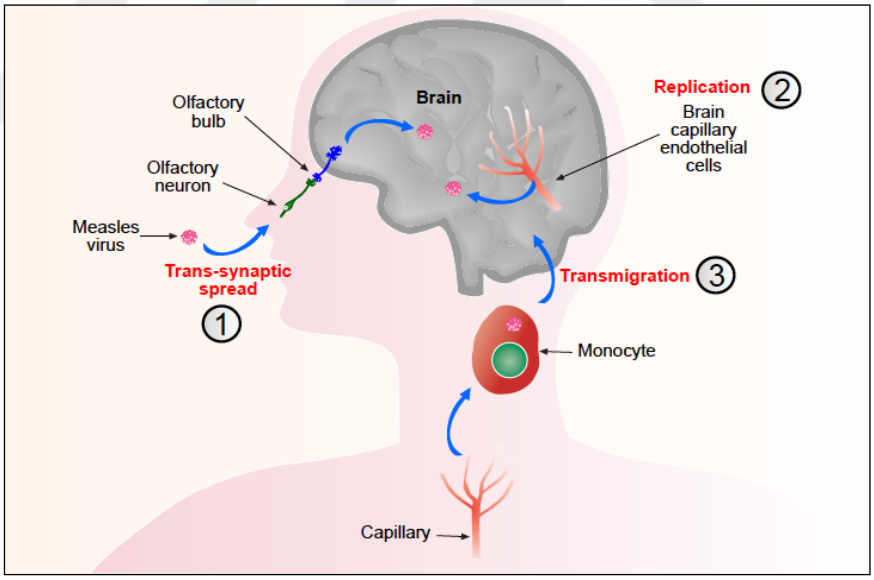


Figure 2.7. Potential routes of measles virus dissemination to the brain [43]

After penetration of measles virus into the brain, perivascular macrophages, microglial cells and neurons constitutes primary target cells. Budding of virus particles from the cell membrane and cell death are not resulted by infection of neurons. But then virus can spread between neurons in a cell-to-cell manner yet this process occurs highly slowly. Frequently, latent measles virus' brain complications only manifest theirselves much later after primary infection [43].

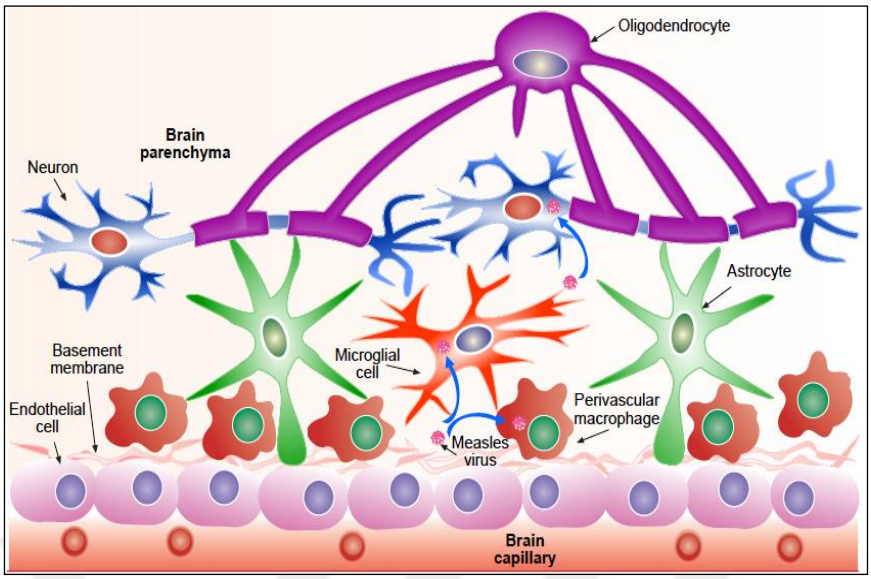


Figure 2.8. Measles virus infection of macrophages, microglial cells and neurons [43]

It has been known that measles virus is able to spread between neurons in a cell-to-cell manner, however, this process takes place very slowly. Initial penetration of neurons may still be dependent on a specific receptor remaining to be identified. Nevertheless, following neuron infection, virus can then spread to adjacent neurons at the synapse from the dendrite to the axon (retrograde). It is suggested that the transport of virus from infected neurons to adjacent uninfected neurons is mediated by microfusion events at the cell membrane at which viral F proteins bind neurokinin-1 receptors expressed on neurons which facilitates membrane fusion. Viral ribonuclear protein complexes containing viral RNA accumulate at the cell membrane and can transmit to the adjacent cell. Incomplete assembly of virus particles exist at the membrane and budding of virus does not take place in neurons. In this way, detection of free virus by antibody is prevented and leads viral latency in the brain [43].

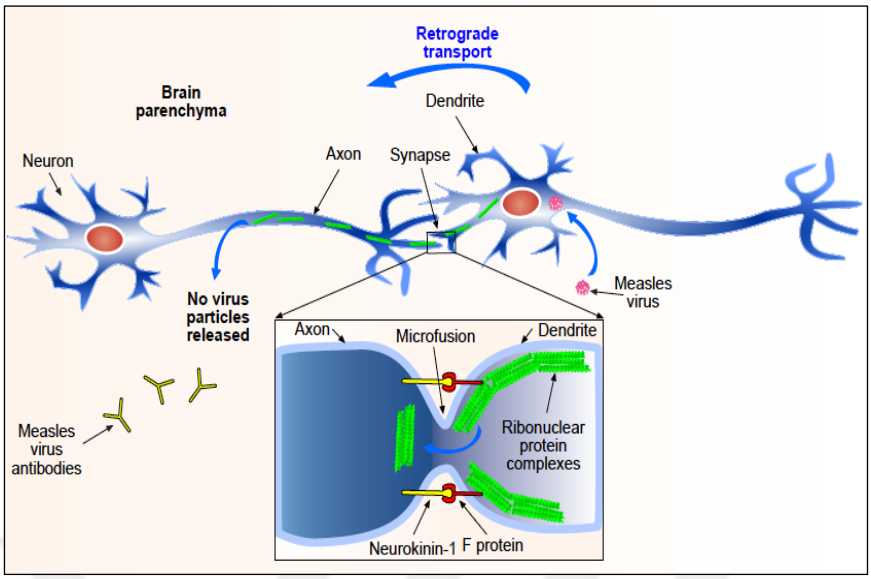
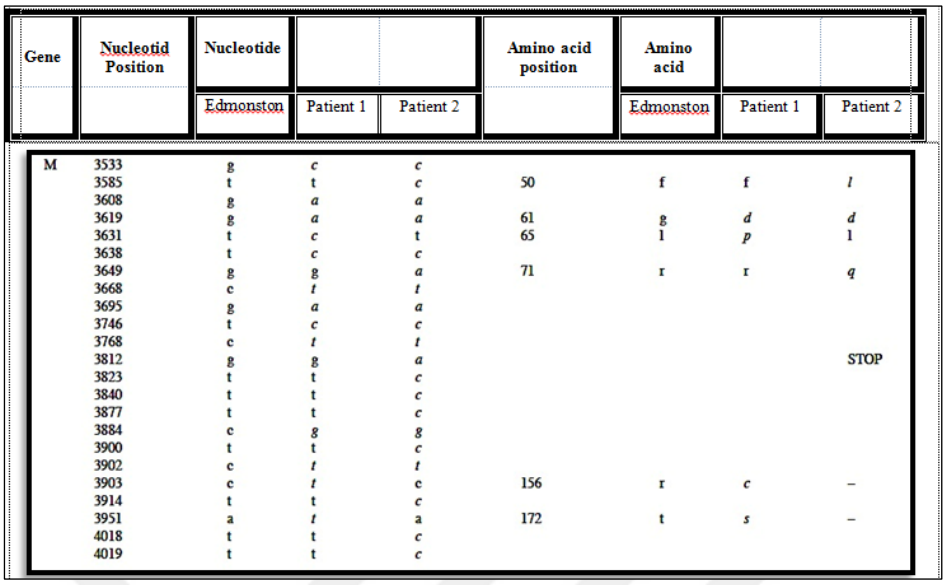


Figure 2.9. Immune evasion of measles virus by cell-to-cell spread in neurons [43]

In the measles virus genome obtained from autopsy material of SSPE patients, the gene sequences out of area where mutations accumulated, have been found to be compatible with the gene sequence of genotype belonging the time when patient encountered primarily with the virus, thus, these data support the role of virus with which body encountered in primary infection period, in causing SSPE.

In SSPE, many mutations have been described in the virus M protein. F and H proteins of the enveloped proteins are mostly protected, yet more mutations are encountered in the H protein. Due to these three proteins having a role in budding from infected cells and in creating fusion with uninfected cells, persistent infection is considered to be associated with these two processes [26, 34, 44, 45].

In the study carried out with two patients and released in 2003 in Crotia, it was revealed that in the *M* gene of virus obtained from one of the patients, there was an additional premature termination codon (amino acid number 125) created by a mutation (nucleotide number 3812) in the protein coding region. As mentioned before earlier, this could impair expression of functional protein alter the course of the disease in this patient [32, 46].



Gene	Nucleotid Position	Nucleotide			Amino acid position	Amino acid		
		Edmonston	Patient 1	Patient 2		Edmonston	Patient 1	Patient 2
M	3533	g	c	c				
	3585	t	t	c	50	f	f	l
	3608	g	a	a				
	3619	g	a	a	61	g	d	d
	3631	t	c	t	65	l	p	l
	3638	t	c	c				
	3649	g	g	a	71	r	r	q
	3668	c	t	t				
	3695	g	a	a				
	3746	t	c	c				
	3768	c	t	t				
	3812	g	g	a				STOP
	3823	t	t	c				
	3840	t	t	c				
	3877	t	t	c				
	3884	c	g	g				
	3900	t	t	c				
	3902	c	t	t				
	3903	c	t	c	156	r	c	–
	3914	t	t	c				
	3951	a	t	a	172	t	s	–
	4018	t	t	c				
	4019	t	t	c				

Figure 2.10. Nucleotide and amino acid differences between reference strain prototype Edmonston and SSPE viruses detected in clinical samples of two SSPE cases [32]

Nonetheless wild-measles virus is highly sensitive to immune response. With an effective response, antibody is created against all proteins and in many cases, viruses rambling freely in the extracellular environment are removed by hypersensitivity reactions and life-long immunity is obtained. When the immune response is ineffective, wild virus gets rid of it and continues to create damage. On the other hand, if it destroyed some part of viral proteins, in this case there could be a few possibilities.

If nucleocapsid proteins are damaged, the organism cannot multiply and virus becomes inactive.

If envelope proteins are damaged, organisms may survive, but this situation will not last long, virus becomes inactive after a short time.

However, if only the M protein is damaged, virus will remain dormant for years. At the end of this period, the virus gains virulence again and will start damage into large neurons of cerebral cortex where it is hidden substantially. In spite of functionality of cellular immunity and high antibody titers, in SSPE, immunological control of infection in the central nervous system remains unsuccessful. The immune system in most of normal

infants is not perfect enough to form sufficient or effective antibodies enough to completely break up measles virus with six proteins. Considering most patients (75%) experienced the measles virus at early ages, the partial response developed by immature immune system is considered to cause persistent infection. Rather than host immune system, it is considered to be associated with specific cell receptor areas enabling binding of measles virus [47, 48, 49, 50].

2.3.4.1.1. Host related factors of SSPE Development

- **Age of measles infection**

Measles infection in children less than 2 years is a risk factor in developing SSPE [40, 51]. As immune system is not fully developed yet, it is suggested that antibody response and cellular response do not occur at a level enough to destroy the virus in these children [11]. Moreover, since MV could not be destroyed by nascent immune system, it is suggested that mutations in MV are more likely to develop in these children.

- **Measles Development in spite of the Vaccine**

It has been reported that adults who had been vaccinated in childhood have also experienced measles. For this reason, it has been understood that the second dose of the vaccine is necessary in children whose immune systems being not developed or failing to occur an antibody titer at a sufficient level in first vaccination [52]. Normally, the rate of immune response development after two dose of vaccine is 99% [51].

- **Genetic Susceptibility**

The susceptibility of SSPE in those who had undergone measles may be based on genetic differences. In a study conducted in patients with SSPE in Japan, it has been put forward that a polymorphism (- 589T) shown in promoter area of IL-4 gene cause a genetic susceptibility for SSPE. In this study, it has been suggested that response of Th2 remains dominant long term due to height of IL-4 and therefore the virus could not be destroyed, and persisted in the patient [53]. That genetic polymorphism may be at least responsible in part for the variable susceptibility to SSPE is suggested by substantive worldwide variability in the occurrence of the disease in different populations and ethnic groups [25].

- **Socio-Economical Status**

Being affected in development of immune system, having a high risk in contracting measles due to poor hygienic and nutritional circumstances, and being not vaccinated of some children are likely to promote susceptibility of the SSPE [40].

2.4. PATHOLOGY AND CLINIC

2.4.1. Clinical picture of SSPE

The mutated MV invades the nervous system in a dormant form until its activation six to seven years after the initial measles infection in those immunocompetent patients.

The clinical revealing of SSPE is usually insidious and personality changes, school failure and bizarre behavior are among the forms of displayed (Stage I). The transition from stage I to II occurs when the typical periodic or quasiperiodic axial myoclonic jerks causing recurrent falls appear. Step by step, generalized rigidity with extrapyramidal features and unresponsiveness become visible and are considered as typical symptoms of Stage III. Stage IV is the terminal stage of the disease and is identified by minimal conscious state and later akinetic mutism associated with persistent high fevers and bouts of generalized sweating due to an autonomic failure. In most patients the transition from one stage to next one is insidious and they die of the disease over the period of 1–3 years [54]. In some cases, the clinical course is quite protracted, with a longer stage I. It has been reported that, in approximately 10 % of the patients, rapid and devastating course may be observed (Fulminant SSPE) [55].

Childhood onset mental and behavioral regression accompanied by myoclonic jerks, an EEG with generalized bilateral rhythmic synchronous bursts of spike-wave or slow wave complexes, often time locked with the myoclonic jerks, elevated measles antibodies in the blood and CSF having no concomitant evidence of clinical measles, elevated IgG and presence of IgG oligoclonal bands in the CSF are the major diagnostic clues.

The distinctive feature of neuropathological findings are composed of perivascular inflammatory cuffing (the accumulation of lymphocytes or plasma cells in a dense mass

around the vessel), cortical and subcortical white matter astro-microgliosis (accumulation of microglial cells as a reaction to injury to the parenchyma of the central nervous system, a distinctive feature of nonsuppurative encephalomyelitis) , neuronopahgia and Cowdry type A eosinophilic intranuclear inclusion bodies in the brain (Figure 2.11.). In typical cases the initial behavioral changes are seen between the ages of eight–eleven years, about six–seven years after contracting measles. The interval between measles immunization and the onset of SSPE changes between six–seven years.

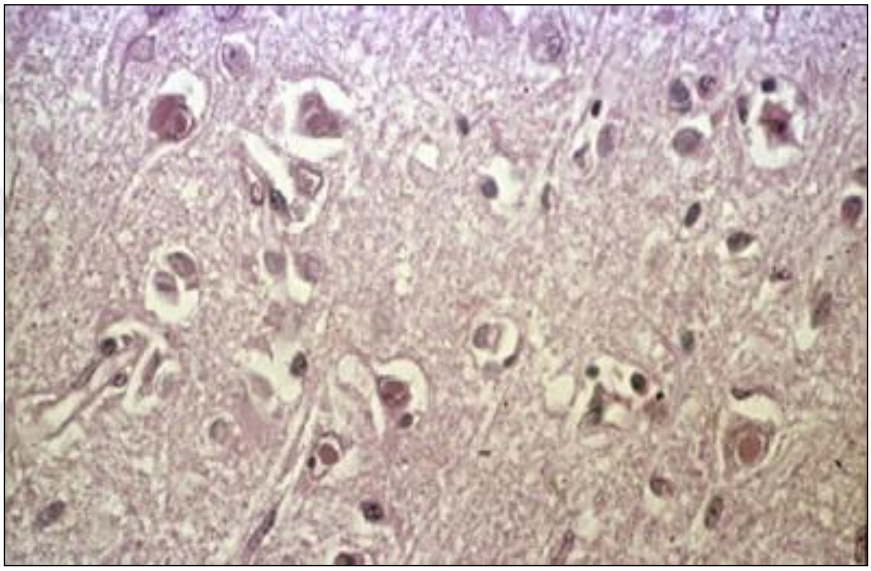


Figure 2.11. Large intranuclear eosinophilic Cowdry type-A inclusions which compress the chromatin against its nuclear membrane [25].

Focal seizures with secondary generalization and generalized tonic –clonic seizures, in addition to myoclonic jerks, may also be observed [56].

With the criteria noted above, patients with childhood onset SSPE can be easily diagnosed. Nevertheless, this is not that easy in the adult onset form which may be present in about 1–12.7% of the patients [54]. Those patients generally present at an average age of 25 years with visual agnoxia or blurring of vision as myoclonus may be absent. Interestingly, there was no history of measles in about 30% of patients in one of the series [57].

2.4.1.1. Clinical Staging of SSPE

Clinical staging of SSPE that has been done by Jabbour and Gascon is as follows.

Stage 1A: Behavioral, cognitive and personality changes (decrease in school performance, attention deficit disorder / hyperactivity, social maladjustment). There is no difficulty in walking.

Stage 1B: Myoclonic spasms: non-periodic, focal. Intact independent walking. The mental / behavioral symptoms similar to Stage 1A.

Stage 2A: More significant mental and behavioral disorders. Myoclonic spasms: periodic, generalized and synchronous, frequent.

Stage 2B: Apraxia, agnosia, language disorders. Motor symptoms-spasticity, ataxia. Ambulation is provided with assistance.

Stage 3A: Speaking is reduced, visual impairment exist. He / She sits independently, can stand but cannot walk independently. Myoclonic spasms are multifocal, frequent, spasm interval is short (3-5 seconds), duration is long (3-4 seconds). Seizures may occur.

Stage 3B: There is no spontaneous speaking. Understanding is corrupted, blindness could be developed. Myoclonic spasms are similar to those in Stage 3A. He / She is bedridden. Dysphagia exists. A nasogastric tube feeding may be needed. Delta background activity in the EEG is available, and periodic complexes may not be observed due to this background activity. Movement disorders can occur (Chorea-ballismus-athetosis).

Stage 4: There are no myoclonic spasms. Low voltage background activity in EEG is available. There are no periodic complexes. Patient is in neurovegetative status [58, 59].

2.4.1.2. Pathophysiology

An impaired immune response to MV, even it is not an immunological disease plays a substantial role in SSPE. During the initial two years of life the immune system is still relatively immature [60]. At about next six–seven years, the infants who suffer from measles at their first two years are at a higher risk to develop SSPE. The normal host develops a cell mediated response to the invading virus in the incubation period of measles. The activation of Th1 lymphocytes, release of INF-α and IL-2, which are capable

of eradicating MV particles from the infected cells, are included in this response.

A humoral antibody mediated long term immunity for future measles infection is set by the action of Th2 lymphocytes, secretion of IL-4 and to a lesser extent IFN-α and IL-2 [61] following the typical papulo-macular skin rush. In contrast, patients with SSPE have an inadequate cellular response to common mutagens due to genetic polymorphism.

2.5. DIAGNOSIS

Diagnosis of SSPE is based on some certain clinical symptoms and laboratory data.

Table 2.2. Diagnostic criteria of SSPE [42]

Diagnostic criteria of SSPE	
1. Typical clinical **picture**	Typical signs like myoclonus with progressive, subacute mental deterioration
2. Typical EEG pattern	Periodic, stereotyped, high voltage discharges
3. CSF	Increased gammaglobin or oligoclonal pattern
4. Measles antibodies	Increased titre in serum (≥ 1: 256) and/ or CSF fluid (≥ 1:4)
5. Brain biopsy	Suggestive of panancephalitis
Definitive; criteria 5 with more 3 criteria , **probable**; three of the five criteria	

2.5.1. Detection of Measles Virus in Clinical Specimens

2.5.1.1. Serological Tests for Diagnosis of Measles

Despite the fact that a number of laboratory method for the diagnosis of measles are currently administered, detection of measles virus (MV)-specific serum IgM antibodies remains the gold standard [62, 63]. General laboratory confirmation is performed by

enzyme immunoassays (EIAs), by determination of IgG seroconversion or a four-fold rise in IgG titer between acute-and convalescent-phase sera. However, the latest and the most reliable method for diagnosis of measles and in that case SSPE, is isolation of MV or detection of MV-RNA from clinical specimens [64, 65].

2.5.1.2. Molecular tests for detecting Measles Virus

Despite IgM EIAs is still a recommended laboratory method in routine diagnosis of acute infection, methods such as RT-PCR, that can detect MV-RNA in a variety of clinical samples [65, 66, 67, 68] can substantially serve as a valuable, alternative procedure particularly in cases in which the results of serologic testing are inconclusive, inconsistent or not available.

Reverse transcription polymerase chain (RT-PCR) based methods which are more sensitive, reproducible and stable have been described for the detection of MV genomic RNA [69]. Different clinical specimens can be used in these assays such as throat swabs, urine, oral fluids [63, 70, 71].

Nowadays, WHO highly recommends collecting urine and/or nasopharyngeal samples as well as sera for IgM testing from representative cases in order to perform molecular epidemiologic studies of MV [65, 72].

In addition to that, genotyping MV are based on sequence analysis of PCR products derived either directly from clinical specimens or from viral isolates in cell culture and this technique is the only diagnostic method that can distinguish between wild-type infection and vaccine-associated cases [65, 73, 74].

According to the results acquired from a reported measles outbreak in 1998 (about 650 cases) in Croatia, it was revealed that clinical samples support the view that the vaccine strain is not associated with SSPE causing measles virus [32, 75].

2.5.1.3. Real-time PCR for detection of Measles Virus in Measles and SSPE diseases

The development of fluorescence-based real-time detected PCR techniques allowed the quantitative detection of RNA sequences as well as qualitative detection [63, 76, 77].

These methods provide detecting of large amounts of samples in a short period of time with a minimal risk of cross-contamination. In the diagnosis of chronic infections, quantitative aspect of the assay is especially significant [63, 77, 78].

As for applying real-time PCR assays in diagnosis of SSPE, this method provides much more reliable results compared with conventional PCR methods thanks to its higher specifity in detecting genetic materials even in low viral load conditions which refers to low concentration of viral particles which is expectable in SSPE cases, especially in non-CNS clinical specimens.

2.5.1.4. Investigating MV-RNA in whole blood and other clinical specimens by Real Time PCR in SSPE Cases

In previous researches; plasma, peripheral blood mononuclear cells (PBMC), CSF and brain tissue were generally used as clinical samples for detection and molecular characterization of MV by various PCR method in SSPE cases. Unfortunately MV-RNA could not be detected in any of those materials excluding their brain tissue. In patients serologically and clinically diagnosed with SSPE, brain materials were usually positive, which makes brain tissue the most reliable clinical sample in diagnosis of SSPE disease.

However, the study performed by Miki and his colleagues in 2002, was also taken as a base for this study, reported that RNA transcribed from *N* gene of MV genome was successfully amplified by real-time PCR method from PBMCs of two patients of 19 patients [32, 79].

These results shed a light on possibility of utilizing peripheral blood materials alongside of brain tissue in detecting and amplifying MV-RNA with molecular techniques in SSPE cases.

In our country, especially in South-East Anatolia Region in which SSPE case incidence substantially higher, that carrying out brain biopsy after autopsy is quite difficult due to traditional beliefs makes using blood specimens as a target material also much more advantageous and rapid option in diagnosis of SSPE.

In this study peripheral whole blood samples collected from 28 patients who were serologically and clinically diagnosed with SSPE were used for molecular diagnosis by real-time PCR. Peripheral whole blood is beneficial in terms of obtaining viral material from MV located in PBMCs as well as MV circulating free in the blood stream. Since it is determined that N encoding genomic RNA commonly exists in genome of the measles virus causing SSPE and N RNA is consequently the most abundant viral transcript among the others such as H, M and F in SSPE infected cells [65, 80]; N RNA was amplified in the reaction in accordance with the studies performed by this time.

Recent researches have also showed that MV virus can rarely be located on various organs besides of blood and CNS tissues in SSPE patients [81, 82, 83]. In a study performed with several organs including lung, hearth and liver MV-RNA could successfully be isolated from appendix and thymus as only non-CNS tissues [83].

2.5.1.5. Advantages of Real Time PCR as a method compared to Standard PCR

Despite the fact that both standard PCR and real-time PCR follow a similar procedure, real-time PCR has many more advantages compared to standard PCR. Ability to identifying amplified sequences during the PCR process is one of the primary advantages of real-time PCR. Real-time PCR measures the amount of the product during the exponential phase. However standard PCR measures product during the plateau phase which makes measurement less effective due to the fact that measurements taken during the plateau phase do not always precisely determine the quantity of starting material.

Standard PCR requires post-PCR analysis such as agarose gel electrophoresis which identifies the product either by size or sequence. Running gel electrophoresis is a time-consuming and manual technique, although it is less expensive. In addition to that, since molecules of the same or similar weights cannot be easily differentiated, it has low specifity. Hence, gel electrophoresis alone is not sufficient for endpoint analysis for most laboratory purposes. It requires extra methods in order to confirm results. Probe hybridization or ELISA detections are usually used for characterization of the product by its sequence. These methods are relatively more reliable and informative. Nevertheless, it is time-consuming and expensive. Real-time PCR eliminates these needs in that it achieves

amplicon recognition by monitoring the accumulation of specific products during each cycle.

That the entire process including amplification and analysis occurs in the same tube is another advantage of real-time PCR, which decrease possibility of contaminating the product. This distinguishes the real-time PCR from standard PCR where the PCR product is moved and placed into other systems [84].

2.5.1.6. Amplicon Detection in the real-time Thermal Cycler

PCR product generation and recognition are combined by real-time PCR thermal cyclers into an integrated format that allows for the subsequent analysis of the procured data. In order to achieve these two above mentioned duties, conventional PCR thermal cycler technology is incorporated with integrated fluorimeters and detectors by real-time PCR devices and the ability to both excite a fluorochrome and detect the emitted light are provided through these. However, different providers of such fittings are there to range in cost depending upon their features. This represents a considerable investment for many laboratories whereas it can result in savings while the analysis of PCR products can be carried out without running agarose gels, standing for a savings in both time and money when processing a large number of samples.

As the real-time PCR reactions are surveyed at each period by measuring fluorescence, most real-time thermal cyclers need to be adjusted for the definite tube, microtiter plate, microtiter plate seal used and the PCR reaction volume. For this reason, it is important to determine the type of the real-time thermal cycler to be used in the experiment before setting up the reactions [84].

As they provide higher throughputs, Roche (Light Cycler), Biorad and Applied Biosystems of Life Technologies have the outstanding and the most preferred real- time PCR systems [85].

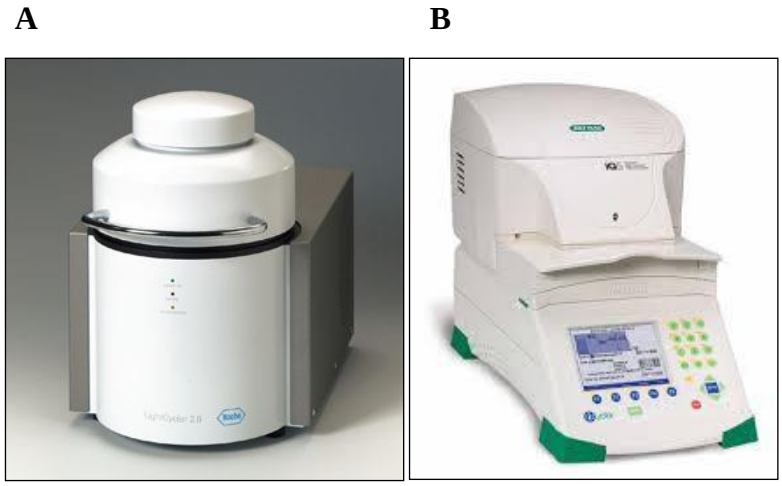


Figure 2.12. Roche LightCycler 2.0 (A) and Bio-Rad iQ5 Systems (B)

2.5.1.7. Fluorescent dyes for monitoring real-time amplification

Finding out the PCR product in real-time comprises the use of a fluorescent dye. These can be either nonspecific dyes, such as fluorescent DNA binding dyes (e.g., SYBR Green I) or strand-specific probes (such as Taqman or Molecular Beacons), that use a phenomenon recognized as fluorescent resonance energy transfer (FRET) to differentiate between diverse products [87]. Nonspecific fluorescent dyes from the SYBR Green family are most commonly used for the preliminary optimization of the real-time PCR amplification because they are more economical in proportion to strand-specific probes and easier to optimize. A single, specific DNA fragment has to be attained during PCR amplification When using this type of detection, because any supplemental nonspecific DNA fragment accumulation will contribute to the measured fluorescence. If nonspecific bands are amplified in the PCR reaction or if the amplification of more than one target sequence will be monitored in a single PCR reaction, strand-specific probes need to be designed [84].

- **Nonspecific fluorescent probes**

Even though various types of nonspecific probes available (e.g., Molecular Probes's YoPro and YoYo), SYBR Green that is the minor groove binding dyes is particularly used widespread. The SYBR Green 1 maximum emission values are around 520 nm and most real-time PCR thermal cyclers possess optics to detect at this wavelength. Since no probe is needed, their relative affordability and generic nature make them ideal for optimizing a

great deal of PCR reactions, and can provide quantitative data proper for many practices (such as expression studies). However, they can be used to control the amplification of any double-stranded DNA sequence. Strand-specific methods are still recommended (e.g., TaqMan or Molecular Beacons) for some experiments such as detection of allelic variations.

The SYBR Green family of dyes has a basic principle which is that they sustain a 20- to 100-fold increase in their fluorescence on binding dsDNA that is detected by the real-time PCR machine's detector. Hereby, there will be a relative increase in the fluorescent signal since the amount of dsDNA mounts up in the reaction mix. On the other hand, this simplicity means that they may bring out false positive signals; i.e., as the SYBR® dye ties up to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences. Consequently, it is vital to have well-designed and optimized linings that do not amplify non-target sequences, and that melt curve analysis be performed [84].

- **Step-by-step method**

1.SYBR® dye immediately binds to all double-stranded DNA that are in the sample when it is added to a sample. The aimed sequence which creates the PCR products is amplified by DNA polymerase during PCR. Then SYBR® dye binds to each new copy of double-stranded DNA.More PCR products are created since the PCR progresses. SYBR® dye binds to all double-stranded DNA, so the result is a mount up in fluorescence intensity adjusted to the amount of PCR product generated [88].

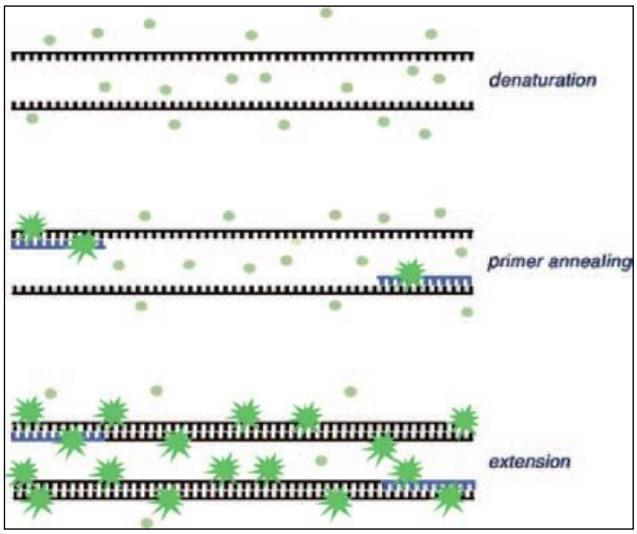


Figure 2.13. SYBR Green during PCR amplification [84]

- **Strand-specific fluorescent probes**

Fluorophore-coupled nucleic acid probes are likely to be used as a detection system. In a sequence-specific manner, these strand-specific probes interact with the PCR products and obtain data about a particular PCR product as it accumulates. Nevertheless, one commonly used example will be explained in order to introduce the basic principle behind strand-specific probes and to emphasize some of the advantages of this approach upon the use of nonspecific probes.

Hydrolysis probes are included here as one of the most widely used strand-specific approaches. They are named so because their utility is based upon the 5_ nuclease activity of Taq polymerase. Another well-known example is TaqMan probes that have been used extensively in a wide range of researches. TaqMan probes are sequence-specific oligonucleotides with two fluorophores marked at both end.

The quencher is the term for one fluorophore and the other is the reporter. When the reporter fluorophore is actuated by a laser at the suitable wavelength, it absorbs that light and then releases a particular wavelength of light. The quencher will absorb the emitted light by FRET all the time it remains in close affinity. The quencher is chosen according to its ability to specifically absorb the emitted spectra of the reporter and should be placed in

the probe to optimize the capture of that light.

The probe is created to anneal to the accumulating product at an internal site as shown in Figure 2.14.. Characteristically the 3_ end of the probe is obstructed to suppress extension due to annealed probe. As product piles up, the dual-marked primer binds to the aimed sequence and gets degraded by the 5_ nuclease efficiency of the Taq polymerase. As shown in Figure 2.14., this is the consequence of the Taq enzyme matching the probe during amplimer extension. Providing less absorption of the light emitted by the reporter, degrading the probe separates the quencher from close proximity to the reporter. Hereby, there is a corresponding mount up in fluorescence that is associated with the particular amplification of the aimed sequence. Differently form which is seen with other strand-specific approaches that rely on the measurement of the direct hybridization of probe to the target during each cycle, a cumulative signal is produced. The TaqMan method is dependent upon a probe that can be efficiently hydrolyzed because it is very sensitive. In order to secure efficient cleavage, the probe should have a Tm 5◦ to 10◦C greater than the amplimer to enable its binding quantitatively to the template before the extension. However, designing such a probe can be problematic with AT rich targets.

Actually, one of the benefits of the strand-specific probe systems is that multiple probes can be bound to, or multiplexed, and thus information about various target sequences can be provided from one reaction. This can be very beneficial while both controls and target sequences are amplified under the same circumstances. Another advantage is when there are two potential PCR products that can be obtained from the same primer set. A strand-specific probe can be used in order to help distinguish the two products. Both the hydrolysis probe approach explained above and other sequence specific probe systems have been widely used for genotyping and they can identify point mutations, SNPs, and allelic variants which are not easily possible with SYBR Green I or SYBR Gold [84, 89, 90, 91, 92].

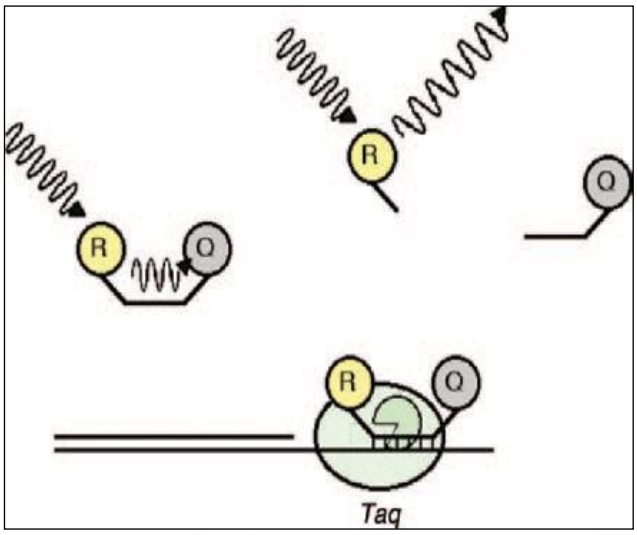


Figure 2.14. TaqMan probe used for sequence-specific amplification of DNA fragments [84]

2.5.1.8. Melting point (Tm) analysis to verify amplification specificity

Before continuing real-time PCR analysis, that the PCR amplification is specific; the actual real-time PCR conditions should be confirmed again. This can be done using the melting curve analysis tools of the system. As a quality control step, to perform melting point analysis after each real-time PCR run is important. It is also useful for optimizing primer-annealing temperature when it is necessary.

Melting point analysis distinguish target sequences from PCR artifacts such as primer dimers or products originated from non-specific primer bindings. This feature is based on the observation that the temperature at which a DNA duplex will denature depending upon length and nucleotide composition. Fluorescence measurements are performed while the temperature of the reaction products raises (for example, from 60◦ to 95◦C). At the low temperature, the amplicons are all double stranded which causes SYBR Green dye to bind the target amplicons and thus, produce a strong fluorescence signal. When the temperature increases, The PCR products undergo denaturation which causes to decrease in fluorescence. As the Tm of a particular ds DNA product is reached, a rapid decrease in the fluorescence over a short temperature range is detected by the instrument and shown as Tm peaks on the screen. These peaks represent the points at which the maximum rate of change in fluorescence is detected. Different amplicons can generate different peaks

centered on different temperatures and, fortunately, PCR artifacts generally have lower melting temperatures than the target amplicon [84].

In this study, melting point analysis was constituted the basis of investigation of N RNA and conducted in order to determine melting temperature of the target N RNA amplicon and distinguish from PCR artifacts or misproducts.

2.6. TREATMENT

The treatments are generally palliative and mostly symptomatic because of SSPE as a fatal disease. Oral Inosiplex (IsoprinosineR), is derived from inosine and p-acetaminobenzoic acid salt of N, N -dimethylamido-2-propanol helps to regulate the immunological stimulation by having an immunomodulator effect.

Reports are suggesting that long term administration of the drug led to a 20% remission rate and or 4 years survival [25,93] Isoprinosine in combination with weekly intrathecal INF-α was suggested as the best treatment by some authorities, however resultant adverse effect rate because of intrathecal administration decreased the usage of this combination as a type of treatment.

Long term administration of the Isoprinosine is determined to be safe and has not any significant adverse side effects. Its immunomodulating effect was also proven by applying to 11 patients with SSPE failed to produce INF after incubation with the common mutagens PHA and Poly (I-C) (polyinosinic:polycytidilic acid).

Applied for a couple days, daily oral Isoprinosine treatment has been seen as to lead to a significant increase in INF production by PBL of the patients after incubation with the same mutagens [25, 94]. Besides, some researchers have reported that Ribavirin in combination with intraventricular IFN [25, 95], anti CD 20 antibodies [25, 96], Thymic Humoral Factor, Amantadine, steroids and plasmapheresis also have beneficial effects in selected non- randomized trials [25, 96].

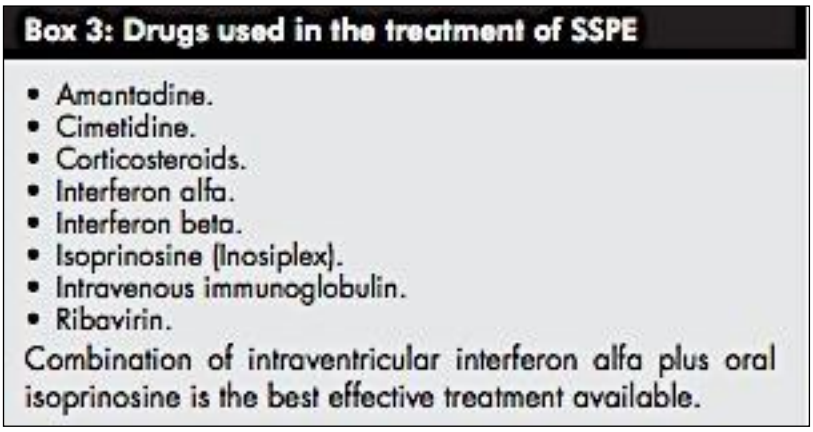


Figure 2.15. Drugs used in the treatment of SSPE [42].

3. MATERIAL

3.1. REAGENTS AND COMMERCIAL KITS

- Roche High Pure Viral Nucleic Acid Kit
- RevertAid First Strand cDNA Synthesis Kit; Thermo Scientific
- LightCycler® DNA Master SYBR Green I; Roche
- Taq Polymerase PCR kit; Thermo Scientific
- Sybr Gold
- 6X Loading Dye; Fermentas
- DNA Ladder; 50 bp, Invitrogen
- Agarose; Prona Basicale

3.2. LABORATORY TECHNICAL EQUIPMENT

Table 3.1. Laboratory technical equipment

Equipment	Brand
Micropipettes 1000, 200, 100, 10, 2.5 µl	Thermo E. C. Finnpipette
Eppendorf tubes (pcr, 0.5, 1.0 ml)	Eppendorf
PCR Thermal Cycler	Applied Biosystems, Gene Amp PCR System 5700
PCR Thermal Cycler for Gradient PCR	Biorad, T100
Real Time Light Cycler	Roche, 2.0
Real Time Light Cycler Centrifuge adapter	Roche, 2.0
Cooler for +4 ° C storage	Sanyo Medicool, MPR-513
Refrigerator for -20 ° C storage	Sanyo Biomedical Freezer, MDF-4333
-86 ° C Freezer	Sanyo, MDF-U52V

Vortex	IKA, MS2 Minishaker
Microcentrifuge	Hettich Zentrifugen, Mikro 120
Spin-down	Labnet-Spectrafuge
Analytical balance	Denver Instrument, APX-200
Heater	ETG, MBT250
Water bath	Memmert, WB14
Autoclove	Systec, V-95
Microwave	Bosh, Micro Combi
UV Cabinet	Cleaver Scientific LTP, UV Light
Biological Safety Cabinets	Thermo E. C., Holten LaminAir
Spectrophotometer	Implen Nanophotometer
Gel Electrophoresis System	Thermo E. C., Midicell Primo EC300
Agarose gel visualition tools	Chemi Doc (for PC), Biorad® Gel Imager
UV protector for direct gel visualition	Biorad

3.3. CHEMICALS

- EDTA; Scharlau AC0940
- Tris; Ultra Pure MP Biomedicals Inc.
- Boric Acid; Scharlau AC0577

3.4. BIOINFORMATIC TOOL

Table 3.2. Bioinformatic tools

Tool	Internet Site
Entrez Nucleotide (NCBI)	http://www.ncbi.nlm.nih.gov/nucleotide/
Entrez Blast (NCBI)	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Integrated DNA Technologies (IDT) – Oligo Analyzer	http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/
Biology Workbench Nucleotide Tool, CLUSTALW (Multiple Sequence Alignment)	workbench.sdsc.edu

4. METHOD

4.1. PATIENT GROUPS and CONTROLS

Twenty-eight whole blood specimens collected from SSPE patients who were serologically and clinically diagnosed were kindly provided by Prof. Dr. Güher Saruhan Direskeneli (Istanbul University, Faculty of Medicine, Department of Physiology). Drawn blood specimens formerly transferred to Blood Collection Tube with EDTA and divided into 1.5 ml Eppendorf tubes were stored at -86°C freezer by following long term storage condition rules of biological materials until thesis experiments initiated. Schwarz strain of Measles Vaccine and cell cultured measles strains from a patient kindly provided by Associate Prof. Gülay Korukluoğlu from Public Health Agency of Turkey were used as positive control and whole blood samples collected from healthy donors provided from Yeditepe University Hospital were used as negative controls. Yeditepe University Ethical Committee approval was also received before performing study.

4.2. POLYMERASE CHAIN REACTION (PCR)

Experiment steps were performed respectively as indicated at flow-chart below (Figure 4.1.)

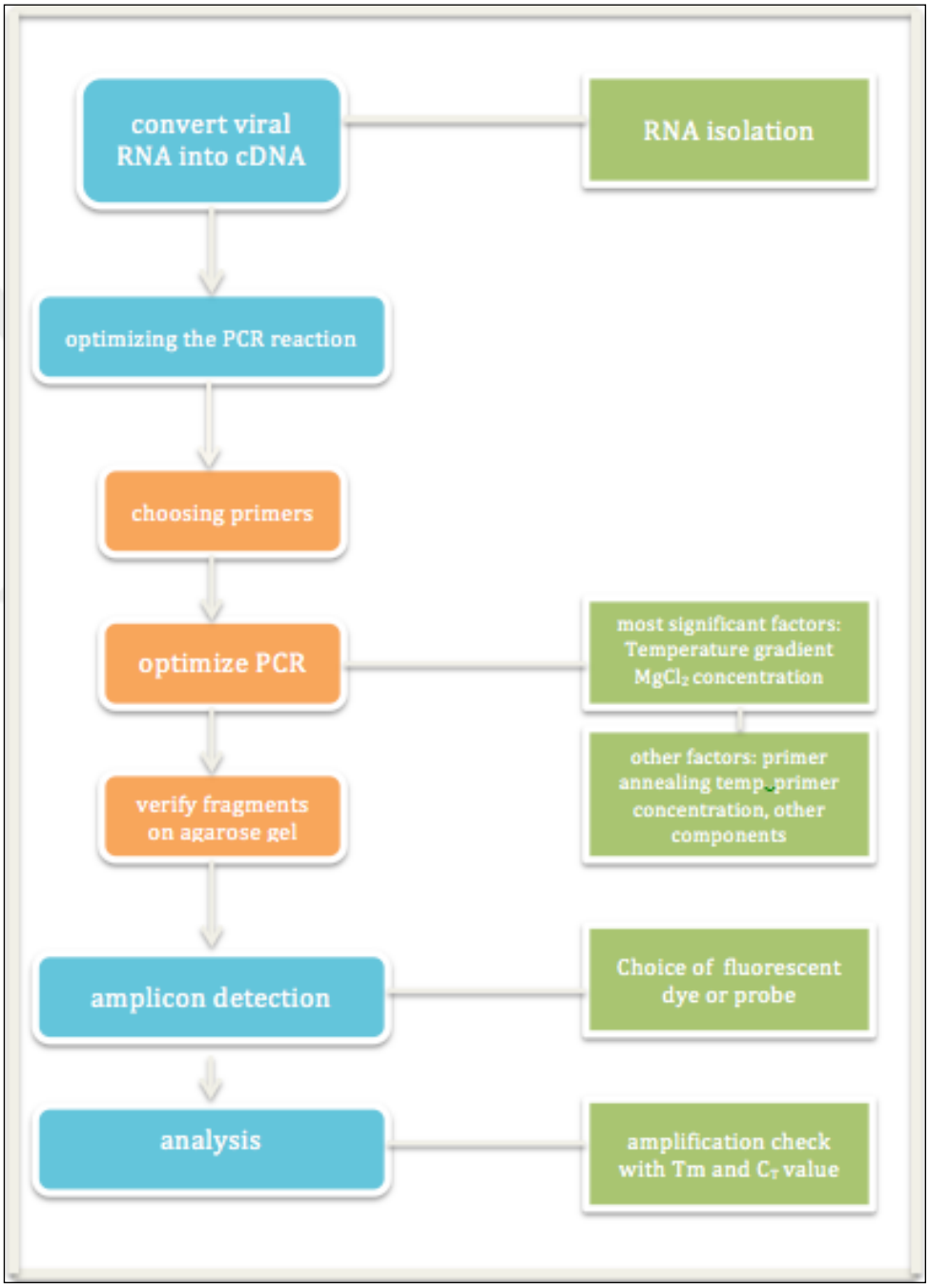


Figure 4.1. Experimental flow-chart

4.2.1. Viral RNA isolation

Whole blood specimens stored at -86°C freezer were held at -20 °C for 24 h in order to prevent samples from heat shock before viral RNA isolation. Extraction was performed with Roche High Pure Viral Nucleic Acid Kit according to the manifacturer instructions. Basically 200 µl whole blood samples were treated with 200 µl Working Solution composed of Poly (A) carrier RNA and Binding buffer to extract more stable and pure viral RNA material. 50 µl Proteinase K were added to eliminate protein contamination. Samples were vortexed and incubated for 10 min at 72 °C. After incubation, 100 µl Binding buffer added. Samples were collected in spin column tubes with silico semi membrane provided with the kit centrifuged in 8000g. Samples in spin columns were washed once with 500 µl Inhibitor Removal buffer and twice with 450 µl Wash buffer in order to remove any remeaning residual contaminants and then samples were centrifuge in 8000 g. Eventually 50 µl Elution buffer was added due to extricate and collect RNAs binding filter membrane of the column tubes. Extracted viral RNAs were aliquoted into eppendorf tubes placed on ice. Viral RNA concentration was calculated by Implen Nanospectrophotometer using 260/280 wavelength. Extra aliquots which were not used for cDNA synthesis were stored at -86°C freezer.

4.2.2. Synthesis of cDNA by Reverse Transcription

cDNA was synthesized from isolated viral RNA using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. 1 µg total viral RNA, 1 µl random hexamer primers and necessary amount of nuclease free water up to 12 µl were added to PCR tubes in order to prepare the first mixture and tubes were incubated at 65 °C for 5 min by using Gene Amp PCR System 9700 thermal cycler. After then 4 µl Reaction Buffer, 2 µl 10 mM dNTP mix, 1 µl Ribolock RNase Inhibitor and 1 µl RevertAid M-MuLV Reverse Transcriptase were prepared in PCR tubes as second mixture and added to first mixture. Tubes were incubated at 42 °C for 60 min and 70 °C for 5 min respectively by using Gene Amp PCR System 9700 thermal cycler. Synthesized cDNA were stored at – 20º C for further use.

4.2.3. Primer Confirmation

N gene primers, which were acquired from the publication released by H.S. El Mubarek and his colleague in 2003 [42], were preferred due to its sequence alignment which were already performed in currently active measles virus genotypes stated by WHO.

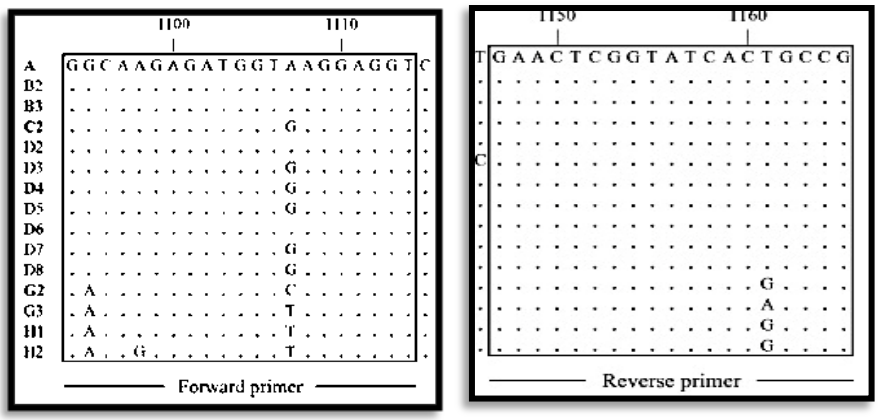


Figure 4.2. *N* gene sequence alignment currently active measles virus genotypes (reference strains as indicated in WHO, 2003) [42]

Primers quality considering their GC contents, melting temperatures and intensity of forming hairpin, dimer structures were checked by using IDT Oligo Analyzer. Primers gene specifity also determined by using NCBI Blast tool (Entrez Blast).

Since Schwarz strain (genotype A, accession number: U03668) of Measles Vaccine derived from Edmonston wild type [97] strain could be provided as positive controls, alignmet of *N* gene Schwarz strain vaccine sequence and Edmonston Wild Type/ USA/ 1954 *N* gene sequence (genotype A, accession number: U01987) which is generally selected to design primers were performed in order to determine overlapping ratio of sequences of the strains and localize primers on these sequences. NCBI Nucleotide tool (Entrez Nucleotide) were used in order to access the sequences. Multiple Sequence Alignment were performed using Biology Workbench CLUSTALW tool. Only three base differences were detected, which can be ruled out.

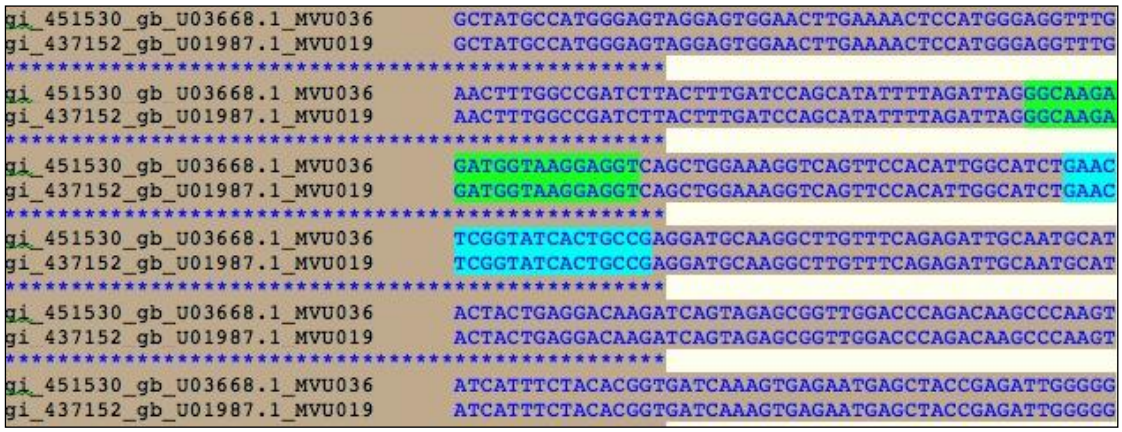


Figure 4.3. Multiple Sequence Alignment of Shwarz strain and Edmonston Wild Type/ USA/ 1954 and localized forward (green) and reverse (blue) *N* gene primers.

4.2.4. Primer Optimization with Standard PCR

For standart PCR , dNTP, distilated nuclease free water, 10X Taq buffer, $MgCl_2$, forward and reverse primers, Tag DNA Polymerase enzyme were mixed in an eppendorf tube. Template cDNA were added afterwards in different lab to avoid contamination. Total reaction volume was 20 µl. All the preparations were performed on ice and in biological safety cabinets. Various amounts of components were tested in order to determine the most efficient PCR protochol.

Table 4.1. The content of PCR mixture

Component	Volume	Final Concentration
Template	1 µl	varies
10X buffer	5 µl	1X
10Mm dNTP	1µl	0,5 mM
$MgCl_2$	4 µl	2 mM
Forward Primer	0,4 µl	0,2 µM
Reverse Primer	0,4 µl	0,2 µM
DNA polymerase	0,3 µl	
dH_2O	Up to 20 µl	

Table 4.2. Experimental Protocol for PCR

Predenaturation	95° C	3 min
Denaturation	95° C x30	30 second
Annealing	varies	30 second
Extension	72° C	30 second
Last extension	72° C	10 min

Table 4.3. Sequences of *N* gene forward and reverse primers

Gene Name	Forward primer	Forward primer	Expected Product Lengt
N	5’ GGC AAG AGA TGG TAA GGA GGT ’3	5’ GAA CTC GGT ATC ACT GCC G ’3	71 bp

The PCR products checked on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold. Samples were run at 100 volt for 45 minutes using electrophoresis system and gel was visualized under ultraviolet with Biorad® Gel Imager with Chemi Doc tool.

4.2.5. Real-time PCR Assay with LightCycler® DNA Master SYBR Green I

For real-time PCR, initially, 10 µl LightCycler® FastStart Enzyme was pipetted into Reaction mix and re-labeled as LightCycler® DNA Master SYBR Green I. PCR grade H_2O, $MgCl_2$, PCR primer mix and LightCycler® DNA Master SYBR Green I were mixed in an eppendorf tube, then transformed to LightCycler® capilleries placed on LightCycler® Centrifuge Adapters formerly held at 4°C . Template cDNA were added afterwards in different lab to avoid contamination. Total reaction volume was 20 µl. cDNA, $MgCl_2$ and accordingly H_2O concentration optimization were performed in order to validate the most efficient Real Time PCR protocol for detecting N RNA. All the preparations were conducted in biological safety cabinets.

Real Time PCR was administered in LightCycler 2.0 Instrument (Roche). 530 nm wavelength channel were chosen for fluorescence and absorption measurements of Sybr Green.

Table 4.4. The content of real-time PCR mixture

Component	Volume	Final Concentration
Template	2 µl	Varies
$MgCl_2$	Varies (0.8-1,6 µl)	Varies (2-3 mM)
PCR Primer mix	2 µl	0.5 mM of each
Master SYBR Green I	2 ul	1X
dH_2O	Up to 20 µl	

Table 4.5. Experimental Protocol for real-time PCR

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95° C	10 min	None
Amplification					
Quantification	45	Denaturation	95° C	10 min	None
		Annealing	52° C	4 sec	None
		Extension	72° C	7 sec	None
Melting Curve					
Melting Curves	1	Denaturation	95° C	0 sec	None
		Annealing	65° C	15 sec	None
		Extension	0.2° C	0 sec	Continuous
Cooling					
None	1		40° C	30 sec	None

The PCR products were checked on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold. Samples were run at 100 volt for 1 h using electrophoresis system and gel was visualized under ultraviolet with Biorad® Gel Imager with Chemi Doc tool.

5. RESULTS and DISCUSSION

5.1. REAL-TIME and STANDART PCR OPTIMIZATION RESULTS

5.1.1. *N* gene Primers Annealing Temperature Optimization by Standard PCR

After primers have been synthesized, their annealing temperatures should be optimized. Primers of which annealing temperatures are stated and acquired from various publications may also need optimization since reactions are carried out with different kits or systems may cause differences in annealing temperatures. Optimization can be performed utilizing a temperature gradient analysis to determine the optimal annealing temperature. A temperature gradient analysis allows to conduct several PCR reactions at different temperatures simultaneously. Thus, temperatures can then be compared and which annealing temperature gives the best product can be determined [84].

In our study, *N* gene primers annealing temperature were determined via gradient gel PCR and confirmed with standard gel PCR using Thermo Scientific Taq Polymerase Kit. The PCR products were checked on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold. Samples were run at 100 volt for 45 min using electrophoresis system and gel were visualized under ultraviolet with Biorad® Gel Imager with Chemi Doc PC tool. As shown in Figure 5.1., in order to assign *N* gene primers annealing temperature, temperatures varied between 50°C-56°C were tested via gradient PCR. MV vaccine cDNA was used as template. According to the electrophoresis results of gradient PCR, annealing temperature was determined as 52° C. Then as indicated in Figure 5.2, it was confirmed with standard PCR.

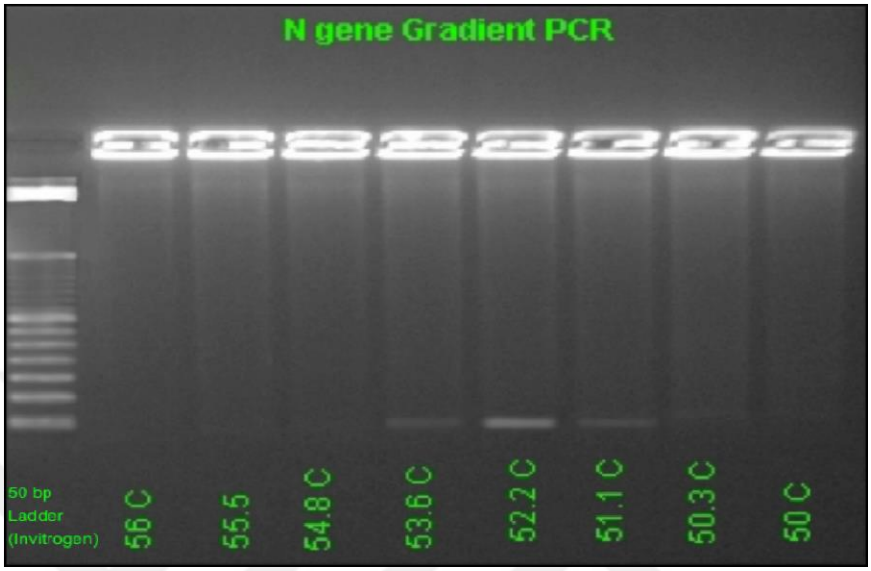


Figure 5.1. *N* gene Standard Gradient PCR gel electrophoresis result

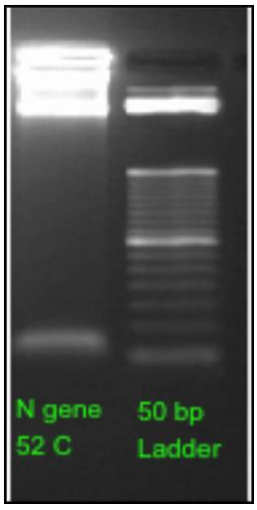


Figure 5.2. *N* gene Standart PCR result

5.1.2. *N* gene Primers Concentration Optimization for Real Time PCR Assay with LightCycler® DNA Master SYBR Green I Kit

Primer concentration is one of the main elements which directly effects PCR efficiency. Increasing primer concentration can increase the detection of the PCR products, however, primer concentrations that are too high may cause the production of nonspecific products such as primer-dimers or smears. If the signal is weak or nonspecific products are too problematic, testing varying amounts of each primer is helpful. Typically, optimal final concentrations will be between 0.05 and 0. 9 μM [84].

In this study, *N* gene lyophilized primers were resuspend and prepared as 100 μM stock solution and 10 μM working solution before experiments initiated [97]. Then, as recommended by instructor's manual of LightCycler® DNA Master SYBR Green I Kit, a primer mix was prepared as each primer concentration to be 0.5 μM and 2 μl primer mix was used for each reaction.

5.1.3. $MgCl_2$ Concentration Optimization for Real Time PCR Assay with LightCycler® DNA Master SYBR Green I Kit

$MgCl_2$ concentration is a very important factor for all kinds of PCR since it directly influences the productivity and fidelity of the enzyme in the reaction. Its impact ranges from no detectable product if too low, to a multitude of nonspecific products and PCR artifacts if too high. An optimal concentration for $MgCl_2$ is typically between 1 to 5 mM.

In this study, $MgCl_2$ concentration were determined for real-time PCR assay with LightCycler® DNA Master SYBR Green I. cDNA synthesized from MV vaccine used as template. 0.8 μl (2 mM), 1 μl (2.25 mM), 1.2 μl (2.5 mM), 1.4 μl (2.75 mM), 1.6 μl (3 mM), and 1.8 μl (3.25 mM) $MgCl_2$ were tested respectively and 1 μl (2.25 mM) $MgCl_2$ showed the most accurate amplification curve result regarding to its C_P value which is 25 as shown in Figure 4.3 and Figure 4.4. On LightCycler systems C_P (Crossing point) value is equivalence of C_T (treshold cycle) on other real-time PCR systems. Since C_T values are generally evaluated as 20; high, 30; low, 40; unsignificant or no expression, the value we choose, representing any parameter, should be ranged between 20-30, if possible. It is also

important to choose the lowest C_T value between 20-30 as lower C_T values point out a more efficient production of the product [84].

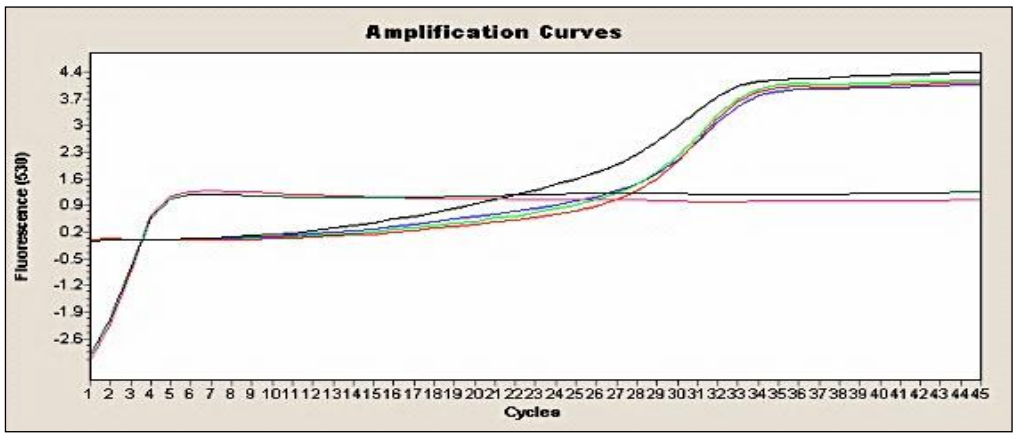


Figure 5.3. Amplification curves of various $MgCl_2$ concentrations obtained from Real Time PCR assay

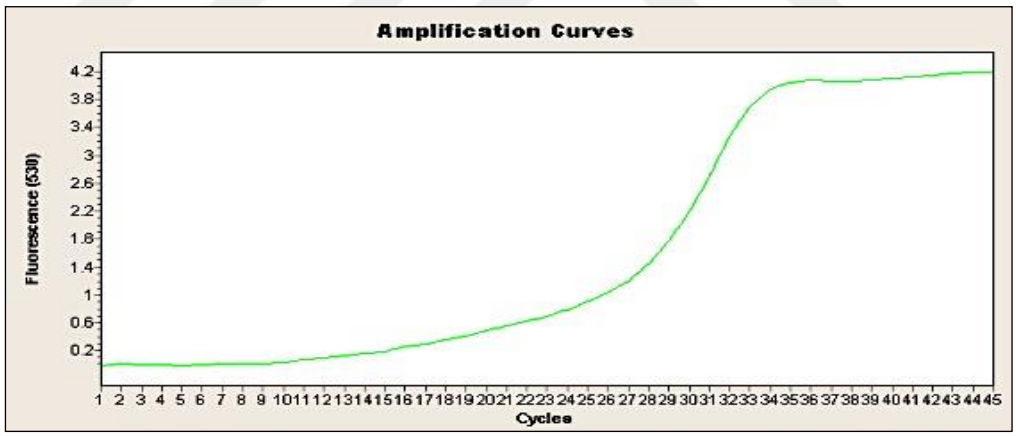


Figure 5.4. Amplification curve obtained by using 1 µl $MgCl_2$ alone

Samples which represent various $MgCl_2$ concentration were also confirmed on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold (Figure 5.5.). Samples were run at 100 volt for an hour using electrophoresis system and gel was visualized under ultraviolet by Biorad® Gel Imager and Chemi Doc PC tool. As seen in the Figure 5.6., 1 µl $MgCl_2$ showed the clearest band among the other bands representing different concentrations in gel electrophoresis, as supporting the real-time results.

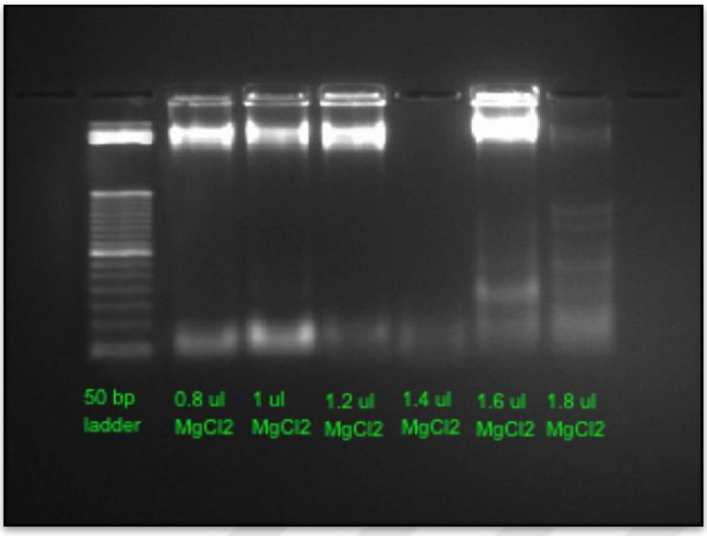


Figure 5.5. Gel electrophoresis result of different $MgCl_2$ concentrations

5.1.4. Template Concentration Optimization for Real Time PCR Assay with LightCycler® DNA Master SYBR Green I Kit

All the $MgCl_2$ concentrations showed double melting peaks as indicated in Figure 5.6., which referred to unspecific primer bindings or primer dimers. Those unwanted products could have been arisen from using unsuitable template concentration as well as unsuitable $MgCl_2$ concentration. However, since different concentrations for $MgCl_2$ already were tested, template concentration optimization option were decided to try.

At the beginning high concentration of cDNA (1 µg) had been decided to use considering a big part of measured RNA actually consisted of Poly A carrier RNA provided by viral isolation kit, and therefore low amount of viral RNA might in fact had been extracted from whole blood samples. Nevertheless, excess or too little amount of cDNA usage might lead to occure those problems mentioned above. Hence, in order to obtain a single peak, real-time assay was repeated with 1 µl $MgCl_2$ and 1:10 diluted template (100 ng) and it was revealed that using 100 ng cDNA as template still caused double melting peaks formation. However, one of the melting peak's d/dt value which represents absorbance was very lower than the value of correspond peak of 1µg template. Theron, same assay was tested with 1 µl $MgCl_2$ and 30 ng cDNA as recommended by instructor's manual of LightCycler® DNA Master SYBR Green I Kit.

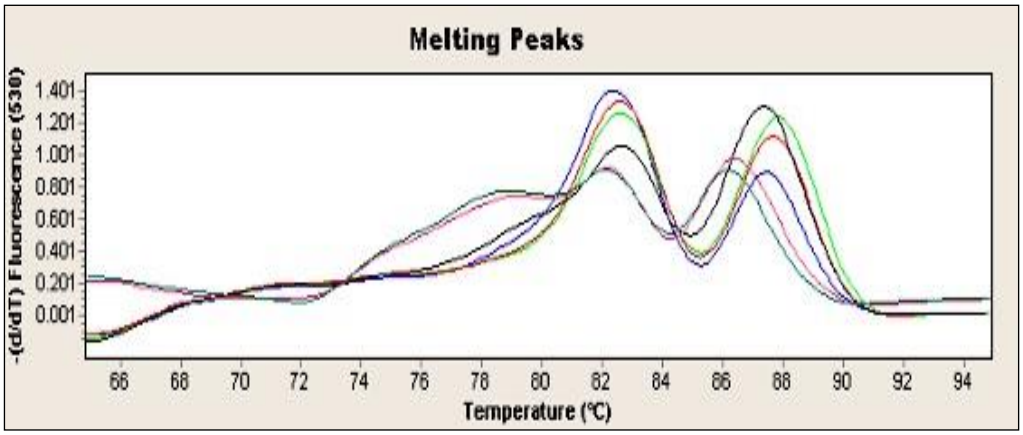


Figure 5.6. Double melting peaks of various $MgCl_2$ concentrations obtained from real-time PCR

As shown in the Figure 5.7. single melting peaks were obtained by using 30 ng MV cDNA (red) and cell cultured measles strains (green), which are positive controls, as templates with 1 µl $MgCl_2$. It was also revealed that Tm value specific to our interested N RNA sequence was approximately 82.5 ° C, which played a key role investigating N RNA in following PCRs.

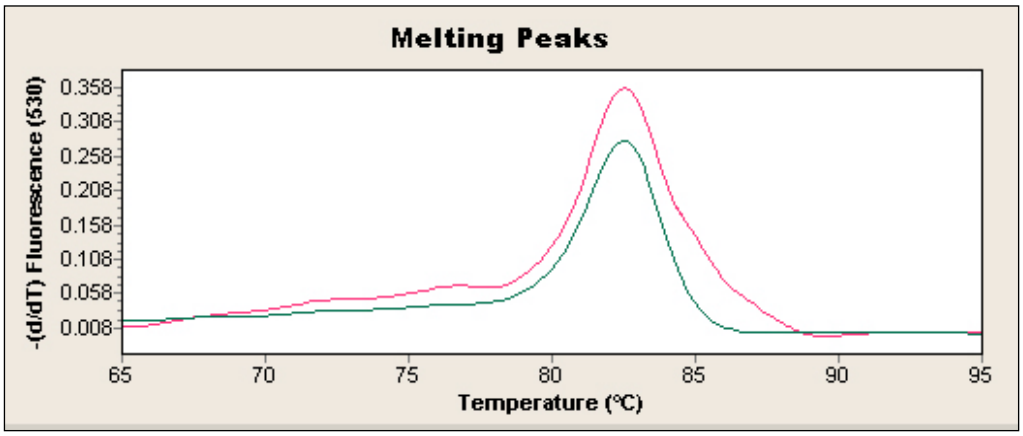


Figure 5.7. Melting peaks acquired from 30 ng MV cDNA and 30 ng and cell cultured measles strains, which are positive controls, with 1 µl $MgCl_2$

5.2. MV-RNA INVESTIGATION RESULTS IN SSPE PATIENTS

Whole blood specimens of twenty patients were examined by separating into groups. For each reaction two positive controls, one negative control (different healthy donors's cDNA for each reaction) and dH_2O were used. Only one patient blood specimen (no: 6761) showed positive result which indicated that, target N RNA could be extracted by RNA isolation from patient blood and detected and amplified by real-time PCR assay. As shown in Figure 5.8.; melting peaks of MV vaccine (dark green) and of cell cultured measles strains (dark blue) as positive controls and of positive patient (black) are intersected at approximately 82.5° C. Measles vaccine showed one more small peak which might resulted from unspecific primer binding. As cDNA used as template became denatured due to undergoing defrosting and freezing numerous times for each reaction, cDNA concentration in 2 µl might have started to decrease (lesser than 30 ng) and this might have led to primer dimers or unwanted products since optimum template concentration impaired. However that small peak could be neglectable, for the melting peak specific to N RNA sequence was clear. Negative control cDNA (grey) has two melting peaks of which Tm values are 80° C and 86° C respectively. dH_2O (orange) does not show any melting peak.

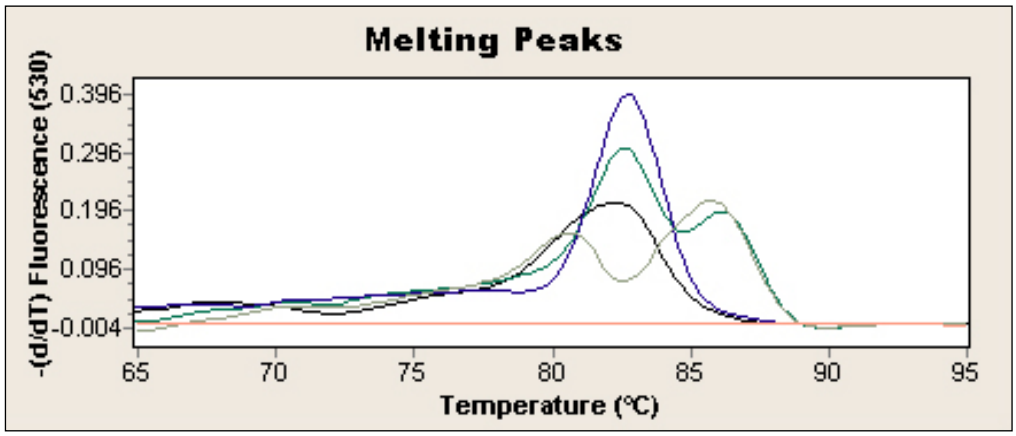


Figure 5.8. Melting curve analysis of real-time PCR assay with positive patient (no: 6761)

Melting peak of N RNA sequence of positive patient alone could be seen in Figure 5.9..

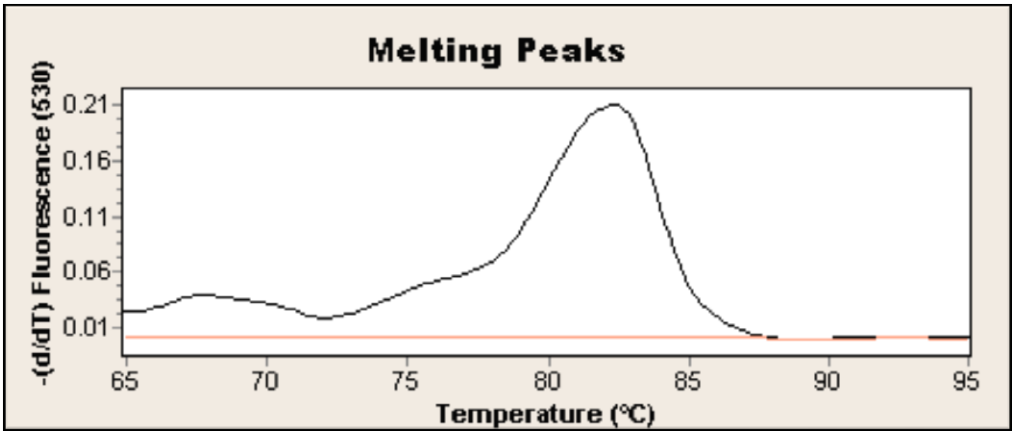


Figure 5.9. Melting peak of positive patient alone

As seen in Figure 5.10., according to amplification curve analysis data, C_P values of positive patient (black), Measles vaccine (dark green), cell cultured measles strains (dark blue) and (-) cDNA (grey) are 30.98, 33.17, 28.59 and 29.25 respectively, which imply that C_P values of the samples indicates sufficent gene expression. Thus, the assay can be considered as a significant and an efficient real-time PCR.

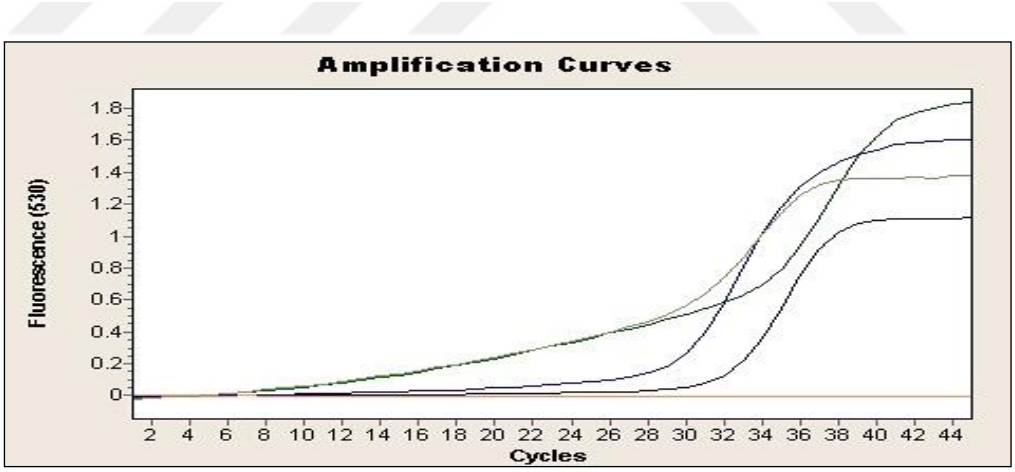


Figure 5.10. Amplification curve analysis of real-time PCR assay with positive patient

This real-time PCR assay results was also checked on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold. Samples were run at 100 volt for an hour using electrophoresis system and gel was visualized under ultraviolet by Biorad® Gel Imager and Chemi Doc PC tool.

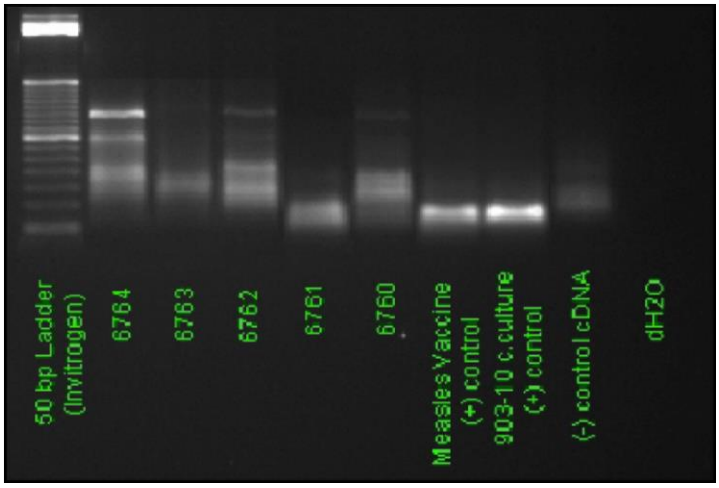


Figure 5.11. Gel image of real-time PCR assay results with positive patient (no: 6761)

As shown in Figure 5.11., while positive patient's (no: 6761) and positive controls' (measles vaccine, cell cultured measles strains) bands appears at the same level, which points out the length of expected product (71 bp) ; other patients' (no: 6764, 6763, 6762, 6760) and healthy donors' bands appeared at higher levels. Negative patients seems to have similar bands at the same level in correlance with common melting curve values of theirs in real-time PCR assay. Positive patient's and controls' same leveled bands also are associated with the specific Tm value of their melting curves (~ 82.5° C). dH_2O has no bands, revealing no expression just as indicated in real-time PCR assay. In the light of these findings, it can be concluded that real-time PCR results are consistent with gel electrophoresis results.

As clearly seen Figure 5.11., bands represented gene expression of negative patients and healthy donors formed as a consequence of non-spesific bindings of primers. Impairment of optimum cDNA concentration caused by denaturation is responsible for this formation. It is also suggested that during viral RNA isolation, human small RNAs (non-coding RNAs) could have been extracted with viral RNAs and primers might bind these RNA sequences. However this assumption could only be acceptable for human specimens.

Next, positive patient data was additionally checked with three negative controls (healthy donors) in another real-time PCR assay of which result is shown in Figure 5.12.. As positive controls; MV vaccine (light blue), cell cultured measles strains (light green) and

positive patient (pink) melting peaks intersect at approximately 82.5° C as they did in the previous real-time Assay as seen in Figure 5.8.. Each samples including positive controls and positive patient have double melting peaks, probably indicates unspesific primer binding due to the fact that cDNAs might have started to denaturate. Negative control cDNAs are dark blue, grey and orange, which have a common melting peak with the value of approximately 86 ° C.

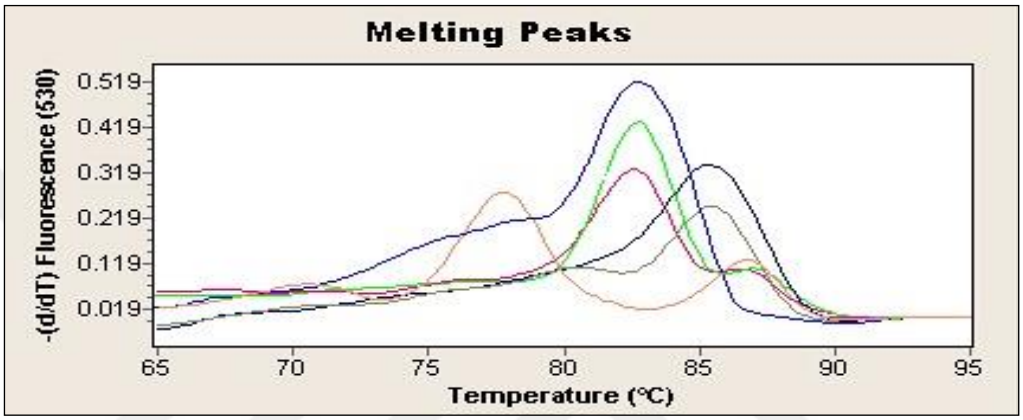


Figure 5.12. Melting Curve analysis data of positive patient and two positive and three negative controls

Positive patient result and negative results with positive controls can be seen separatedly in Figure 5.13. and Figure 5.14., respectively.

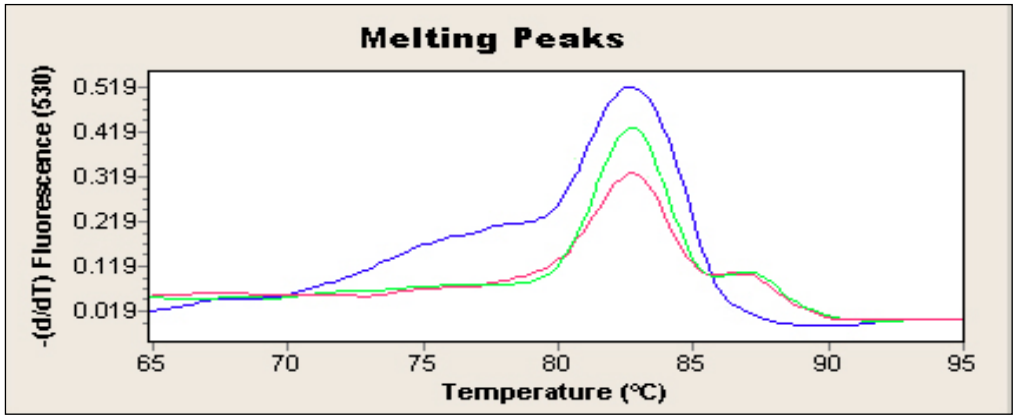


Figure 5.13. Positive patient result with only positive controls

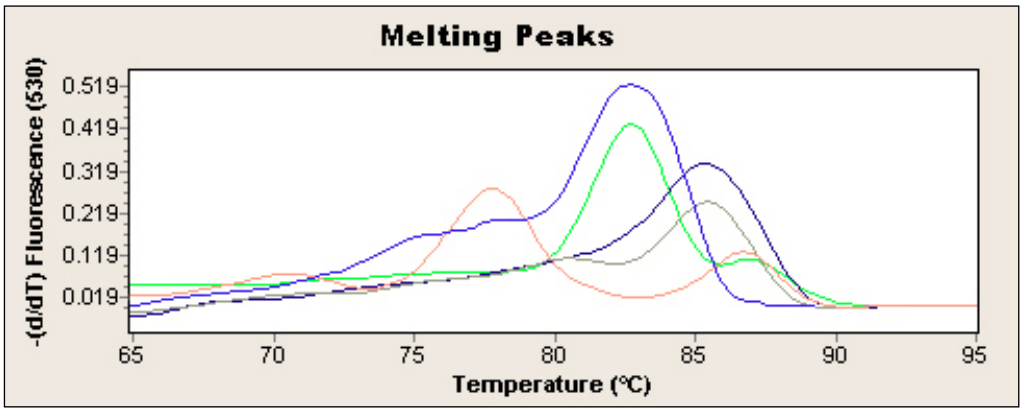


Figure 5.14. Healthy donors results (negative controls) with only positive controls

As stated in Figure 5.15., C_P values of patient (black) is 27.70, Measles vaccine (light blue) is 22.53, cell cultured measles strains (green) is 27.78 and of three negative controls are (dark blue) 24.51, (grey) 27.28, (orange) 25.08 respectively, indicating a significant and an efficient real-time PCR.

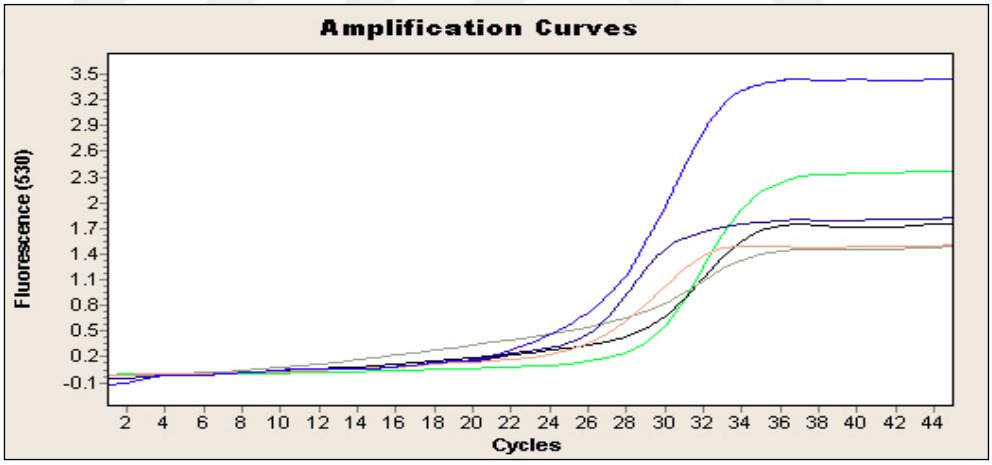


Figure 5.15. Amplification curve analysis of positive patient, positive controls and negative controls.

As shown in Figure 5.16., these real-time PCR assay results were additionally checked on 2% agarose gel prepared with TBE buffer and agarose staining with Sybr Gold. Samples were run at 100 volt for an hour using electrophoresis system and gel was visualized under ultraviolet by Biorad® Gel Imager and Chemi Doc PC tool. In accordance with real-time PCR assay, bands belonging to positive controls (MV vaccine and cell cultured measles strains) and positive patient are in the same level in the gel image, indicating expected

product length (71 bp) whereas negative controls bands appear at higher level, also being in correspondence with previous real-time PCR assays and their gel electrophoresis results.

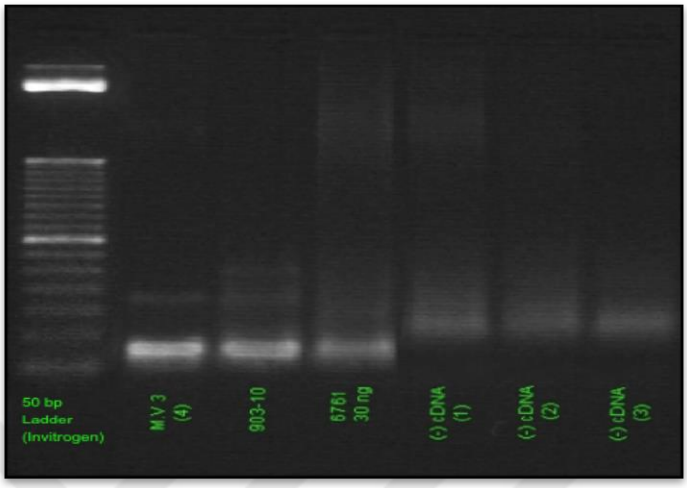


Figure 5.16. Gel image of real-time PCR assay results of positive patient, positive controls and negative controls.

6. CONCLUSION and RECOMMENDATION

The primal aim of this study was the validation of the most convenient real-time PCR method to detect and amplify MV-RNA in blood specimens collected from SSPE patients clinically and serologically diagnosed. *N* (nucleoprotein) gene belonging to MV genome was the interested gene since it is revealed that N RNA is the most abundant transcript among the others, and additionally its whole gene sequence has been published.

As a pilot study, twenty-eight patients' specimens were examined and only one patient's blood specimen gave positive result. In the study carried out by Miki and his colleagues in 2002, which we took as base, two patients had given positive results among nineteen patients.

The differences in this ratio may result from various parameters. One of the consideration is that substantial MV-RNA in our blood specimens could be denatured during the years in storage. Even if blood specimens were stored with EDTA at - 80° C, RNAs might not survive due to their unstable single strand structure after a certain period of time. In order to prolong RNA's stability special blood tubes such as PAX gene blood tubes which are recently developed for particularly inhibiting nucleic acid denaturation could be utilized. Another suggestion is that there was no RNA existed in blood specimens except one patient's. Since MV virus is generally located in brain in SSPE disease, the time of phlebotomy plays a key role in existence of RNA in blood. If blood samples are collected during the period which all MV viral particles have not yet passed through the blood brain barrier and still been circulating in the blood stream, it is possible to detect MV-RNA in blood samples.

In PCR false negatives may occur due to reaction failure especially because of presence of inhibitors in clinical specimens; therefore, only negative reactions accompanied by positive internal controls are accepted as true negatives. Correspondingly our results would be of much value if a multiplex real-time PCR including internal control, which is more expensive, could be optimized.

Also the different sensitivity of the PCR detection methods are possible explanations for these two different results. Extension of the concept of the molecular analysis could be useful to gain more precise and reliable data regarding of RNA existence. In this study due to economical conditions, one certain region of *N* gene was examined. However, as a further research, development of a more sensitive nested real-time PCR which allows detection of different regions of the interested gene with various primers and multiplex real-time PCR which provides to amplify sequences belonging to different genes simultaneously in one reaction may assist us to obtain more positive results. With these assay different regions of *N* gene can be tried to amplify and *M, H* or/and *F* genes detection can also be add in research. As an another approach, development of a quantitative real-time PCR assays with optimized standards can give us much more accurate results in investigating MV-RNA compared to less developed existence analysis based on melting curve analysis.

As a further research, sequence analysis will be useful in order to investigate which genotype of MV virus causes SSPE in the cases. In this study we used primers which amplify 71 bp sized product. However, primers which amplify more than 100 bp product in length can be used to achieve a convincing sequence analysis.

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