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HERPES SIMPLEX VIRUS SEQUENCE VARIATION IN THE PROMOTER OF THE LATENCY ASSOCIATED GENE AND CORRELATION WITH CLINICAL FEATURES

Doctoral dissertation

Biomedical Sciences, Medicine (06 B)

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HERPES SIMPLEX VIRUSO SU LATENCIJA SUSIJUSIO GENO PROMOTORIAUS SEKŲ ĮVAIROVĖ IR SĄSAJA SU KLINIKINIAIS POŽYMIAIS

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CONTENT

ABBREVIATIONS

bp	base pairs
CI	confidence interval
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
HIV	human immunodeficiency virus
HPT	homopolymer tract
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HSE	herpes simplex encephalitis
IgG	immunoglobulin G
kb	kilobase pairs
LAT	latency associated transcript
LAP	latency associated promoter
nt	nucleotide
OR	odds ratio
ORF	open reading frame
p	level of statistical significance
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SSR	simple sequence repeat
STI	sexually transmitted infections

1. INTRODUCTION

1.1. The rational of the study

Herpes simplex virus (HSV) is one of the most common infectious pathogens in humans. The first information about HSV infection came from ancient Greeks: Hippocrates described the cutaneous spread of herpes simplex lesions. It was named herpes, i.e. „creeping or crawling“ in Greek (Scott, 1986). In ancient Rome, The Emperor Tiberius prohibited kissing in Rome as at that time so many people were infected with labial herpes.

Herpes simplex virus as a cause of herpes infection was not identified until the 1940s. Since the 1960s the two types of HSV (i.e., HSV-1 and HSV-2) are distinguished by antigenic differences in their envelope proteins (McGeoch et al., 1986; Beauman, 2005; Wagner et al., 1997).

HSV causes recurrent orofacial and genital infections (Bloom et al., 2010), sometimes disseminated or visceral diseases. Genital herpes simplex infection is the leading sexually transmitted disease (Malvy et al., 2005) most often caused by HSV-2 (Gupta et al., 2007) although HSV-1 also can be detected in genital lesions (Lowhagen et al., 2002; Pebody et al., 2004). HSV-1 usually is found in orofacial lesions and is supposed to be associated with the developement of Alzheimer disease (Letenneur et al., 2008). Herpetic keratitis is one of the main cause of infectious origin blindness worldwide (Millhouse and Wigdahl, 2000). HSV-1 is the most common cause of sporadic encephalitis which can be fatal whithout early treatment (Whitley and Roizman, 2009). HSV infection continues to be a public health problem in both developed and developing countries (Slomka, 2000).

HSV is transmitted during close contact with a sore or fluid from an infected person, including birth-acquired herpes that newborn gets from the infected mother during birth (Whitley and Roizman, 2009).

Herpes simplex virus has the capacity to invade CNS, replicate in neurons and establish a latent infection (Whitley and Roizman, 2009). After establishment of latency HSV persists in sensory neurons for the lifetime of

the host (Perng et al., 2002). From this site virus can reactivate and be transported to the skin where virus-laden vesicles occur (Gupta et al., 2007). Some patients get more frequent and intense reactivations than others (Whitley and Roizman, 2009; Arduino and Porter, 2008).

Nevetherless infection with herpes simplex virus is a widespread disease, only a few patients present with typical vesicular and ulcerative lesions. Up to 80 % of cases of genital herpes have no typical clinical picture or are asymtomatic and therefore are not diagnosed (Corey and Holmes, 1983). It was established that most individuals (both with orofacial and genital infection) shed HSV without any clinical signs in amounts sufficient for virus transmission. Therefore unrecognized infections and subclinical viral shedding are the main causes for HSV transmission. Infection with both viruses can cause clinically indistinguishable lesions (Wald, 2006), however, recurrence frequency (Solomon et al, 2003; Lafferty, 2002; Benedetti et al, 1999, Wald et al, 2002) and subclinical viral shedding in genital herpes is greater for HSV-2 than HSV-1 disease (Engelberg et al, 2003; Faber et al, 2011; Ashley et al, 1999; Pereira et al, 2012) and it is opposite in orofacial infection (Wald et al., 2004).

Genital herpes infection enhances the acquisition and spread of human immunodeficiency virus (HIV) (Ryan and Kinghorn, 2006; Gupta et al., 2007; Paz-Bailey et al., 2008) making mucosa more susceptible to HIV (Xu et al., 2006).

Introduction of polymerase chain reaction (PCR) into the routine laboratory diagnostics improved diagnosis of HSV infection; especially quantitative PCR (qPCR) which enabled both detection and quantification of HSV DNA. PCR also enables typing of HSV which is especially important in genital herpes as HSV-1 has a more favourable outcome than HSV-2.

HSV infection is an incurable disease. Available antiviral drugs inhibit HSV replication but do not affect the latent reservoir of HSV (Knipe and Cliffe, 2008).

It is well known that herpes simplex virus establishes latency and can reactivate and cause recurrent disease, however the mechanisms by which latency occurs, are unclear (Perng et al., 2002). It was discovered that HSV genome was not completely quiescent during latency; all genes are supressed except the latency associated transcripts which are transcribed from latency associated gene (LAT) (Stevens et al., 1987; Rock et al., 1987). Numerous studies have been performed with a purpose to detect LAT region responsable for latency and reactivation HSV but still many facts have to be determined (Wagner and Bloom, 1997; Perng and Jones, 2010).

The expression of LAT is under the control of the LAT promoter (LAP). It is established that HSV-1 and HSV-2 LAT promoter mutants have lower levels of spontaneous reactivation rates than wild type HSV in rabbits (Perng et al 1994, 1996), mice (Perng et al, 2001) and guinea pig (Krause 1995) models. All previous LAT promoter studies have been conducted using small animal (mouse, rabbit, guinea pig) models. Especially little work has been done with HSV-2 (Wang et al 1997). However, the variation in the LAT promoter has not been studied in viruses from clinical samples, and it is not known whether this variation has any bearing on the tendency for reactivation and anatomical site in humans.

This study consists of the clinical and experimental parts and deals with HSV DNA detection, quantification and typing of HSV in skin lesions, risk factors for genital herpes and sequence variation in the LAT promoter region in clinical samples from patients with HSV infection. Samples from genital and orofacial lesions were obtained in Lithuania (Vilnius) and were used in the clinical part of the study meantime both Lithuanian and Swedish (Uppsala) clinical samples were analyzed in the experimental part.

1.2. Aim of the study

To evaluate the sequence variation in herpes simplex virus latency associated gene promoter by developing and applying molecular methods and correlate with clinical features.

1.3. Objectives of the study

Clinical part of the research

1. To type and quantify the amount of herpes simplex virus in skin lesions of suspected herpetic origin with the qunatitative real time PCR method

2. To evaluate the possible risk factors for genital herpes

Experimental part of the research

3. To develop new PCR assay for HSV-1 and HSV-2 LAT promoter analysis; to clone difficult to sequence due to G+C richness LAT promoter regions.

4. To estimate the variability of LAT promoter sequences; to evaluate relation between HSV strains regarding the rate of recurrence, anatomical site of HSV infection and geographical distribution

1.4. Scientific novelty of the study

Detection, quantification and typing of the HSV DNA in the skin lesions was never performed in Lithuania before. For the first time we evaluated risk factors for genital herpes in Lithuania.

This is the first so extensive study of the LAT promoter region from the clinical samples worldwide. For the first time we mapped the variability of the HSV LAT promoter region, without the possible confounding effects of cell culture. We developed a new PCR assay and sequencing methods for the LAT promoter region and analyzed 145 clinical samples. The analysis of the LAT promoter variability in Lithuanian HSV strains was performed regarding the rate of recurrences, anatomical site of HSV infection. For the first time we performed phylogenetic analysis and compared HSV LAT promoter region in HSV strains obtained from Lithuania and Sweden.

1.4. Practical value of the study

Our results show that HSV-2 is the main cause of genital herpes and that some patients are unaware of having HSV (i.e., are seropositive but have never experienced clinically appearent episodes of herpes simplex infection) therefore shedding and transmission of HSV can occur.

Since the clinical diagnosis of HSV infection (especially in genital herpes) is not always specific and the type of HSV affects prognosis and subsequent couseling, virological and type-specific serological tests should be used routinely to confirm HSV diagnosis and to distinguish HSV-1 from HSV-2 infection.

Informing patients about the risk of transmission and sexual behaviour is important and could help to prevent the spread of disease and neonatal complications. The recognition of atypical features of HSV infection may help to avoid unnecessary and costly investigations and treatments for others though clinically similar-appearing disorders.

The experimental part of this study showed variable repeated sequences of DNA in LAT promoter region within and between HSV strains. When these sequences are deposited in GenBank they will be a lasting resource for further research of HSV pathogenesis, aspects of latency, evolution and may be a basis for future HSV therapies.

1.5. Defended statements of the dissertation

1. HSV-2 is the main cause of genital herpes; HSV-1 is found more often in orofacial region.

2. Higher number of lifetime sexual partners, early age at first intercourse, history of sexually transmitted infections (STI), practice of oral sex are the risk factors for the aquisition of genital herpes.

3. LAT promoter region contains hypervariable repeats which differ between and within strains. There was no relation between variation in

the LAT promoter region and frequency of recurrence, the site of infection and geographical distribution.

2. LITERATURE REVIEW

2.1. Herpes simplex virus

HSV belongs to the family *Herpesviridae*, subfamily *Alphaherpesviridae*. The HSV virion is composed of a double-stranded linear DNA, an icosahedral protein capsid, tegument and a lipid envelope. The HSV viral genome is approximately 152 kilobase pairs (kb) long and encodes more than 80 genes (Perng et al., 2002). The base composition of G+C is 68% for HSV-1 and 69% for HSV-2. HSV DNA consists of two stretches of unique sequences known as the unique long (UL) and unique short (US) regions which are flanked by large inverted long (RL) and short (RS) repeats (Bloom, 2004; Whitley and Roizman, 2009).

2.2. Epidemiology of herpes simplex virus infection

Seroprevalence of HSV-1 is 60%-90% throughout the world (Smith and Robinson, 2002). Seroprevalence of HSV-1 is decreasing in industrialized countries due to better socioeconomic conditions (Malkin, 2004) still reaching up to 90 % and over in Africa. Most acquisition of HSV-1 occurs in childhood and adolescence (Smith and Robinson, 2002) via orofacial route (Malkin, 2004).

Seroprevalence of HSV-2 varies significantly from country to country, ranging from 7% to 80 %. It depends particularly on geographic area, population group, sex and increases with age (Smith and Robinson, 2002). The rates of HSV-2 were found to be highest in Africa reaching up to 80 % in certain populations, lowest in Asia. The rates of HSV-2 infection are much lower in southern part of Europe, especially in Spain, than in northern Europe and North America (Malkin, 2004; Smith and Robinson, 2002). Recent study

from Sweden showed a decrease in HSV-2 seroprevalence among pregnant women which is supposed to be due to changes in sexual behaviour over time (Berntsson et al., 2009).

2.3. Risk factors for genital herpes

HSV-2 is indicative for genital herpes although HSV-1 also can cause genital infection. According to the seroepidemiological studies performed in the developed and developing countries, risk factors associated with genital herpes could be divided into two categories: biological (age, gender, race) and behavioural (sexual debut, number and choice of sexual partners, etc.) (Wald, 2004). Risk factors for acquisition of genital herpes infection include:

- Female gender: women are more tend to have genital herpes than men due to the anatomical features and usually older sexual partners (Lafferty, 2002; Jonsson et al, 2006);
- Age: the risk increases with age, i.e. likely reflects number of sexual partners (Pebody et al., 2004; Rodriges et al., 2009);
- Race: HSV-2 seroprevalence is higher in black race comparing with whites (Rodriges et al., 2009);
- Higher number of lifetime sexual partners: the risk increases with the number of sexual partners (Faber et al, 2011; Pereira et al, 2012);
- History of sexually transmitted infections: this indicates that sexual partners are at higher risk of genital herpes (Faber et al, 2011; Rodriges et al., 2009);
- Early age at first intercourse: related with duration of lifetime sexual activity (Rodriges et al., 2009);
- Sexual contact with commercial sex workers (Faber et al, 2011; Rodriges et al., 2009);

- HIV infection (Ryan and Kinghorn, 2006; Gupta et al., 2007; Xu et al,. 2006)
- Low level of education or lower socioeconomic status

HSV-1 genital infection mostly is associated with younger age at first intercourse age (Cowan et al., 2002), oro-genital sex (Nieuwenhuis et al., 2006), female gender.

2.4. Herpes simplex virus infection clinical picture and viral shedding

HSV-1 and HSV-2 are the primary agents of recurrent oro-facial and genital herpetic infection (Wald., 2004). HSV-1 usually is associated with oro-facial infections and HSV-2 with genital infections, but either type can infect a person anywhere on the skin (Beauman 2005). The number of genital HSV-1 infections has been increasing (Xu et al., 2006; Pebody et al., 2004; Lowhagen et al., 2000; 2002; Faber et al, 2011; Coyle et al., 2003; Thompson, 2000; Lafferty et al., 2000; Kortekangas-Savolainen and Vuorinen, 2007).

As already mentioned above, HSV is acquired through direct contact with the secretions on mucosal surfaces of an infected person. After initial infection virus migrates through the nerves to the trigeminal, vagus or sacral nerve root ganglia where remains latent and can reactivate resulting in recurrences or asymptomatic viral shedding (Gupta et al., 2007; Beauman, 2005).

HSV causes wide spectrum of diseases (Figure 1): primary and recurrent mucosal and skin infections, keratoconjunctyvitis, neonatal herpes, HSV encephalitis, widespread skin and visceral HSV infections in immunocompromised persons, herpetic eczema, herpetic whitlows, herpes gladiatorum, association with erythema multiforme.

Clinically appearent HSV infection is subdivided into **first episode and recurrent** infection. The manifestations of the disease depend upon the involved anatomical site and whether the clinical episode is due to

primary infection or recurrence. Typical symptoms of HSV infection include grouped vesicular lesions on erythematous base in the skin or mucose membranes of the mouth, lips or genitals.

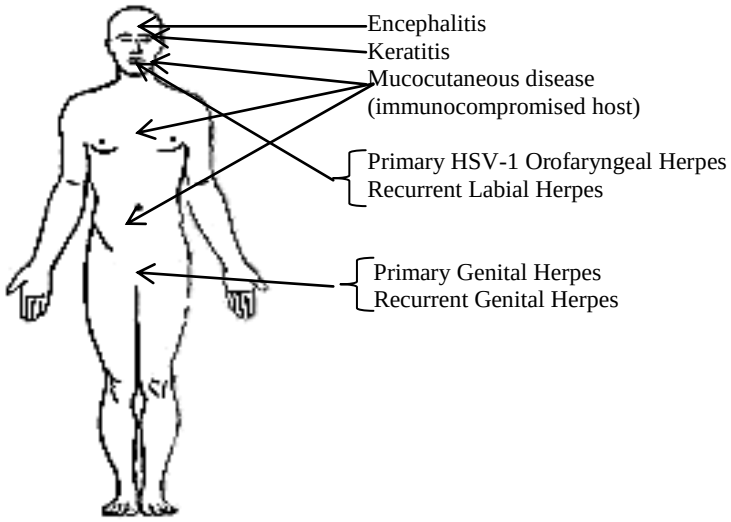


Figure 1. Sites of HSV infection and disease

During the **first episode of HSV infection** vesicular and ulcerative lesions are usually associated with systemic symptoms and may last up to 21 days (Ashley and Wald, 1999). **The first episode** is divided into a true primary herpes and a non primary first episode herpes. **True primary herpes infection** is defined as the first herpes episode when a patient has never previously been exposed to HSV and has no preexisting antibodies to any of the two herpes simplex viruses (Page et al., 2003). Primary infections have more numerous and scattered vesicles and more systemic symptoms. Up to 80% of individuals with primary infection develop constitutional symptoms. (Beauman, 2005). **Non primary first episode herpes infection** is usually mild, of shorter duration and is defined as the first clinical manifestation of HSV infection with IgG antibodies to the other type - for example new infection with HSV-2 with pre-existing infection and antibodies to HSV-1 (Ashley and Wald, 1999; Page et al., 2003; Beauman, 2005). Following initial infection, HSV becomes latent in the sensory ganglia and can reactivate

causing **recurrent infection** (Ashley and Wald, 1999). Classic recurrences usually are short-lived and present with few lesions that heal within 7 days.

Orofacial herpes infection. The incubation period ranges form 2 to 12 days. Primary symptomatic disease, gingivostomatitis, usually occurs in children and is characterized by vesicles and shallow ulcerations on the erythematous base on the buccal and gingival mucosa. Primary HSV oro-facial infection of adolescents and adults cause pharyngitis (Arduino and Porter, 2008). Fever, sore throught, difficulty to swallow liquids due to the pain, fever, malaise, headache, mialgia, submandibular lymphadenopathy are quite common. The episode lasts up to 2-3 weeks.

After primary infection some patients experience recurrences of HSV infection. Recurrent episodes usually are milder and of shorter duration than the initial episode. The majority of the patients report burning, itching, tingling few hours up to 2 days before lesions appear. Vesicles usually appear at the vermilion border of the lip and persist for 48 hours on average progressing to the pustular or ulcerative and crusting stage of the disease. The episode lasts up to 8-10 days. (Whitley and Roizman, 2009)

Genital herpes. After the incubation period of 3-7 days grouped papules and intraepidermal vesicles appear on external genitalia and ulcerate. Lesions can appear on buttocks, upper thighs, perianal region. In women herpetic cervicitis is quite common in primary episode of herpes infection (Gupta et al., 2007). Local symptoms include pain, itching and burning. More than half of the patients report fever, general malaise, headache, myalgias, tender inguinal and femoral lymphadenopathy, dysuria which can lead to urinary retention (Gupta et al., 2007). Primary genital herpes usually is more severe and more often associated with complications in women rather than men. This is due to anatomical features. Complications include extragenital symptoms and aseptic meningitis. Lesions heal after 2-3 weeks.

As in oro-facial HSV infection, recurrent episodes usually are less severe than the initial episode. (Beauman, 2005). Lesions heal in 5-10 days

without antiviral treatment (Gupta et al., 2007) Fever and constitutional syptoms are uncommon.

Recurrences can be provocated by various factors such as physical or emotional stress, fever, infection, exposure to heat, cold, ultraviolet light, menstruation, fatigue, nerve or tissue damage, sexual intercourse (Beauman, 2005)

The clinical spectrum of herpes infection ranges from **asymptomatic to severe disease** (Ryan and Kinghorn, 2006). Most primary infections are asymptomatic. Asymptomatic oro-facial HSV infection occurs twice as often as symptomatic disease. Progression of oro-facial HSV recurrences stop at the prodromal or papular stage approximately in 25% of cases (Tovaru et al., 2011).

Many individuals are unaware of having genital herpes. Only 20 percent of patients has typical clinical picture of genital herpes. Other 20 percent are truly asymptomatic and 60 percent of patients has atypical or unrecognized herpes which is diagnosed as other infections or dermatoses (Corey and Holmes, 1983). Atypical symptoms of genital herpes include fissures, localized erythema, linear ulcerations, excoriations (Gupta et al., 2007), vaginal and urethral discharge, back pain, dysuria, perianal, vulvar fissures (Wald, 2004).

HSV recurrences. In general, infection with both viruses can cause clinically indistinguishable lesions, however HSV-1 reactivates more frequently in the orofacial area compared with the HSV-1 genital infection. HSV-2, opposite to HSV-1, recurrs more often from genital area (Solomon et al., 2007) and recurrences from oral cavity are rare (Wald et al., 2004; Lafferty et al,. 1987).

The frequency of **orofacial** HSV-1 recurrences vary among individuals, usually in healthy persons recurrences are rare (Whitley and Roizman, 2009; Arduino and Porter, 2008). Savair et al. in a study of recurrent labial herpes found that 51.2 % of students had at least 2 recurrences annually (Sawair et al., 2010).

In **genital herpes**, about 40% of patients infected with HSV-2 had 6 or more recurrences per year and about 20% - 10 or more recurrences. (Benedetti et al., 1994). HSV-1 causes few or none recurrences after the first year of infection (Wald, 2006). Men tend to have more recurrences than women. The patients with prolonged and severe first episode of genital herpes have more recurrences (Benedetti et al., 1994). One-third of infected persons have eight-nine recurrences per year, one-third have two to three recurrent episodes and remaining one - third – between four and seven episodes (Whitley and Roizman, 2009). Over time episodes of active disease usually reduce in frequency (Benedetti et al, 1994).

Asymptomatic viral shedding – excretion of infectious virus from oro-facial or anogenital sites in the absence of the lesions or symptoms (Ashley and Wald, 1999; Miller and Danaher, 2008; Al-Dujaili et al., 2011). It frequently occurres both from oral mucosa/saliva and genital mucosa/skin (Lowagen et al., 2000; Al-Dujaili et al., 2011; Wald et al., 1995).

Miller and Danaher (Miller and Danaher, 2008) reported that at least 70% of the population shed HSV-1 asymptomatically in the oral cavity at least once a month. The mean duration of shedding was between 1 and 3 days. Oral HSV-2 shedding is rare and ususally occurs without clinical signs or can occur during new or recurrent genital HSV-2 infection (Wald et al., 2004).

In **genital herpes**, subclinical viral shedding is greater for HSV-2 than HSV-1 disease (Engelberg et al, 2003; Faber et al, 2011; Ashley and Wald, 1999; Pereira et al, 2012). Unrecognized infections (Malkin et al., 2004) and asymptomatic virus shedding are major factors in transmitting genital herpes to the sexual partners (Wald, 2004; Pebody, 2004; Ashley and Wald, 1999). Subclinical viral shedding was detected in more than 80% of HSV-2 seropositive persons who reported no lesions (Beauman, 2005). Only 10% - 25% of persons who are HSV-2 seropositive report a history of genital herpes, which suggests that most infected persons have unrecognized symptomatic or completely asymptomatic infections. However, once patients are informed about their seropositivity to HSV, more than 50% identify clinical symptoms

of recurrent genital herpes that previously were ascribed to other conditions (Wald et al., 2000). Wald et al. (Wald at al., 2000) reported that seropositivity for HSV-2 is associated with viral shedding even in persons with no history of genital herpes.

Neonatal infection. The major risk for fetus is primary genital infection of the mother. Such episode prior to 20 weeks‘ can lead to spontaneous abortion, meantime infection which develops later in gestation can cause neonatal HSV disease, severe intrauterine growth retardation. Neonatal HSV infection can be divided into three categories: a) localized skin, eye and mouth disease; b) encephalitis with or without skin lesions; c) disseminated disease with multiple organs involvment including CNS, liver, lungs, skin, eyes, mouth, adrenal glands and other organs (Whitley and Roizman, 2009).

Herpes simplex keratoconjunctyvitis usually is caused by HSV-1. It is associated with either unilateral or bilateral conjunctyvitis and preauricular lymphadenopathy; later on a geographic ulcer of the cornea can develop. Recurrent episodes usually involve one eye. Progressive disease can lead to opacification of the cornea, visual loss or even rupture of the globe (Whitley and Roizman, 2009).

Skin HSV infections. The most common skin infection caused by HSV is eczema herpeticum in persons with atopic dermatitis. Lesions can be localized as seen in herpes zoster or disseminated, as in Kaposi‘s varicellalike eruption. Herpetic whitlow, infection of the digits, mostly is common among medical personal. Herpes gladiatorum, disseminated HSV infection, is found among wrestlers. Darier‘s disease and Sezary syndrome are other skin diseases associated with extensive skin HSV infection. HSV can be a cause of erythema multiforme: in this case HSV DNA is found up to 80 % in skin lesions (Whitley and Roizman, 2009).

HSV infection in immunocompromised host. Patients after renal, hepatic, bone marrow, heart transplantation are at great risk to develop severe HSV infection most often involving respiratory and gastrointestinal tracts.

Reactivation in such persons can occur in multiple sites. Resistance to antiviral drugs can develop (Whitley and Roizman, 2009).

CNS HSV infection. Herpes simplex encephalitis (HSE) is the most common cause of fatal sporadic encephalitis which is characterized by severe destruction of temporal and frontal lobe tissue, including amygdala, limbic mesocortices and hypocampus. About 2/3 of HSE cases occur due to HSV reactivation from latency (Perng and Jones, 2010). HSV can involve all anatomic sites of nervous system, including meningitis, radiculitis. Patients with primary genital herpes quite often develop aseptic meningitis (Whitley and Roizman, 2009).

2.5. Laboratory diagnosis of herpes simplex infection

Because of the wide clinical spectrum of genital herpes, clinical picture, alone, usually is not sufficient to diagnose this infection therefore HSV isolation remains a definitive diagnostic method and laboratory confirmation is very important (Gupta et al., 2007; Corey and Holmes, 1983; Wald and Ashley-Morrow, 2002; Paz-Bailey et al., 2008).

HSV diagnosis could be established by detection of HSV in lesions by viral culture, HSV DNA polymerase chain reaction (PCR) and HSV antigen detection by direct immunofluorescence assay (IFA) or by enzyme immunoassay (EIA) (Gupta et al., 2007; Geretti, 2006). The Tzanck and Papanicolau cytological examination should not be used in diagnosing HSV infection due to modest sensitivity and specificity (Corey and Holmes, 1983). The presence of multinucleated giant cells and intranuclear inclusions is indicative of HSV infection, but not diagnostic (Whitley and Roizman, 2009).

Identification of the **type of HSV** is important for diagnosis of genital herpes (Ashley and Wald, 1999; Wald, 2006), appropriate clinical management (Solomon et al, 2003) and complete patient prognosis-counselling (Strick and Wald, 2006; Ramaswamy et al., 2005) because HSV-1

has a more favourable outcome than HSV-2 (Engelberg et al, 2003; Ryan and Kinghorn, 2006).

The sensitivity of any laboratory procedure for detecting herpes simplex virus infection depends on the stage of the disease (Corey and Holmes, 1983; Scoular et al., 2002)**.**

Viral culture, HSV inoculation onto human foreskin fibroblasts or Vero cells which are susceptible to HSV cytopathic effect, for a long time was used as the "gold standart" in detecting HSV (Slomka et al., 1998). But it's sensitivity is low, especially in crusted (up to 27%) (Scoular et al., 2002; Corey and Holmes, 1983; Geretti, 2006) and recurrent lesions (25-50%) (Gupta et al., 2007). Another problem is that this method is rather slow and needs special collection and transport conditions (Scoular et al., 2002; Gupta et al., 2007). The live HSV should be transported to the laboratory at 4ºC within a short time. The final result could be obtained within *5-7* days (Beauman, 2005).

Polymerase chain reaction (PCR), a method for direct detection of HSV by amplifying DNA sequences using HSV-1 or HSV-2 specific primers (Scoular et al., 2002).

The invention of PCR by Kary Mullis in 1984 was considered as a revolution in science (Deepak et al,. 2007)**.** Introduction of PCR into routine diagnostic has gained the dominant role in diagnosis of many disases (Drago et al., 2004), including HSV infection.

Real Time PCR has represented a further step forward - detection and quantification of the amplified DNA as the reaction progresses in real time (Drago et al., 2004). Real Time-PCR enables both detection and quantification of a targeted DNA molecule. This made a huge influence to the 21st century biological science due to its tremendous application in genetic variation of between and within organisms, quantitative genotyping, diagnosis of diseases (Deepak et al,. 2007) and was a new approach compared to standard PCR, where the product of the reaction was detected at its end. PCR detects only DNA, not infectious virus (Al-Dujaili et al., 2011).

HSV DNA PCR is proved to be be up to 4 times more sensitive than viral culture to detect HSV on mucosal surfaces (Wald, 2004; Filén et al., 2004) including asymptomatic viral shedding (Ryan and Kinghorn, 2006; Slomka et al., 1998; Wald et al., 2003; Filén at al., 2004; Wald, 2006). Compared with antibody serology tests, PCR is 1000-10,000 times more sensitive in detecting HSV (Al-Dujaili et al., 2011). PCR now is considered as a rapid, most sensitive (82%) and specific (78%) method for HSV diagnosis, including typing (Scoular et al., 2002; Strick and Wald, 2006; Al-Dujaili et al., 2011), useful in clinical as well as in epidemiological work (Slomka et al., 1998) and research.

Further PCR variants used in this study are discussed.

Nested PCR, a variant of PCR, is used to increase the specificity of DNA amplification. Two sets of primers are used in two successive reactions. In the first PCR, one pair of primers is used to amplify DNA products, which may contain products amplified from non-target areas. The products from the first PCR are then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

Nested PCR is especially uselful for detection of low copy number strains and previously was regarded as the gold standart for HSV detection in cerebrospinal fluid in CNS infecions. Later on, real time PCR was showed to be even more sensitive than nested PCR (Drago et al., 2004).

Touchdown PCR (step-down PCR), another variant of PCR in which the annealing temperature is gradually decreased in later cycles. The annealing temperature in the early cycles is usually 3-5°C above the standard melting temperature (Tm) of the primers being used. The annealing temperature progressively is decreased over the course of successive cycles while in the later cycles it becomes 5-10°C below the Tm. The initial higher annealing temperature leads to greater specificity for primer binding, while the lower temperatures permit more efficient amplification at the end of the reaction (Bachmann et al., 2003; Korbie and Mattick, 2008; Don et al., 1991).

Touchdown PCR is very useful in amplifying difficult templates (for example, G+C rich DNA) and can also be used to enhance specificity and product formation (Korbie and Mattick, 2008).

Direct immunofluorescence assay (IFA) and enzyme immunoassay (EIA). IFA detects viral antigen on smears, EIA – on swabs. IFA showed lower sensitivity and specificity in comparison with virus culture. (Geretti, 2006). EIA is higly specific but has lower sensitivity regarding viral culture and HSV PCR (Slomka et al., 1998).

Serologic assessment. HSV antibodies form from 2 weeks up to 3 month after infection (Gupta et al., 2007) and remain indefinitely (Beauman, 2005). Anti-HSV-2 antibodies are indicative for genital herpes. Anti-HSV-1 serotesting cannot differentiate between anogenital and orofacial herpes infection. (Beauman, 2005; Wald and Ashley-Morrow, 2002; Geretti, 2006) therefore type-specific serologic tests based on glycoprotein G should be the test of choice (Strick and Wald, 2006). Serology can be useful when lesions are negative or not sampled for virus (Song et al., 2006), when atypical herpes is suspected or lesions appear herpetic but may have other etiology; or to establish the diagnosis of HSV infection when no active lesion is present (Strick and Wald, 2006; Ashley, 2001). In the case of primary infection type-specific serology can help to distinguish between true primary and an initial episode of HSV infection (Munday et al., 1998).

2.6. The life cycle of HSV

The life cycle of neurotropic herpes simplex virus involves both lytic (productive) and latent (non-productive) infection (Kent et al., 2003).

The latency reactivation cycle is divided into three steps: establishment of latency, maintenance of latency and reactivation (Wagner and Bloom, 1997; Jones, 2003; Perng and Jones, 2010) (Figure 2).

Establishment of latency includes acute infection and virus entry into sensory neuron (Jones, 2003). Replication of virus is not essentially required for establishment of latency (Jones, 2003; Perng and Jones, 2010).

HSV usually is acquired by direct contact with the mucosa or the skin where replicates in the epithelial cells and undergoes typical lytic life cycle (Bloom et al., 2010; Held et al., 2011). After replication in the epithelial cells virus enters the nerve termini of the sensory neuron and travels intra-axonally to the sensory ganglia. After getting into neuron virus continues either productive (lytic) replication or repression of lytic genes and establishment of latency (Bloom et al., 2010). During latency the viral genome is present as a circular episome and is associated with cellular histones forming chromatin in latently infected neurons (Jones, 2003).

About one third of sensory neurons in a ganglion harbour latent HSV genomes (Bloom et al., 2010; Perng and Jones, 2010).

During **productive infection** more than 80 viral genes are expressed in a cascade pattern: the products of each class gene initiate the expression of the subsequent class (Bloom et al, 2010). Three classes or sets of lytic genes, immediate early (IE), early (E) and late (L) are distinguished. IE genes are expressed from 2- 4 hours post infection (Knipe and Cliffe, 2008). These include infected cell proteins (ICP0, ICP4, ICP22, ICP27, ICP47). All these proteins exept ICP47 have regulatory function. ICP4 represses IE gene expression and activates expression of most E and L genes. ICP0 activates gene expression of all classes of genes and is required for complete reactivation from latency with infectious virus production (Jones, 2003; Perng and Jones, 2010; Knipe and Cliffe, 2008).

HSV tegument protein, VP16, interacts with cellular factors, including the protein Oct-1 and HCF1, and enhances the transcription of IE genes. IE gene products activate expression of the E genes. The E gene products are involved in viral DNA replication, after which the L genes, structural and capsid associated genes, are expressed (Bloom et al, 2010; Perng and Jones, 2010; Knipe and Cliffe, 2008).

E genes are the target for currently available antiviral drugs which interfere with viral replication enhancing faster healing of the skin lesions and

lowering the chance of transmission to others. Reservoir of latent HSV is resistant to contemporary antiviral chemotherapy (Knipe and Cliffe, 2008).

Maintenance of latency. After establishment of latency in sensory neurons the virus persists for the lifetime of the host. The HSV genome exists in an episomal form within the nucleus in the neurons (Millhouse and Wigdahl, 2000). During latent infection no infectious virus is produced: viral gene expression is suppressed except latency associated transcript (LAT) (Bloom et al., 2010; Jones, 2003; Knipe and Cliffe, 2008). However, several studies showed minimal low expression of other genes (including ICP4, thymidine kinase) than LAT (Bloom et al., 2010; Jones, 2003; Held and Derfuss, 2011; Millhouse and Wigdahl, 2000). During this stage infectious HSV is not detectable by standart virus isolation methods (Perng and Jones, 2010). Host defense mechanisms are inefective to eradicate the infection (Millhouse and Wigdahl, 2000). Latent virus serves as a reservoir for transmission to other host following reactivation (Chen et al, 1995).

Reactivation can be provocated by various factors such as physical or emotional stress, fever, infection, exposure to cold, sunlight, immunosuppression, nerve or tissue damage and other stimuli which activate viral gene expression. (Jones, 2003; Perng and Jones, 2010). During reactivation viral lytic gene expression is abundant causing production of infectious virus, recurrent disease and virus transmission (Perng and Jones, 2010). It is not clear if neuron during reactivation of HSV survives or is killed (Perng and Jones, 2010) (Figure 2).

Establishment of latency

- Entry of the viral genome into a sensory neuron and acute infection
- Viral gene expression extinguished except for LAT gene
- **LAT inhibits apoptosis, productive infection**

Maintenance of latency

- Lasts for the life of the host
- HSV is not detected by standard virus isolation procedures
- Lytic genes required for acute infection are supressed
- **LAT is abundantly expressed in neurons, inhibits apoptosis and productive infection**

Reactivation

- External stimuli (stress, immunosuppression, etc.)
- Viral lytic gene expression occurs in a cascade (IE>E>L)
- Recurrent infection – virus transmission
- **LAT expression late in the productive cycle**

Jones C. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clin Microbiol Rev. 2003

Figure 2. Major steps during the latency-reactivation cycle

2.7. Latency associated transcripts

Maintenance of latency and reactivation is largely controlled via a special viral genetic region, the latency associated gene, which is abundantly transcribed during HSV latency and express a family of transcripts - Latency Associated Transcripts (LATs). HSV-1 and HSV-2 LATs are the major gene products in latently infected cells. Detection of LATs in a subpopulation of sensory neurons during the latency phase in murine ganglia in 1987 and observation that HSV genome is not totally quiescent during latency was the

first major discovery in understanding molecular aspects of HSV latency (Stevens et al., 1987; Rock et al., 1987; Millhouse and Wighdal, 2000).

HSV is a classical example of latent infection in humans and small animal models (Allen et al., 2011). HSV-1 and HSV-2 genetically are very similar (Bloom et al., 2010) but HSV-1 is studied better and serves as a model for both viruses (Wagner and Bloom, 1997). Due to HSV tropism to the neuronal cells it is difficult to investigate herpes simplex virus infection. Thus, most of the information about HSV latency is derived from small animal models of infection (Held et al, 2011). The mechanism by which LAT sequences influence establishment of and reactivation from latency is not fully understood (Jones, 2003; Cliffe et al., 2009).

Structure of LAT. The LAT is situated in the viral long repeats that flank the unique long region of the viral genome and is therefore present in two copies per genome (Perng et al., 2002; Wang et al., 1997; Krause et al., 1991). The primary LAT transcript (minor LAT) is 8.3 kb length and is unstable. Splicing yields a family of HSV-1 and HSV-2 LAT RNAs including a stable 2 kb LAT intron which is referred to as “stable” or “major” LAT and an unstable 6 kb LAT. In HSV-1, a very stable 2 kb LAT can be further spliced into a less abundant 1.5 kb LAT intron (Bloom et al., 2010; Jones, 2003; Rock et al., 1987; Millhouse and Wigdahl, 2000; Wang et al., 1995; Krause et al., 1991) (Fig. 3 shows a scheme of gene transcription). Correct splicing is important for establishment and maintenance of latency (Perng and Jones, 2010). The LAT region run in an antisense direction to the immediate-early gene transactivator ICP0, the HSV key neurovirulence factor ICP34.5 and the 3’end of the immediate-early gene transactivator ICP4 (Krause and Stanberry; 1995).

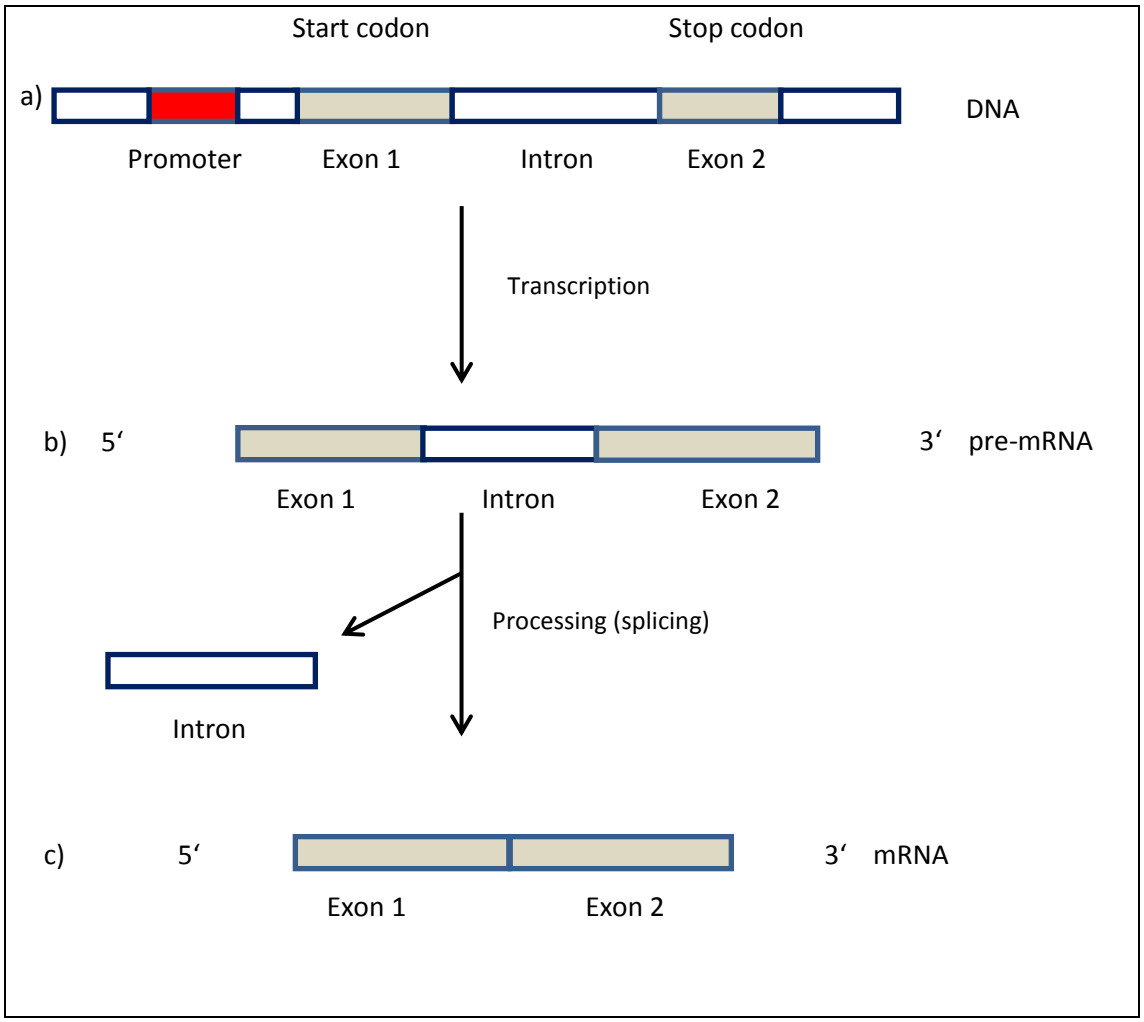


Figure 3. Schematic simplified mechanism of gene transcription
a) Genes consist of coding sequences (exons) and non coding sequences (introns). Promoter is a region of DNA that initiates transcription of a gene
b) During transcription the DNA is copied into a complementary messenger RNA which is called the primary transcript
c) RNA splicing (a modification of pre-RNA) removes introns from pre-mRNA and connects exons together

LAT promoter. The expression of LAT is under the control of the LAT promoter (LAP), which thus is a key for the establishment of herpesviral latency. The LAT promoter is a segment of DNA occurring upstream from a LAT gene coding region.

HSV-1 and HSV-2 are genetically related viruses and their LAT promoters show similar sequence elements, but the LATs sequences differ between both viruses (Yoshikawa et al., 1996).

Most data about the LAT promoter are known from studies of HSV-1 (Wang et al., 1995). Two active LAT HSV-1 promoters are distinguished: LAP1 and LAP2 (Chen et al., 1995, Goins et al., 1994). LAP1 promoter lies appoximately 700 bp upstream of the 5‘ end of the 2-kb LAT (Chen et al., 1995, Dobson et al., 1989). This classical promoter has a TATA box and is critical for LAT expression in latency (Wang et al., 1997; Millhouse and Wigdahl, 2000).

The second, putative, promoter, LAP2, lacks a TATA box and other features of traditional promoter, and is located between LAP1 and the LAT intron (Chen et al., 1995, Goins et al., 1993). LAP1 promoter is the principal promoter during latency, while LAP2 is more active during acute infection (Chen et al., 1995) or when LAP1 activity is suppressed (Farrel et al., 1994, Millhouse and Wigdahl, 2000). Sequence elements homologous to HSV-1 LAP1 and LAP2 have also been found in HSV-2 promoter region (Krause at al., 1991; Yoshikawa et al., 1996). A schematic presentation of the HSV-1 genome is shown in figure 4. This map is essentially the same for HSV-2.

Functions of LAT. As LATs are the only transcripts abundantly transcribed during latency, they were supposed to play an important role in establishing, maintaining latency and reactivating from latency. The LAT gene is expressed in latency and late in the productive cycle (Kent et al., 2003)(Figure 2).

LAT has several functions: suppresses lytic genes (ICP0, ICP4, ICP34.5) promoting assembly of heterochromatin on viral lytic-gene promoters, inhibits neuronal apoptosis (Perng and Jones, 2010; Knipe and Cliffe, 2008; Wang et al., 2005), affects the type of neurons in which HSV establishes latency (Bertke at al., 2007, 2009), plays a role in HSV virulence (Perng et al., 2002). Inhibiting of apoptosis correlates with promotion of spontaneous reactivation (Bloom, 2004).

Supression of viral lytic genes. It was shown that LAT downregulated viral lytic gene expression and replication during productive and latent infection (Wang and Zhou, 2005). A proposed mechanism of lytic

gene silencing is the association of heterochromatin with the viral genomes (Kent et al., 2003; Knipe and Cliffe, 2008; Cliffe et al., 2009). Besides, LAT is complementary to ICP0 and overlaps ICP0 transcript giving rise an idea that LAT inhibits ICP0 expression by an antisense mechanism (Jones, 2003; Perng and Jones, 2010).

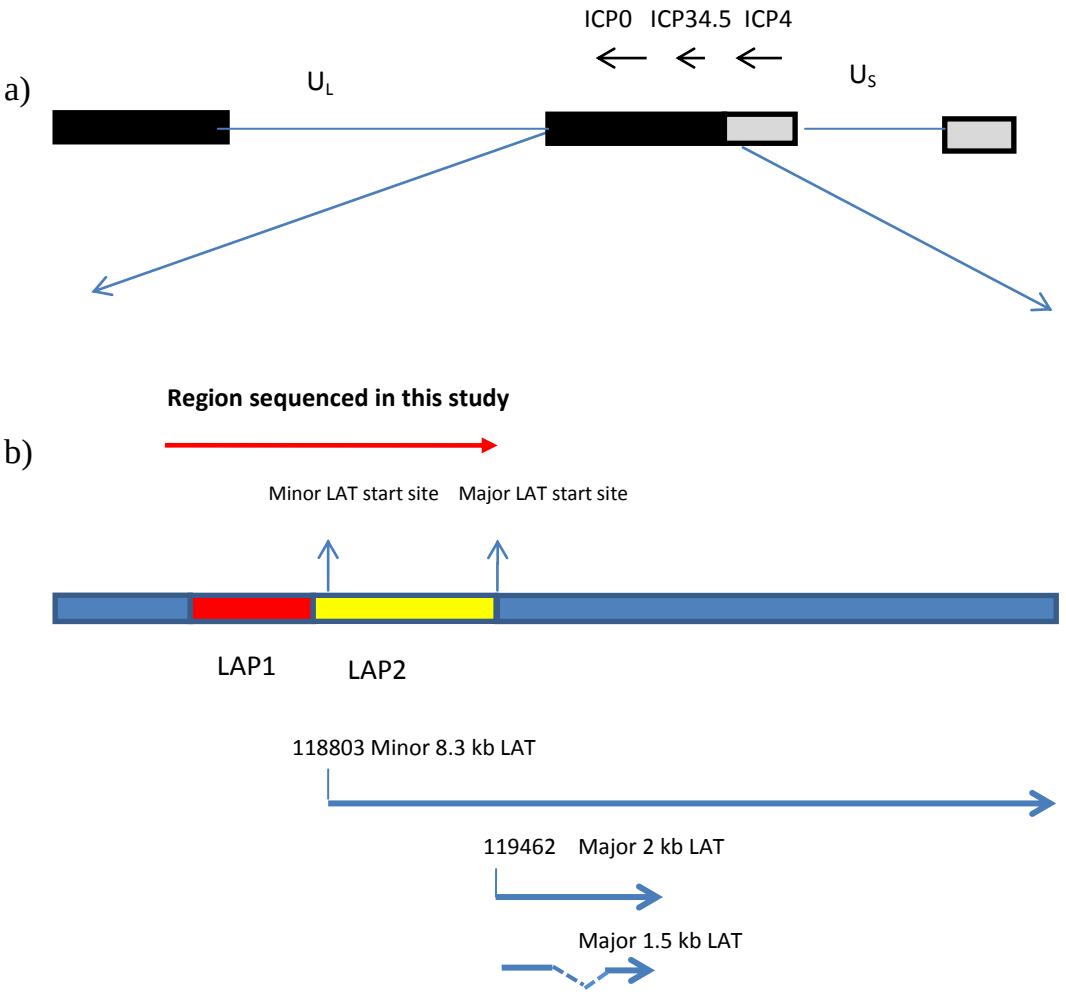


Figure 4. Schematic structure of the HSV-1 genome and the HSV-1 LAT region:
a) HSV genome is depicted at the top. U_L and U_S denote the unique sequences of the long (L) and short (S) components of the genome. Locations of the viral genes ICP0, ICP34.5 and ICP4 which are encoded on the oposite strand and overlap with the LAT. The boxes depict repeat sequences (Perng 2010)
b) The expanded region shows the repeat region. The map of the LAT region: LAT promoters LAP1 and LAP2. The primary 8.3 kb LAT transcript, 2 kb and 1.5kb LAT introns
Red arrow indicates the region sequenced in this study

The combination of DNA and proteins in nucleus form chromatin which is found in two forms: euchromatin and heterochromatin. Euchromatin is a less condensed form of chromatin that participates in the active transcription of DNA. Heterochromatin is a tightly packed form of DNA and is is the result of silenced genes (Cliffe et al., 2009).

The basic structural unit of chromatin is nucleosome, i.e. DNA wrapped around a complex of histone proteins (Gemayel et al, 2010). The positioning of the nucleosomes along chromatin at the promoter influences gene transcription by making DNA more or less accessible to transcription factors (Yuan et al, 2005). HSV DNA is not associated with histones inside the virion. Upon entry into the host cell nucleus, host cell mechanisms assemble chromatin on HSV DNA. During latency viral genome, as mentioned above, is circular and is associated with nucleosomes (Knipe and Cliffe, 2008).

HSV encodes gene products that modulate chromatin structure towards either euchromatin (active) or heterochromatin (inactive) (Wang and Zhou, 2005). It was hypothesized that HSV uses LAT gene to promote the formation of heterochromatin, to decrease the amount of active euchromatin on viral lytic-gene promoters and shut down the expression of these genes. Therefore, during latent infection the LAT gene is associated with "active" euchromatin whereas the lytic genes are associated with "inactive" heterochromatin (Knipe and Cliffe, 2008). This histone modification mechanism allows HSV to be in a quiescent latent form which is not detected by host immune system (Wang and Zhou, 2005). Downregulation of lytic genes leads to large number of latently infected neurons.

Inhibition of apoptosis. LAT inhibits apoptosis, a programed cell death. During apoptosis, host organism can eliminate cells which are infected by viruses or carry damaged DNA. That's why many viruses, including HSV, are able to block apoptosis during viral infection of host cells (Hay and Kanourakis, 2002). Studies showed that LAT can block the extrinsic caspase-8-dependent apoptotic pathway and the intrinsic caspase-9-dependent pathway, the two major apoptotic pathways in mammals (Bloom, 2004), as well as

caspase-3 activation by CD8 T cell lytic granules (Jiang et al., 2011). LAT also promotes neuronal survival by the induction of T cells exhaustion in latently infected mouse trigeminal ganglia. Exhaustion is a T cell response to long term exposure to the specific antigen. The exhausted T cells have significantly impaired proliferation and function to produce cytokines IL-2, TNF-α (Allen et al., 2011). The ability to block apoptosis is very important to the virus to survive during latency. When LAT is not present the cell continues with productive infection causing destruction of neurons and thereby reduce the pool of latent HSV genomes (Held and Derfuss, 2011; Kent et al., 2003).

Induction of spontaneous reactivation. Inhibition of apoptosis seems to be the most important function of LAT. The antiapoptotic activity of LAT correlates with promotion of spontaneous reactivation from latency (Jones, 2003; 12 Jiang et al., 2011). It is established that HSV-1 and HSV-2 LAT promoter mutants have lower levels of spontaneous reactivation rates than wild type HSV in rabbits (Perng et al. 1994, 1996), mice (Perng et al., 2001) and guinea pig (Krause et al., 1995) models.

Virulence. Studies have shown that a region within the 5‘ end of the primary 8.3-kb LAT transcript alters HSV virulence when is deleted (Zhu et al., 1999; Perng et al., 2001). Perng et al. (Perng et al., 2002) suggested that antisense to LAT (AL) transcript may encode a protein which could play a role in virulence of HSV.

Site specific reactivation. The clinical picture caused by HSV-1 and HSV-2 is similar whereas HSV-1 reactivates more efficiently from trigeminal ganglia, HSV-2 – from lumbor-sacral ganglia. HSV is a neurotropic virus and there is an evidence that HSV-1 and HSV-2 latency may be restricted to different subtypes of sensory neurons. A study performed by Bertke et al. (Bertke et al., 2009) showed that HSV-1 preferably established latency in neurons positive for A5 markers, whereas HSV-2 demonstrated a preferance to KH-10 neurons. It was supposed that LAT region may play a key role in this process (Bloom et al., 2010).

MicroRNAs encoded within the LAT region. Recently, in addition to LAT, several short microRNAs (miRNA) were found to be encoded within the primary LAT locus (Perng and Jones, 2003). MicroRNAs are a family of 21-24 nucleotides (nt) noncoding RNAs that regulate gene expression based on sequence similarity to their targets. Thus, in latently infected neurons HSV LAT encodes miRNAs that may enhance establishment and maintenance of viral latency by post-transcriptionally downregulating viral gene expression (Umbach et al., 2008).

HSV1 LAT genome region encodes for six miRNAs. Two of them (miR-H1 and miR-H6) are located in the promoter. One of miRNAs, miR-H6, inhibits ICP4 protein levels, another miRNA, miR-H2-3p, inhibits ICP0 proteins, i.e., supress these two important lytic genes (Perng and Jones, 2010). It was supposed that these miRNAs enhance the maintenance or establishment of latency but are not crucial for antiapoptotic effect (Held et al., 2011). Both miR-H3 and miR-H4-3p downregulate expression of ICP34.5, key viral neurovirulence factor, by an ”antisense” mechanism (Umbach et al., 2008).

Two small RNAs (s-RNA1 and s-RNA2) are encoded within HSV1 LAT sequences. These sRNAs are supposed to suppress ICP4 protein expression and inhibit apoptosis (Shen et al., 2009). These small LAT RNAs are not true micro-RNAs because the mature miRNA bands that migrate between 21 and 23 nt were not detected (Peng et al., 2008; Shen et al., 2009).

HSV2 LAT genome region encodes for 5 miRNAs. MiR-I/miR-H3 and miR-II/miR-H4 efficiently silence ICP34.5, a HSV key neurovirulence factor. MiR-III downregulates the expression of ICP0, a key viral transactivator (Tang et al., 2008, 2009). Recently, two novel HSV-2 miRNAs derived from the LAT, miR-H7 and miR-H9, were detected. These miRNAs are located on the strand opposite transactivator ICP0 (Umbach et al., 2010).

Other transcripts in HSV-1 LAT region. Several transcripts have been identified within HSV-1 LAT promoter and the first 1.5 kb of LAT coding sequences. The upstream of LAT (UOL) transcript is expressed in the LAT direction from the sequences of LAT promoter and is supposed to

encode a protein. The presice function of UOL is not known (Naito et al., 2005). In addition, the antisense to LAT transcript (AL) covers transcriptional start site and core LAT promoter, appears to encode a protein (Perng et al., 2002). Further examination revealed two antisense to LAT open reading frames (ORFs) (AL2 and AL3) within the first 1.5kb of LAT coding sequences. AL3 is expressed during productive infection and in trigeminal ganglia of latently infected mice. It is not known whether AL2 is expressed during productive infection or latency. It is not clear if AL2 and AL3 play a role in reactivation-latency cycle (Jaber et al., 2009; Perng and Jones, 2010) (Figure 5).

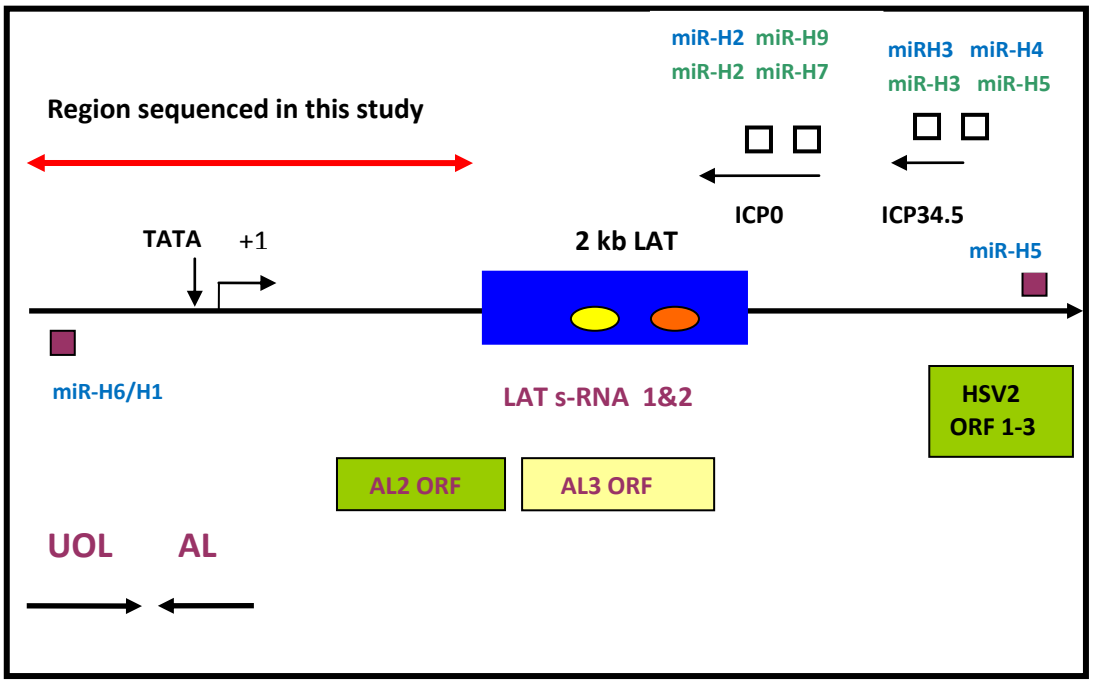


Figure 5. Expanded schematic diagram of the HSV-1 and HSV-2 LAT locus. The blue rectangle represents the stable 2 kb LAT intron. The arrow at +1 depicts the start of 8.3 kb primary LAT transcription. Red arrow indicates the region amplified and sequenced in this study

HSV-1. The positions of the UOL transcript, AL transcript, the two LAT small RNAs (s-RNAs) located within the first 1.5 kb of the LAT coding sequences and ORFs located on the opposite strand to LAT (AL2 and AL3) are shown

Micro-RNAs encoded in the HSV-1 LAT locus are written in blue (miR-H6/H1are encoded in promoter region)

HSV-2. Micro-RNAs encoded in the HSV-2 LAT locus are written in green. ORF1, ORF2 and ORF3 are also shown

Other transcripts in HSV-2 LAT region. Three ORFs have been detected in the HSV-2 LAT starting 750 bp dowstream 2kb LAT (Krause at el., 1991) (Figure 5).

HSV LAT, IE proteins and miRNAs are supposed to be the main viral factors in controling latency-reactivation (Held at al., 2011).

2.8. Repetitive DNA

Until 1970s DNA was thought to be a very stable entity with extremely low occurence of mutations. In the 1970s it was found that DNA is much more dynamic, especially due to a high degree of instability in repeated sequences (Ramel, 1997) which can be found in every genome (Ouyang et al, 2012), in both protein coding and non-coding regions (Usdin, 2008).

Tandem repeat is DNA sequence which forms a „unit“ that is repeated several times (i.e., in tandem) head to tail (Gemayel et al, 2010). The sequence CGACGACGA, for example, is a TR composed of trinucleotide repeat CGA repeated three times.

In general, two classes of repetitive DNA sequences are distinguished: repeats up to 1-9 bp (mostly defined as being 1-6 bp) generally known as microsatellites or simple sequence repeats (SSRs), or short tandem repeats, and those with 10-100 bp sequence repeats referred as minisatellites (Gemayel at el 2010; Kashi and King, 2006). Many of these DNA regions with repeated sequences show inter-individual hypervariability in their repeat number and also are called variable number of tandem repeats (Gemayel at el 2010; van Belkum et al, 1998). Homopolymer tracts (HPTs) form the most numerous class of repeated sequences in most genomes (Kashi and King, 2006) and are composed of variable number of one nucleotide repeat (for example, GGGGGG).

Tandem repeats previuosly were regarded as „junk“, i.e. non functional, DNA (Vinces et al., 2009; Kashi and King, 2006). Indeed, SSRs are extremely unstable (Gemayel at el., 2010) and are supposed to be hot spots

in length mutations (Davis et al., 1999). Rates of mutations are often up to 100,000 times higher comparing with average mutation rates in other parts of the genome (Gemayel at el., 2010). SSRs undergo more insertion-deletion mutations due to the changes of repeated units number comparing with nonrepetitive sequences.

The instability of repeated DNA mostly has been attributed to DNA polymerase slippage (Gragg et al., 2002). Homopolymer regions DNA polymerase can cause insertion/deletion errors by adding/omitting one or more extra nucloetides to/from a sequence. An insertion error occurs when DNA polymerase adds one or more extra nucleotides (+1, +2, +3, and so on) to a sequence; a deletion error is made when one or more nucleotides (−1, −2, −3, and so on) are omitted from a sequence (Figure 6).

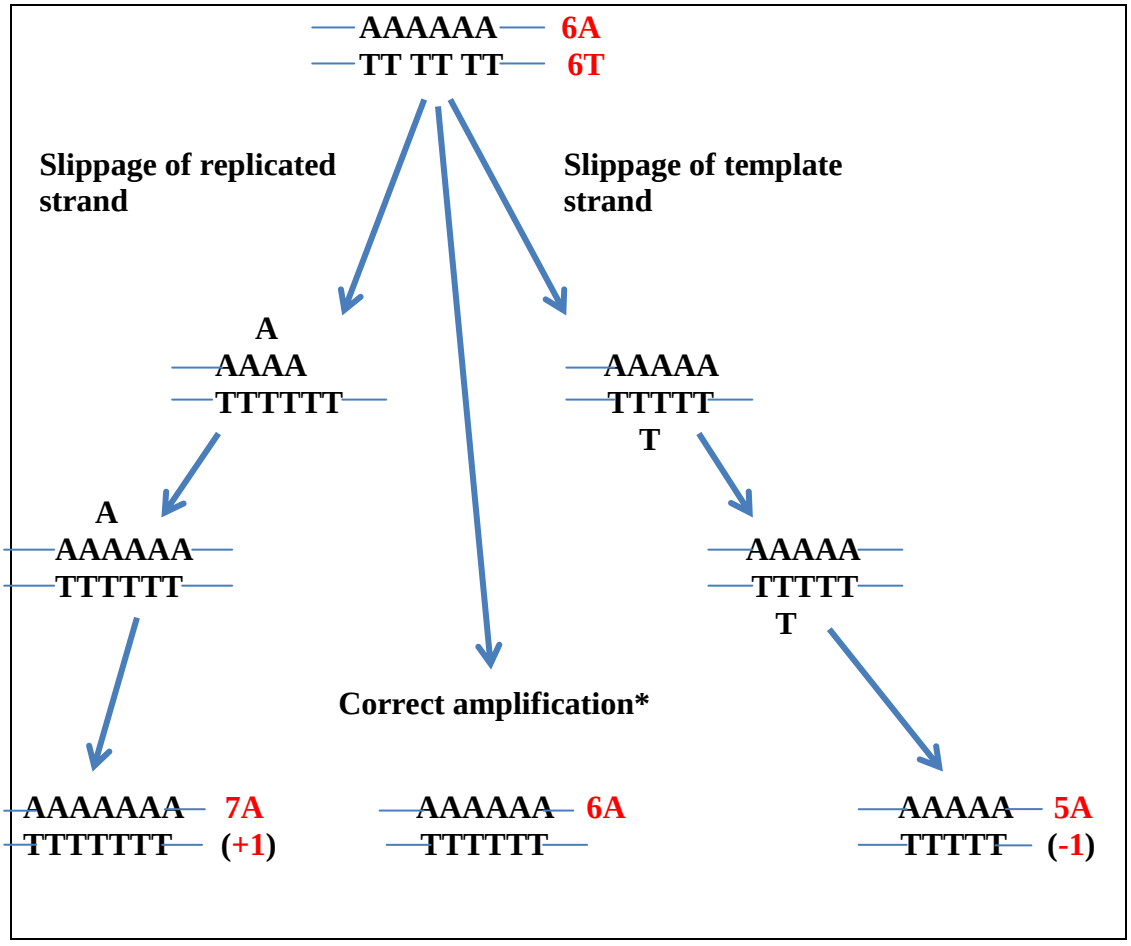


Figure 6. Slipped-strand mispairing: the figure shows a strand of DNA being carried through two rounds of DNA replication (except for correct amplification*)
Slippage is caused by mismatches between DNA strands while being replicated: deletions or insertions of repeat units occur which cause changes of length in sequence repeats

At the same time repeated sequences are prone to reversible changes of DNA (Davis et al 1999). The frequency of mutations depends on the number of repeat units, the length of the repeat unit and base composition. The longer and the purer the repeat tract, the more unstable it is (Gemayel at el 2010).

SSRs play an important role in evolution, regulation of gene expression, repetitive DNA can cause reversible phenotypic changes influencing rapid adaptation to changing environments, can affect structure and function of protein (Gemayel et al, 2010; Ouyang et al, 2012). For example, variability of SSRs in bacteria, especially pathogenic, were shown to influence phases variation, i.e. the reversible high frequency gain or loss of a phenotype which helps rapidly adapt to changing host enviroments (Martin et al., 2005). The expansion and shrinking of tandem repeats play a role in development of several cancers (stomach, lung, colorectal, etc.) (Zhang et al., 2001; Parsons et al., 1995), Alzheimer disease in humans (Linnertz et al., 2012; Lutz et al., 2010; Roses et al., 2010). Several human hereditary neurodegenerative disorders are caused by expansion of trinucleotide repeats. The best known of which is Huntington‘s disease where unstable $(CAG)_n$ repeats lead to the synthesis of the extended polyglutamine tract in the protein (Li et al., 2004; Ramel, 1997; Timchenko and Caskey, 1996).

Regulatory (promoter) regions also contain tandem repeats. Up to 10% - 20% of eukaryotic genes and promoters contain repetitive sequence. Hypervariable repeats in promoter regions affect gene expression and/or gene function therefore promote occurence of different phenotypes (Gemayel et al, 2010). Promoters with variable SSRs (changes in their lenght) show different gene expression within and between individuals. Often a particular size of tandem repeat is required for optimal gene expression: when repeat tract is longer or shorter, gene expression level is decreased (Vinces et al, 2009; Gemayel et al, 2010).

Ouyang et al. (Ouyang et al., 2012) analyzed the presence, location and composition of SSR tracts in HSV-1 genome. In contrast to many

other studied organisms (for example, *Drosophila*, *Arabidosis*, *C. elegans*, yeasts), investigators found that in all SSRs the G and C were very abundant as in all HSV-1 genome. The G+C content more than 90% was detected in mononucleotide repeats. The C/G repeats in HSV-1 were found more frequently in noncoding regions compared to coding sequences. Authors concluded that high G+C content and repeated sequences of HSV-1 genome could affect the genome structure and play a role in virus pathogenesis (Ouyang et al., 2012).

3. MATERIALS AND METHODS

The study protocol (2005-02-25 No7) was approved by the Lithuanian Bioethics Committee and informed consent (Appendix 1) was obtained from the subjects, prior to entry into the study.

The research was carried out at the Center of Dermatovenereology of Vilnius University Hospital Santariškių Klinikos (Lithuania) and Department of Medical Scienes, Section of Clinical Virology of Uppsala University (Sweden) in 2005-2012.

3.1. Study population

3.1.1 Study population of the clinical part of the study

The patients‘ group. Fifty-four patients with typical and atypical symptoms and signs suspected for genital herpes infection (vesicles, erosions, crusts, erythema, dysuria, vaginal discharge, soreness, itching) and 37 patients with lesions suspected for orofacial herpes attending the Center of Dermatovenereology of Vilnius University Hospital Santariškių Klinikos and during the period of June 2005 – March 2011 were enrolled into the study. This group of patients was divided into two subgroups: those having primary herpes and recurrent herpes infection on the basis of the patients’ history about presence or absence of previous episodes.

The control group was composed of fifty subjects without any complains and signs of genital herpes who attended the National Hospital of Tuberculosis and Infectious Diseases of Vilnius University for the purpose of vaccination during the period of August – September 2006.

3.1.2. Study materials of the experimental part of the study

Collection of clinical samples. In total, 146 clinical samples were collected. HSV samples from mucocutaneous lesions were obtained using cotton-tip swabs from 72 patients with suspected oro-facial or genital HSV infection in Vilnius University Hospital Santariškių Klinikos (Lithuania).

Seventy four HSV positive Swedish clinical samples were selected from samples submitted for diagnosis to the Department of Clinical Microbiology of the Uppsala University Hospital. The samples were from the Dermatological clinic of the Uppsala University Hospital (Sweden). Written approvals of this study by the chief of the Dermatological clinic, and by the physicians involved were obtained. Patients gave informed consent to the use of their samples for research and methodological development, according to the policy of the Uppsala University Hospital. The Swedish HSV samples were handled anonymously.

Among 72 patients from Vilnius, 32 HSV-1 samples were obtained from oro-facial and 6 from genital lesions, HSV-2 was detected in 34 genital swabs. Among 74 patients from Uppsala, 30 swabs from the oro-facial region and 6 swabs from genital region were positive for HSV-1, 34 swabs from genital region were positive for HSV-2. Four cerebrospinal fluid samples were taken from Uppsala patients with CNS infection: 1 sample was positive for HSV-1 and 3 were positive for HSV-2. HSV positivity was determined using quantitative real-time PCR (Figure 7).

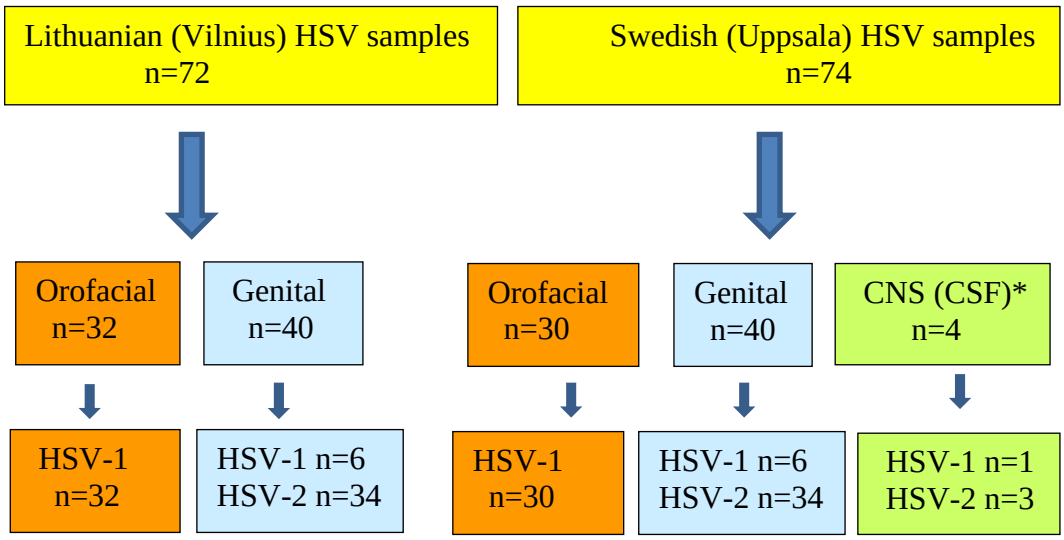


Figure 7. HSV samples (**n=146**) used in LAT promoter analysis.
*CNS – central nervous system, CSF – cerebrospinal fluid

3.2. Course of the research

The clinical part of the study (Lithuanian patients). At the time of the visit patient's history was taken, every patient underwent physical examination and sampling for nucleic acid detection. Quantitative real time PCR (qPCR) was performed for the confirmation of the diagnosis of HSV infection and quantification of virus in the genital lesions. Blood samples for HSV serology were drawn from both patients and controls and questionnaire survey was done.

The experimental part of the study. The analysis of LAT promoter was performed using HSV strains detected in clinical samples of both Lithuanian and Swedish patients.

3.3. Methodology of the study

3.3.1. Methods of the clinical part of the study

Lithuanian patients participated in the clinical part of the study.

Specimen collection. Samples for quantitative PCR from the skin lesions were obtained using cotton-tip swabs. Two specimens from each patient were taken: „wet" swab was inserted into a tube containing 0.4 mL 10

mmol/L Tris-HCL buffer with 10 mmol/L EDTA and kept frozen at -20°C, „dry“ one was kept in an empty tube at room temperature until DNA extraction. It has previously been shown that the user-friendly collection and storage of a simple cotton swabs allows HSV detection (Filén et al., 2004) and this technique was applied in this study. „Dry“ swabs from genital and orofacial lesions were kept as a reserve if repetitive or additional HSV analysis would be needed.

DNA detection. DNA extraction was performed using the QIAgen DNA blood mini kit (QIAgen, Hilden, Germany, Cat. No 51306) according to the manufacturer‘s instructions.

Quantitative real-time PCR for herpes simplex virus detection and typing was performed as previuosly described by Filén et al. (Filén et al., 2004) using RotorGene 2072 thermal cycler (Corbett Research, Northlake, Australia). Briefly, the final reaction volume was 50 µl (TaqMan Universal PCR Master Mix was used (Applied Biosystems, Foster City, Canada, Cat. No 4304437)). Ten microlitres of extracted DNA was added to each PCR tube. Cycling conditions were: 50°C for 3 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 57°C for 60 seconds.

Serology. Blood samples from both patients and subjects of the control group were collected for anti-HSV-1 IgG and anti-HSV-2 IgG serology in order to distinguish first episode of HSV infection from recurrence and to detect asymptomatic carriers of genital herpes infection. Sera were kept at -20°C till analysis. HSV-1 and HSV-2 antibodies were analyzed by ELISA assay (HerpeSelect®, Focus Diagnostics, California, USA, Cat. No EL0910G and Cat. No EL0920G) according to the manufacturer’s instructions.

Questionnaire. All participants were asked to fill in an anonymous questionnaire which included questions on socio-demographic data, history of herpes infection, history of sexually transmitted infections (STI) and sexual habits (Appendix 2). Questionnaire was mostly important for evaluation of risk factors for genital herpes and rate of recurrences of HSV infection.

3.3.2. Methods of the experimental part of the study

Isolation of genomic DNA and quantitative real-time PCR from Swedish samples were performed as described in the „Methods of the clinical part of the study".

Amplification of HSV LAT promoter sequences. The LAT gene promoter region was amplified from HSV DNA obtained from the patients' mucocutaneous lesions and cerebrospinal fluid. For analysis of the LAT promoter an array of PCRs which cover this region of both HSV-1 and HSV-2 has been developed. Primers for amplification and sequencing were analyzed by OLIGO Analyzer 1.0.2. This program was not suitable for designing degenerative primers therefore the final results were completed by hand. All primers were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific GmbH, Ulm, Germany). All reverse primers are written as they were purchased, as the inverse complement. HSV-1 and HSV-2 primer sequences presented in tables 1 and 2. The primers were designed following the target sequences presented in GenBank (GenBank accession No NC_001806 for HSV-1, No NC_001798 for HSV-2).

To amplify the LAT region a touchdown hot start PCR was performed. The PCR amplification was carried out in a final volume of 25 µl containing 300 nm of each primer, 0.2 mM dNTPs, 1xQ solution and 1.25 U Hotstar Taq polymerase in 1xPCR buffer (QIAgen, Hilden, Germany, Catalog No 203203). The cycling conditions were: 15 min of preincubation at 95°C, 12 cycles with 30s denaturation at 98°C, 45s annealing with a progressively lowered temperature from 67°C to 56°C at a rate of 1°C every cycle and a primer extension of 1min 30s at 72°C, followed by 25 additional cycles with an annealing temperature of 57°C. The reaction mixture then was incubated for 5 min at 72°C for a final extension followed by indefinite cooling at 4°C.

For low HSV copy numbers HSV1 and HSV2 samples nested touchdown hot start PCR was performed using described primers under the same cycling conditions. In the first PCR 5 µl of purified HSV DNA was

added to a final volume of 25 µl. In the second PCR 2 µl of PCR product, from the initial run, was added to a final volume of 25 µl.

Table 1. Primers used for **HSV1 LAT region with promoter** amplification and sequencing

Primer (nt position)	Function	Type	Sequence (5´→3´)
117970-117990	1st PCR, seq	F	**GCAAGAACAGACACGCAGAAC**
119635-119615	1st PCR, seq	R	**CGGACGGGTAAGTAACAGAGT**
117982-117999	1st PCR, 2nd PCR, seq	F	**ACGCAGAACGGCTGTGTT**
118142-118160	1st PCR, 2nd PCR, seq	F	**CCAGTCTCCTCGCCTTCTC**
118768-118749	2nd PCR, seq	R	**AGATGACGCAGCAAAAACAG**
118631-118648	2nd PCR, seq	F	**TGCCCGCGAGATATCAAT**
119581-119562	1st PCR, 2nd PCR, seq	R	**GCTGGTGTGCTGTAACACGA**
118252-118273	Seq	F	**GATCCCGACAACAATAACAACC**
118279-118258	Seq	R	**CGTTGGGGTTGTTATTGTTGTC**
118347- 118334	Seq	R	**ACTTCCACTTCCCGTCCTTC**
118543-118523	Seq	R	**CGGCCACATAAACAATGACTC**
118749-118768	Seq	F	**CTGTTTTTGCTGCGTCATCT**
119018-119038	Seq	F	**GGAATGTTTCGTTCGTCTGTC**
119183-119202	Seq	F	**GATRAACAMTCGGGGTTACC**
119202-119183	Seq	R	**GGTAACCCCGAKTGTTYATC**
119288-119303	Seq	R	**AAACCACAGCGCATGC**
119480 -119459	Seq	R	**GCAGGTGTCTAACCTACCTGGA**

Notes: Sequences are shown as ordered for synthesis. IUPAC ambiguity codes are used for degenerative positions where Y is C or T, M is A or C. Sequences are from the HSV1 reference strain 17 (accession number NC_001806). nt, nucleotide; Seq, sequencing; F, forward; R, reverse

Table 2. Primers used for **HSV2 LAT region with promoter** amplification and sequencing

Primer (nt position)	Function	Type	Sequence (5´→3´)
119022-119041	1st PCR, 2nd PCR, seq	F	**AAAGCAACGGGAAAGAGAGG**
120357 -120340	1st PCR, seq	R	**CCGCCTCGTGTTTCTTGA**
119189-119209	2nd PCR, seq	F	**CCTGTGTCATTGTTTACGTGG**
119432-119451	2nd PCR, seq	F	**GTGTTGYTGTGGGCATTTCT**
120065-120045	2nd PCR, seq	R	**GAAATGTGAGGAGAGCGAGAC**
120260-120222	2nd PCR, seq	R	**CTCAGCAGAAGCTCACCTGTA**
119463-119445	Seq	R	**GGATGACGCAGCAGAAATG**
119359-119376	Seq	F	**CTGAGTGCTCTGCGGTTG**
119783-119801	Seq	F	**CGGAGGCTTTTTTMGATTC**
119804-119786	Seq	R	**CGGGAATCGAAAAAAGCCT**

Notes: Sequences are shown as ordered for synthesis. IUPAC ambiguity codes are used for degenerative positions where Y is C or T, M is A or C. Sequences are from the HSV2 reference strain HG52 (accession number NC_001798). nt, nucleotide; Seq, sequencing; F, forward; R, reverse

The PCR products were separated on a 1.5% agarose gel in 0.5xTBE buffer (45 mM Tris-borate 1 mM EDTA, pH 8), stained with

ethidium bromide at 100 V for 1h and visualized by UV radiation. DNAs were isolated using a PCR purification (QIAgen, Hilden, Germany, Catalog No 28104) or gel extraction kit (QIAgen, Hilden, Germany, Catalog No 28704). The latter was used if several DNA bands on gel were present and the band of expected size was excised from the agarose gel.

Sequencing of HSV LAT promoter region. Sequences of amplified DNA were determined using the fluorescent dye terminator method, ABI PRISM® Big Dye Terminator Cycle Sequencing v3.1 Ready Reaction kit (Applied Biosystems, Foster City, CA, USA, catalog No 4337455). The sequencing PCR reaction mixture was prepared according to the manufacturer‘s recommendations except for the addition of 4 µl 1M betaine (Sigma Aldrich Sweden AB, Stockholm, Sweden, catalog No B0300) per reaction. The amplification reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) under following conditions: initial denaturation at 96°C for 2 min and 35 cycles of denaturation at 98°C for 20s, annealing at 50°C for 10s and extension at 60°C for 4 min. The cycle sequencing was followed by ethanol purification according to the manufacturer’s instructions. Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Ca, USA) or at the Uppsala Genome Center on an ABI 3730XL DNA Analyzer (Applied Biosystems). Selected PCR products were sequenced directly using one amplification primer.

Cloning of HSV LAT promoter sequences. Difficult to sequence amplified LAT promoter GC-rich regions were cloned into TOPO®TA vectors and transformed into competent Escherichia coli cells (TOPO 10) using the TOPO™TA Cloning kit (Invitrogen, Stockholm, Sweden, Catalog No K450001). The recombinant plasmids were isolated using QIAprep® Spin Miniprep Kit (QIAgen, Hilden, Germany, Catalog No 27104) according to the manufacturer‘s instructions. The concentration of plasmid DNA was quantified by using the NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). All inserts were sequenced using the ABI PRISM®

Big Dye Terminator Cycle Sequencing v3.1 Ready Reaction kit as described above.

Sequence alignment and phylogenetic analysis. The sequencing data were analyzed using DNA Baser v2.80.0 (HeracleSoftware, Lilienthal, Germany). The sequences were either aligned using the ClustalW2, ClustalW1.83 or MUSCLE programs. Cladograms were generated from a Clustal W1.83 alignment, according to the nearest-neighbor algorithm, and displayed with the help of TreeView (Page, 1986). Phylograms "trees" were constructed after MUSCLE (Edgar RC, 2004) alignment and distance calculation according to the minimum evolution algorithm [implemented in the MEGA 5.1 package, (Tamura et al., 2011]). The robustness of the trees was tested by 1000 bootstraps. The bootstrap percentage of occurrence of a branch is shown next to it.

In an attempt to display the degree of similarity of aligned nucleotide sequences, plots were created using SimPlot program (Lole et al., 1999) by using a moving window of 40 nucleotides. The window was moved in steps of 10 nucleotides.

3.4. Statistical analysis

All different data collected from each patient were analyzed using descriptive statistics. Descriptive statistics of continuous variables were expressed as mean ± SD (standart deviation). For comparison of two groups in respect of a continuous variable t -test or non - parametric Man - Whitney test (in case of the asymmetric distribution) was applied. For the analysis of categorical data χ^2 or Fisher‘s exact test was used.

Bivariate multiple logistic regression method was applied for the assessment of relation between genital herpes and risk factor. The variable indicating the disease (genital herpes) was dependent variable, genital herpes was considered as an event. The variables were selected into the model when the obtained p value was <0.25. However, clinically important variables were included into the model despite their statistical significance. The purpose of

this was to ensure control of confounding factors. χ^2, Hosmer-Lemshov test, classification table were used in the model for the assessment of the compatibility of the model. The significance of the coefficient β was assessed by Wald test.

The statistical significance level was chosen $\alpha=0.05$, the results were regarded as statistically significant when $p\leq0.05$.

Statistical analysis was performed on SPSS 19.0 software (version for windows).

4. RESULTS

4.1. Clinical part of the study

The diagnosis of herpes simplex infection was based on PCR results and serological evaluation. Patients with confirmed HSV diagnosis were compared with the group of controls.

4.1.1. Genital HSV infection

Diagnosis of genital HSV infection. Out of 54 patients with suspected genital herpes, genital HSV infection was diagnosed in 83.3% (45/54) patients: 74.1% (40/54) patiens had a positive PCR for herpes simplex virus; 9.2% (5/54) patients had negative PCR but anti-HSV-2 IgG antibodies, indicative for genital herpes, were detected in their sera.

4.1.1.1. Characteristics of the population

The patients group with confirmed diagnosis of genital herpes consisted of 17 (37.8%) males and 28 (62.2%) females, mean age 35.7±12.3. The control group of 50 subjects consisted of 15 (30%) males and 35 (70%) females, mean age 39.4±10.3.

Table 3 shows demographic data of the patients and controls. The majority of the patients and controls were married or cohabiting, had university

education and were employed. Both study groups were similar regarding gender, age, education and social status.

Table 3. Socio-demographic characteristics of the patients with genital HSV infection and controls

	Patients with genital HSV infection	Controls	p value
Overall	n=45	n=50	
Sex:			
1. Male	17 (37.8 %)	15 (30 %)	0.516[1]
2. Female	28 (62.2 %)	35 (70 %)	
Age:			
1. Mean	35.7±12.3	39.4±10.3	0.122 [2]
2. Range	18-65	20-58	
Education:			
1. University	24 (53.4 %)	22 (44 %)	0.103[1]
2. College	6 (13.3 %)	16 (32 %)	
3. Secondary	15 (33.3 %)	12 (24 %)	
Marital status:			
1. Single			
2. Married or cohabiting	18 (40 %)	9 (18 %)	0.208[1]
3. Widowed or divorced	18 (40 %)	33 (66 %)	
	9 (20 %)	8 (16 %)	
Social status:			
1. Employed	33 (73.3 %)	46 (92 %)	0.081[1]
2.Unemployed	4 (8.9 %)	2 (4 %)	
3. Student	6 (13.3 %)	2 (4 %)	
4. Pensioner	2 (4.5 %)	0 (0 %)	

[1]Fisher's exact test, [2]t-test

4.1.1.2. Risk factors for genital herpes

Table 4 shows the most significant risk factors for genital herpes. Patients as compare to controls had higher number of lifetime sexual partners, reported more cases of STI, oral sex and were younger age at first intercourse.

In spite of there was no significant difference between gender among the patients and the group of controls this variable was included in the evaluation of risk factors as females are more prone to acquire genital herpes than men. Our data showed that there was strong association between genital herpes and history of other STIs and oral sex. History of other STIs incresases the risk of genital herpes almost 8 times (OR 7,76; 95% CI 2.53 - 23.79), oral

sex – 6,6 times (OR 6,57; 95% CI 2.47-14.47). Age at first intercourse was divided into 4 categories. Obtained data showed that the patients in the age age groups of ≤16 and 17-18 years were significantly younger at sexual debut as compare to controls. The number of sexual partners was divided into 4 categories and only the category of 10 or more lifetime partners showed signifficant association with genital herpes infection (OR 5.38; 95 % CI 1.36 - 21.30).

Level of education or socioeconomic status were not included in the evaluation of risk factors as there was no significant difference between the group of the patients and the controls.

Condoms were used more frequently in the group of controls comparing with the patients (19/50 or 38% vs. 9/45 or 20%, p<0.001). The frequency of reported contacts with sexual workers was similar in both study groups: 3 out of 45 (7%) patients and 4 out of 50 (8.0%) controls, p=1.000.

Table 4. Characteristics of the possible risk factors for genital herpes

	Crude OR (95% CI)	p	Adjusted OR (95% CI)	p
Gender	1.42 (0.60-3.33)	0.424	1.07 (0.39 - 2.97)	0.891
History of STI	7.01 (2.84-17.34)	0.000	7.76 (2.53 - 23.79)	0.000
Oral sex	7.07 (2.86-17.47)	0.000	6.57 (2.47-14.47)	0.000
Age at first intercourse				
≥22	1.0	0.005	1.0	0.023
≤16	12.00 (2.16 - 66.55)	0.004	9.11 (1.42 - 58.46)	0.020
17-18	8.55 (2.27-32.22)	0.002	8.32 (1.96 - 35.37)	0.004
19-21	3.32 (0.92 -11.98)	0.067	3.68 (0.91 - 14.89)	0.068
Lifetime number of sexual partners				
1	1.0	0.047	1.0	0.067
2-4	1.40 (0.46 - 4.23)	0.554	1.30 (0.42 - 3.99)	0.651
5-9	3.10 (0.82 -11.44)	0.096	2.38 (0.60 - 9.43)	0.218
≥10	5.57 (1.42 -21.86)	0.014	5.38 (1.36 - 21.30)	0.016

OR odds ratio
Adjusted OR - odds ratio djusted for gender, history of other STIs, age at first intercourse, oral sex, number of lifetime sexual partners
CI - confidence interval

According to the questionnaire data history of labial herpes didn't show any difference between the two study groups: approximately half of the patients (22/45 or 48.9%) and the controls (28/50 or 56%; p=0.541) reported episodes of labial herpes. History of genital herpes was reported by 2 (4%) subjects of the control group.

4.1.1.3. Serological assesment

Sera samples from all study participants were tested for anti-HSV-1 IgG and anti-HSV-2 IgG antibodies using ELISA tests. Anti-HSV1 IgG antibodies were detected in majority of patients and controls. Seropositivity for HSV-2 was more frequent in patients than in controls, and this difference was significant (Table 5).

Table 5. Type specific HSV serology in patients with genital HSV infection and controls

	Patients with genital HSV infection	Controls	p value[1]
Overall	n=45	n=50	
Anti-HSV1 IgG serology:			
Positive	39 (86.7 %)	45 (90 %)	0.751
Negative	6 (13.3 %)	5 (10 %)	
Anti-HSV2 IgG serology:			
Positive	31 (68.8 %)	7 (14 %)	0.000
Equivocal	1 (2.2 %)	0 (0 %)	
Negative	13 (28.8 %)	43 (86 %)	

[1]Fisher's exact test

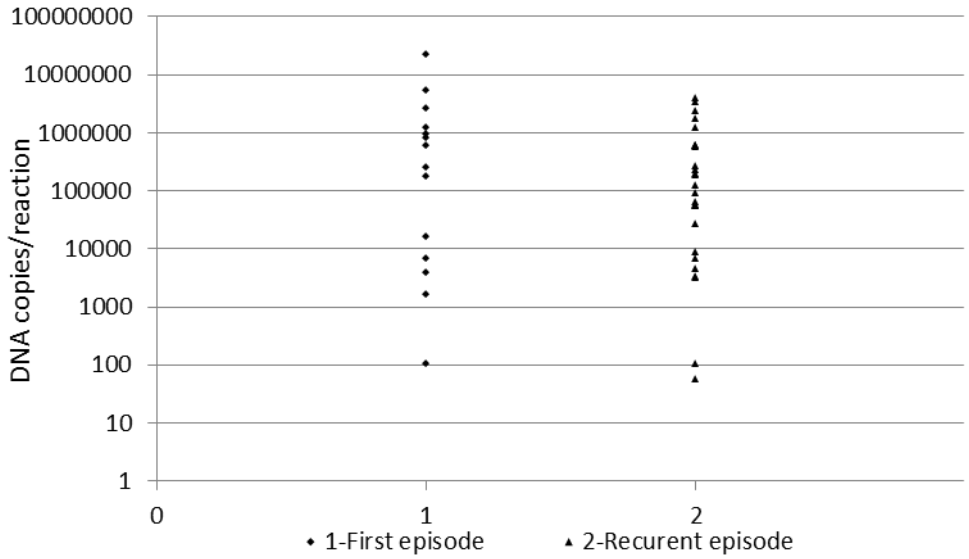
4.1.1.4. Clinical picture, HSV detection and serology

„Wet" swabs from genital lesions were analyzed with quantitative real-time PCR. HSV DNA was detected in 40 (74.1%) patients: in 14 (35%) males and 26 (65%) females. Most of the swabs (85%) were positive for HSV-2. Only 6 swabs (15%) were positive for HSV-1 (Table 6).

Fourteen patients (35%) with positive PCR reported **first episode** of genital herpes lasting from 1 day up to 3 weeks. Four patients (29%) were positive for HSV-1, 10 (71%) patients – for HSV-2.

Twenty-six patients (65%) presented with **recurrent** HSV infection: HSV-1 was detected in 2 (8%) patients, HSV-2 – in 24 (92%) patients. The duration of recurrent lesions varied from 1 to 14 days. The amount of virus in recurrent lesions ranged from 56 to $3.9x10^6$ (median $1.2x10^5$) copies per PCR reaction but the quantity of HSV was lower than during first episode (range 106-$2.2x10^7$, median $2.6x10^5$, Mann-Whitney U test: U=154, p=0.535) (Figure 8).

Figure 8. Number of HSV copies per qPCR reaction in genital lesions



Almost all patients (97.5%) with positive qPCR had vesicles and/or ulcers at the time of sampling. More than half of the patients (65%) had symptoms for less than 5 days. Meanwhile, in the group of the patients with negative PCR 7 (50%) persons presented with atypical symptoms with no signs of ulceration (erythema, dysuria, discharge, soreness, itching) and 12 (85.7%)

patients suffered from lesions for more than 5 days. Meanwhile, in the group of the patients with negative PCR 3 (60%) persons presented with ulceration and all 5 patients suffered from lesions for more than 5 days.

Out of 40 patients with positive DNA, 26 (65%) were HSV-2 seropositive. Five patients with negative HSV DNA had antibodies to HSV-2 (Table 6).

Table 6. Clinical aspects of the patients with suspected genital HSV infection

	Patients with positive PCR	**Patients with negative PCR**
Overall	n=40	n=5
Sex: 1. Male	14 (35 %)	2 (40 %)
2. Female	26 (65 %)	3 (60 %)
Age:		
1. Mean	36.33±12.59	31.0±9.3
2. Range	18-65	21-47
Disease:		
1. First episode	14 (35 %)	3 (60 %)
2. Recurrence	26 (65 %)	2 (40 %)
Vesicles, ulcers:		
1. Yes	39 (97.5 %)	3 (60 %)
2. No	1 (2.5 %)	2 (40 %)
Duration of the onset :		
1. < 5 days	26 (65 %)	0 (0 %)
2. ≥ 5 days	14 (35 %)	5 (100 %)
Type of virus detected in genital lesions		
HSV-1	6 (15%)	
HSV-2	34 (85%)	
Anti-HSV1 IgG serology:		
Positive	34 (85%)	5 (100%)
Negative	6 (15 %)	0 (0%)
Anti-HSV2 IgG serology:		
Positive	26 (65%)	5 (100%)
Equivocal	1 (2.5%)	0 (0%)
Negative	13 (32.5%)	0 (0%)

4.1.1. Orofacial HSV infection

The main focus of the clinical part of this study is on genital HSV infection. HSV-1 samples were collected for LAT promoter studies though here we present the most important data regarding orofacial HSV infection.

Diagnosis of orofacial HSV infection. Out of 37 patients with suspected orofacial HSV infection HSV was detected in 32 (86.5%) samples. Serology test for anti-HSV-1 IgG was positive in 35 patients. Two patients with negative PCR reported recurrent episode although they both were seronegative (both to HSV-1 and HSV-2). Therefore the diagnosis of orofacial HSV infection was established in 35 patients.

4.1.2.1. Characteristics of the population

Among the 35 patients with diagnosed orofacial herpes infection 12 (34%) were male and 23 (66%) were female, mean age 39.1±15.7 (range 19-75). Socio-demographic data are compared with the group of controls and are shown in table 7. There was no significant difference regarding socio-demographic data between the patients and the control group.

Table 7. Socio-demographic characteristics of patients with orofacial HSV infection and controls

	Patients with orofacial HSV infection	Controls	p value
Overall	n=35	n=50	
Sex:			
1. Male	12 (34 %)	15 (30 %)	0.813[1]
2. Female	23 (66 %)	35 (70 %)	
Age:			
1. Mean	39.1±15.7	39.4±10.3	0.931[2]
2. Range	19-75	20-58	
Education:			
1. University	21 (60 %)	22 (44 %)	0.087[1]
2. College	4 (11.4 %)	16 (32 %)	
3. Secondary	10 (28.6 %)	12 (24 %)	
Marital status:			
1. Single			
2. Married or cohabiting	14 (40 %) 16 (45.7 %)	9 (18 %) 34 (68 %)	0.077[1]
3. Widowed or divorced	5 (14.3 %)	7 (14 %)	
Social status:			
1. Employed	27 (77.1 %)	45 (90 %)	0.057[1]
2.Unemployed	4 (11.4 %)	2 (4 %)	
3. Student	1 (2.9 %)	2 (4 %)	
4. Pensioner	3 (8.6 %)	1 (2 %)	

[1]Fisher's exact test, [2] t-test

4.1.2.2. Serological assessment

The majority of the patients with orofacial herpes and controls were seropositive for HSV-1 (97.1% vs. 90%). Both anti-HSV-1 IgG anti-HSV-2 IgG antibodies were detected in 1 patient and 7 controls (table 8).

Table 8. Type specific HSV serology in patients with orofacial HSV infection and controls

	Patients with orofacial HSV infection	Controls	p value[1]
Overall	n=35	n=50	
Anti-HSV1 IgG serology:			
Positive	34 (97.1 %)	45 (90 %)	0.393
Negative	1 (2.9 %)	5 (10 %)	
Anti-HSV2 IgG serology:			
Positive	1 (2.9 %)	7 (14 %)	0.133
Negative	34 (97.1 %)	43 (86 %)	

[1]Fisher's exact test

4.1.2.3. Clinical picture, HSV detection and serology

More than half (68.8%) of the patients with positive qPCR in orofacial swabs had vesicles or ulcers at the time of sampling. Symptoms duration varied from 1 to 4 days; except one patient who had labial herpes for 5 days. All 32 individuals with positive PCR sufferred recurrent episode, except one patient. Although this patient was seropositive to HSV-1 therefore current episode was treated as the first symptomatic reactivation of orofacial herpes (Table 9).

Thirty one (96.9%) patients with orofacial herpes declared symptoms duration less than 5 days meantime in the group of negative PCR lesions lasted for more than 5 days and only crusting lesions were detected (Table 9). The amount of virus in recurrent lesions ranged from 90 to $57x10^6$ (median $5.7x10^5$) copies per PCR reaction (mean $29x10^5 \pm 1x10^7$).

The majority of patients were seropositive for HSV-1 and seronegative for HSV-2. One patient with positive PCR (HSV-1) was seropositive for both viruses (Table 9).

„Dry“ swabs from orofacial and genital lesions. All „dry“ (i.e., without buffer) swabs has been also analyzed by qPCR nevertheless they were collected as a reserve. All PCR results correctly corresponded between „dry“ and „wet“ swabs, exept one. The latter was the sample from genital lesions: „wet“ swab was positive for HSV-1 meantime „dry“ one was negative: maybe this could happen due low copy number of HSV in genital lesions.

Table 9. Clinical aspects of the patients with orofacial herpes infection

	Patients with positive PCR	**Patients with negative PCR**
Overall	n=32	**n=3**
Sex: 1. Male	12 (37.5 %)	0 (0 %)
2. Female	20 (62.5 %)	3 (100 %)
Age:		
1. Mean	40.3±15.8	26.3±5.1
2. Range	19-75	22-32
Disease:		
1. First episode	1 (3.1 %)	3 (100 %)
2. Recurrence	31 (96.9 %)	0 (0 %)
Vesicles, ulcers:		
1. Yes	22 (68.8 %)	0 (0 %)
2. No	10 (31.3 %)	3 (100 %)
Duration of the onset :		
1. < 5 days	31 (96.9 %)	0 (0 %)
2. ≥ 5 days	1 (3.1 %)	3 (100 %)
Anti-HSV1 IgG serology:		
Positive	31 (96.9 %)	3 (100 %)
Negative	1 (3.1 %)	0 (0 %)
Anti-HSV2 IgG serology:		
Positive	1 (3.1 %)	0 (0 %)
Negative	31 (96.9 %)	3 (100 %)

[1]Fisher’s exact test

4.2. Experimental part of the study

HSV1-1 and HSV-2 LAT regions containing the LAT promoter were sequenced in this study. The HSV-1 LAT region includes the UOL (upstream of LAT) gene which is transcribed in the same direction as LAT and is supposed to encode a protein of 96 amino acids (Naito et al, 2005). UOL was also analyzed.

4.2.1. Methodological aspects

In total 146 samples for the LAT promoter region were collected but we failed to amplify one HSV-1 orofacial Lithuanian sample due to low copy number of HSV DNA. Therefore 145 clinical HSV samples were used in LAT study: 74 HSV-1 (61 oro-facial samples, 12 genital and 1 CSF sample) and 71 HSV-2 (68 genital and 3 CSF samples).

For analysis of the LAT promoter an array of PCRs which covers this region of both HSV-1 and HSV-2 has been developed (see results). The LAT region of 1360 bp upstream the 2kb LAT, including LAT promoter, was amplified in HSV-1 and 1141 bp in HSV-2, respectively. Due to the GC-richness (69% and 77% G+C for HSV1 and HSV2, respectively) of LAT promoter, which leads to formation of secondary DNA structure, amplification was not successful or was insufficient under standard PCR conditions. Therefore we used a touchdown hot start PCR method (Bachmann et al, 2003) with Master mix including „Q“ solution, a commercial solution, supplied with HotstarTaq polymerase kit (QIAgen). For low HSV DNA copy number samples a nested PCR technique was used under the same cycling conditions. The amplification conditions were optimized after numerous experiments. In oder to avoid secondary structures of DNA during sequencing, we tested the addition of 1M betaine (Sigma Aldrich) to the BigDye terminator cycle sequencing reactions instead of „Q“ solution. We found that betaine reduced band compression, as reported by Haqqi et al (Haqqi et al, 2002).

Even using modified PCR and cycle sequencing techniques, we failed to get reads from both ends and full double stranded coverage in some samples during sequencing. Sequencing trough homopolymer stretches was the most challenging, as noticed by Kieleczawa (Kieleczawa, 2006). Therefore, difficult to sequence PCR amplicons were cloned into TOPO cells. Between three and five clones were obtained from each sample. However, even when sequencing cloneswe failed to get full double stranded coverage due to homopolymer tracts in a few samples, i.e. especially in samples with 2 long homopolymer tracts located nearby each other **(Poly(T)** and dowstream **Poly(C_n)2** in HSV-1; **Poly(C_n)1** and dowstream **Poly(G_n)2** in HSV-2). In these cases we were able to establish an unambiguous sequence by combining reads from both ends using primers in both forward and reverse orientations.

The LAT promoter region was sequenced for all 145 HSV clinical samples and the HSV-1 sequences were compared with that for strain 17 (table 3) and HSV-2 – for strain HG52 (table 4). The variability in the length of both HSV-1 and HSV-2 LAT promoter regions in different strains was due to the presence of tandem repeats, high G+C content and long homopolymer stretches. We concentrated mostly on mononucleotide repeats.

4.2.2. Homopolymer tracts in HSV-1

After sequencing and alignment of the LAT promoter sequences, five homopolymer tracts (HPTs) with variable nucleotide number were detected in HSV-1 LAT promoter region: two poly(C) (**Poly(C_n)1** and **Poly(C_n)2**), one poly(T) (**Poly(T)**) and two poly(G) (**Poly(G_n)1** and **Poly(G_n)2**) (Figure 9). We focused on homopolymer sequences with a cutoff of 7 mononucleotides per HPT in this work.

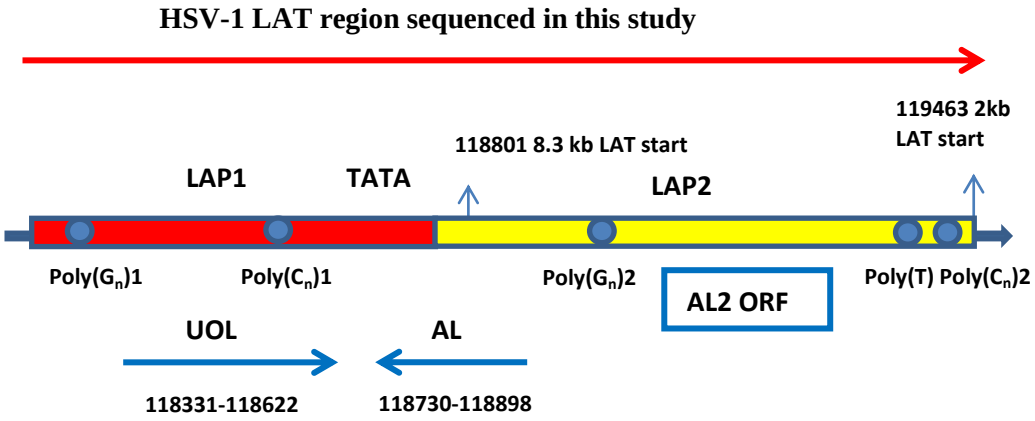


Figure 9. Schematic structure of variable homopolymer tracts (**Poly(G_n)1** and **Poly(G_n)2**, **Poly(C_n)1** and **Poly(C_n)2** and **Poly(T)**) in HSV-1 LAT promoters (LAP1 and LAP2) region

Recurrence rate. Data about recurrence rate of HSV infection was available from Lithuanian patients. Out of 37 HSV-1 Lithuanian samples 32 were taken during recurrent, 5 during first episode of HSV infection (in this case 4 samples from genital and 1 from orofacial region), respectively. The recurrences varied from less than 1 recurrence up to 10 recurrences per year. In this study recurrence rate was divided into 3 categories: very low (≤1-2), low (3-5) and high (≥6) recurrences per year.

Poly(G_n)1. Most of the HSV-1 samples (63/75 84%) had an imperfekt homopolymer sequence „GGGGGAGG“ at nucleotide position 118297-118304 (position 224 in the HSV-1 alignment (Figure 17-18)), same as presented in the reference strain 17. However, due to deletion of deoxyadenosine, 6 variants of sequences with poly(G_n) ranging from 7 to 12 nucleotides were detected in 12 samples (Table 10).

Recurrence rate and Poly(G_n)1 region. Among recurrent samples 3 variants of poly(G) were distinguished where GGGGGAGG dominated and was equally distributed between categories of very low and low recurrences, 6 variants showed 6or more recurrences per year. Both G_9 and G_{12} get into the very low rate category (Table 11).

Table 10. Characteristics of HSV-1 LAT promoter region sequences containing homopolymer **Poly(G_n)1** region

Sequence HSV-1 Poly(G_n)1	Lithuanian	Swedish	Total
GGGGGAGG	34	29	63
G_7	1	1	2
G_8	0	3	3
G_9	1	2	3
G_{10}	0	1	1
G_{12}	1	2	3

Table 11. Variation in HSV-1 **Poly(G_n)1** region and rate of recurrences

Sequence HSV-1 Poly(G_n)1	Recurrences/year			Total
	≤1-2	**3-5**	**≥6**	
GGGGGAGG	12	12	6	30
G_9	1	0	0	1
G_{12}	1	0	0	1
Total	**14**	**12**	**6**	**32**

Poly(C_n)1. Number of „C“ residues in this region (C_{10} region at nucleotide position 118568-118577 of HSV-1 reference strain 17, position 404 in the HSV-1 alignment (Figure 17-18)) varied from 7 to 15 and showed 8 variants. This mononucleotide tract overlaps with UOL in sense direction (Figure 2). In this region we got reads from both ends using forward and reverse primers for sequencing. The majority of poly(C) tracts (57/75 76%) were composed of 7-10 „C“ residues, C_7 being dominant in Swedish samples and C_9 in Lithuanian, respectively (Table 12). Mutations in this region due to expansion and contraction of HPTs could affect upstream of LAT (UOL) protein synthesis.

Table 12. Characteristics of HSV-1 LAT promoter region sequences containing homopolymer **Poly(C_n)1** region

Sequence HSV-1 Poly(C_n)1	Lithuanian	Swedish	Total
C_7	3	11	14
C_8	6	8	14
C_9	13	3	16
C_{10}	6	7	13
C_{11}	1	6	7
C_{12}	4	1	5
C_{14}	4	0	4
C_{15}	0	1	1

Recurrence rate and Poly(C_n)1 region. As already mentioned above, C_9 variant dominated between Lithuanian sequences and there was not significant difference between the categories of recurrence rate (Table 13).

Table 13. Variation in HSV-1 **Poly(C_n)1** region and rate of recurrences

Sequence HSV-1 Poly(C_n)1	Recurrences/year			Total
	≤1-2	3-5	≥6	
C_7	2	0	1	3
C_8	1	4	0	5
C_9	5	3	4	12
C_{10}	3	1	0	4
C_{11}	0	1	0	1
C_{12}	1	2	0	3
C_{14}	3	0	1	4
Total	**15**	**11**	**6**	**32**

Poly(G_n)2. This SSR was composed of imperfect sequence „$G_5TG_2TG_5$“ located at nucleotide positions 118953-18966 of HSV-1 strain 17 (position 915 in the HSV1 alignment (Figure 17-18)) just upstream of AL2 (between AL and AL2). Several samples due to the poly(G) tract reaching up to 12 „G“ were cloned because in some samples with long polyG we couldn‘t get reads from both ends. Sequence „$G_5TG_2TG_5$“ was more prevalent in Lithuanian samples (28/37 75.7%) comparing with Swedish (13/38 34.2%).

There were found 6 variants among 37 Lithuanian sequences and 8 among 38 Swedish, respectively (Table 14).

Table 14. Characteristics of HSV-1 LAT promoter region sequences containing homopolymer **Poly(G_n)2** region

Sequence HSV-1 PolyG_n2	Lithuanian	Swedish	Total
$G_5TG_2TG_5$	28	13	41
G_5TG_{11}	0	1	1
G_5TG_{10}	1	1	2
G_5TG_9	0	2	2
G_5TG_8	3	9	12
G_5TG_7	0	4	4
G_5TG_6	3	7	10
G_5TG_5	1	1	2
G_{12}	1	0	1

All Swedish genital samples had the G_5TG_n genotype while Lithuanian samples of genital herpes contained both G5TG2TG_5 (4 out of 6) and G_5TG_n genotype. All clones from the same patient were with the same fixed number of „G“ residues and were nicely readable (Figure 10).

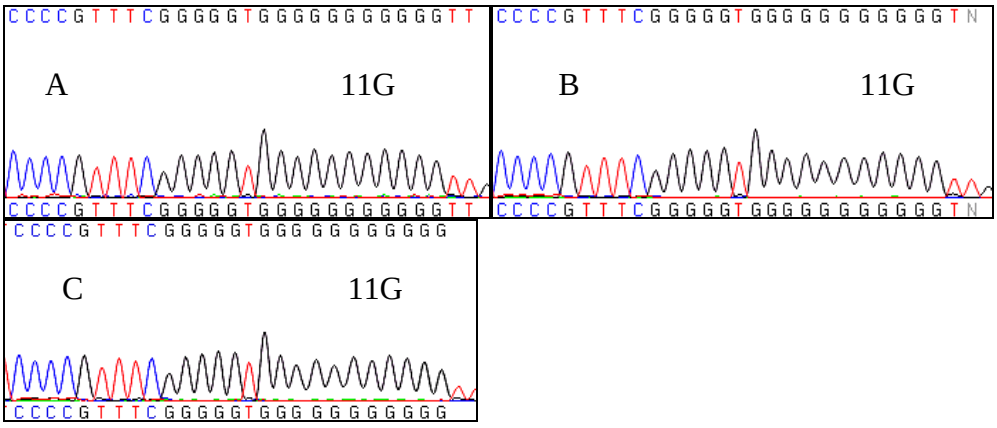


Figure 10. Chromatograms of different clones (A-C) of **Poly(G_n)2** region from the same patient show the same G_5TG_{11} pattern

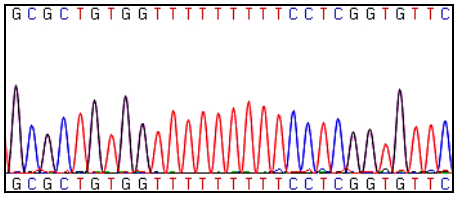
Recurrence rate and Poly(G_n)2 region. The HSV-1 sequence $G_5TG_2TG_5$ was dominant in all three categories of recurrence rate (Table 15).

Table 15. Variation in HSV-1 **Poly(G_n)2** region and rate of recurrences

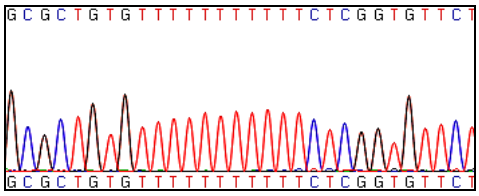
Sequence HSV-1 PolyG_n2	**Recurrences/year**			**Total**
	≤1-2	**3-5**	**≥6**	
$G_5TG_2TG_5$	8	13	3	24
G_5TG_{10}	0	0	1	1
G_5TG_8	2	0	1	3
G_5TG_6	1	1	0	2
G_5TG_5	0	0	1	1
G_{12}	1	0	0	1
Total	**12**	**14**	**6**	**32**

Poly(T_n) and **Poly(C_n)2** regions are located close to each other (46 nucleotides apart) in LAP2, i.e. the TATA-less promoter. Due to the long homopolymer „T“ stretches and/or **Poly(C_n)2** region dowstream poly(T_n) tract, we failed to get reads from both ends therefore 18 Lithuanian and 20 Swedish samples were cloned. **Poly(T_n)** and **Poly(C_n)2** regions due to their vicinity were cloned together.

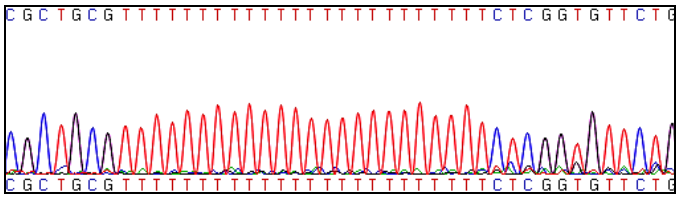
The **Poly(T_n)** (119301-119309 nucleotide position of reference strain 17, position 1271 in the HSV1 alignment (Figure 17-18)) tracts ranged from 9 to 30 „T“ nucleotides. We detected a variable number of „T“ residues in different clones in different samples and even in different clones from the same patient. In spite of this variability, the means of each length category divided samples into two categories (data not shown), those with long poly(T) repeats (16-30 nt) and those with short poly(T) stretches with 9-13 nucleotides. The total number of long poly(T) ≥ 16 nt (ranging from 16 to 30) occured in 27 samples, while 48 samples had short poly(T) sequences. Interestingly, short poly(T) repeats were dominant (32/38 84.2%) in Swedish samples, while poly(T) stretches of Lithuanian samples were mostly long (21/37 56.8%). Long poly(T) regions usually were associated with „GCG“ nucleotides upstream, meantime „GG“ or „G“ were present before short poly(T) with a few exceptions (Figure 11, Table 16).



A



B



C

Figure 11. Chromatograms of HSV-1 **Poly(T_n)** region:
A – short poly(T) with „GG“ upstream
B – short poly(T) with „G“ upstream
C – long poly(T) with „GCG“ upstream

Table 16. Characteristics of HSV-1 LAT promoter region sequences containing homopolymer **Poly(T)** region

Sequence HSV-1 Poly(T)	Lithuanian	Swedish	Total
Long Poly(T) (16-30 nt)	21 (56.8%)	6 (18.8%)	27 (36%)
Short Poly(T) (9-13 nt)	16 (43.2%)	32 (81.3%)	48 (64%)

Each variant is represented by a range of poly-T lengths in different clones even from the same patient but the means of each length category are significantly different.

Recurrence rate and HSV-1 Poly(T) region. Out of 32 patients with recurrent infection long poly(T) profile of the LAT promoter sequences was identified in 18 samples and short poly(T) in 14 samples, respectively. There was no significant difference between recurrence rate and poly(T) profile (Table 17).

Table 17. Variation in HSV-1 **Poly(T)** region and rate of recurrences

Sequence HSV-1 Poly(T)	**Recurrences/year**			Total
	≤1-2	**4-5**	**≥6**	
Long poly(T) (16-30 nt)	7	8	3	18
Short poly(T) (9-13 nt)	5	6	3	14

Poly(C_n)2. This homopolymer „C“ region is located 46 nucleotides downstream of the poly(T) region (119356-119365 nucleotide positions of reference strain 17, position 1345 in the HSV-1 alignment (Figure 17-18)). Six samples, 3 Lithuanian and 3 Swedish, were 14 nucleotides long, 1 Lithuanian samples had 15 Cs, others varied from 7 to 13 nucleotides, mostly 9-11 „C“ residues. All variants of **Poly(C_n)2** region including different clones are shown in the table 18.

Table 18. Characteristics of HSV-1 LAT promoter region sequences containing homopolymer **Poly(C_n)2** region

Sequence HSV-1 Poly(C_n)2	**Lithuanian**	**Swedish**	**Total**
C_7	1	0	1
C_8	0	2	2
C_9	19	18	31
C_{10}	20	22	39
C_{11}	18	23	41
C_{12}	11	9	19
C_{13}	3	0	3
C_{14}	3	3	6
C_{15}	1	0	1

Recurrence rate and Poly(C_n)2. The longest poly(C_{14-15}) stretches were detected in 4 samples from the patients with 1 or less recurrences per year. C_9 - C_{12} were dominant among the variants of Poly(C_n)2 in both Swedish and Lithuanian samples (Table 19).

Table 19. Variation in HSV-1 **Poly(C_n)2** region and rate of recurrences.

Sequence HSV-1 Poly(C_n)2	**Recurrences/year**			**Total**
	≤1-2	**3-5**	**≥6**	
C_7	1	0	0	1
C_9	4	7	2	13
C_{10}	6	5	6	17
C_{11}	9	4	5	18
C_{12}	4	4	2	10
C_{13}	1	2	0	3
C_{14}	3	0	0	3
C_{15}	1	0	0	1
Total	**29**	**22**	**15**	**66**

In contrast to the poly(G) stretches in the **Poly(G_n)2** region**,** we detected a variable number of „T“ and „C“ residues both in different clones from the same patient and in different samples (Figure 5-6), in both **Poly(T_n)** and **Poly(C_n)2** regions (Figure 12-13). Thus, these HPTs displayed both intra- and interindividual variability (see below).

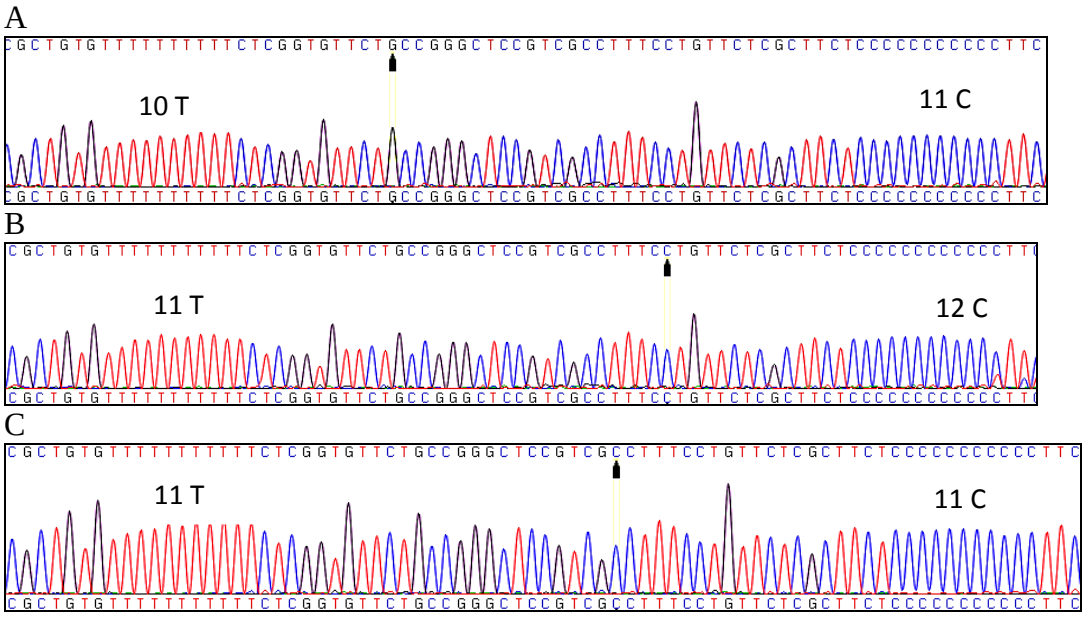


Figure 12. Chromatograms of different clones (A-C) of **Poly(T_n)** and **Poly(C_n)2** region from the same patient show different number of „T“ and „C“ residues

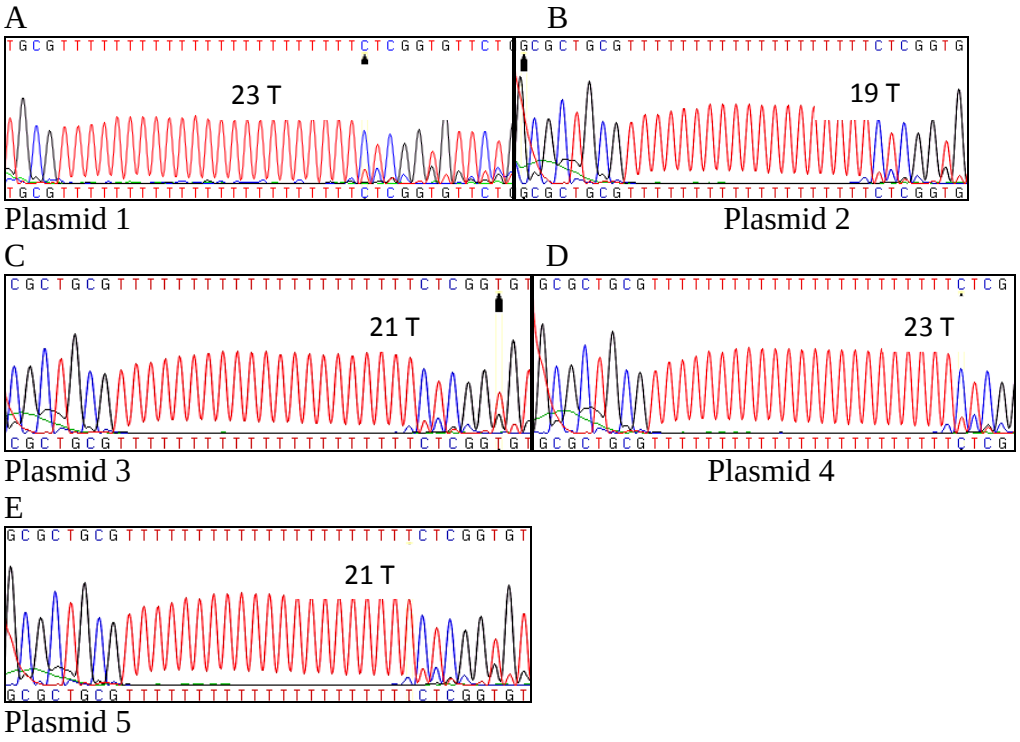


Figure 13. Chromatograms of different clones (A-E) of long poly(T) region from the same patient show 3 variants of „T“ residues (T_{19}, T_{21}, T_{23})

4.2.3. Homopolymer tracts in HSV-2

HSV-2 has been studied much less extensively by others, compared with HSV-1. Three variable homopolymer regions **Poly(G_n)1**, **Poly(C_n)** and **Poly(G_n)2** were detected in the HSV-2 LAT region containing the LAT promoter (Figure 14).

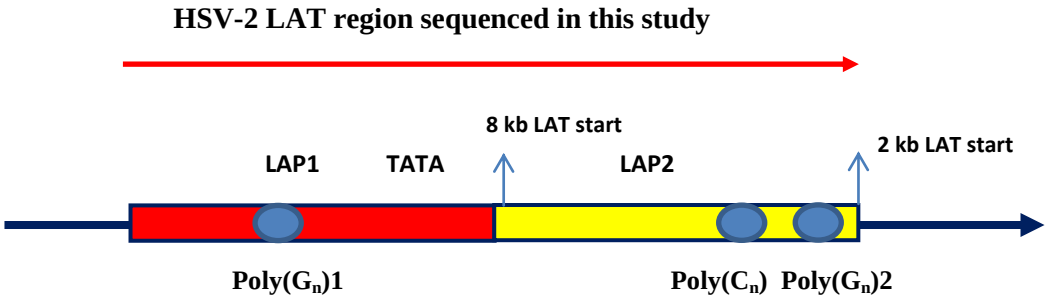


Figure 14. Schematic structure of variable homopolymer tracts (**Poly(G_n)1** and **Poly(G_n)2**, **Poly(C_n)1** in HSV-2 LAT promoters (LAP1 and LAP2) region

Recurrence rate. As in HSV-1, data regarding recurrence rate of HSV-2 are known in the group of Lithuanian patients. Out of 34 Lithuanian patients with HSV-2 genital herpes 25 stated recurrent episode and 9 - first episode of infection. The recurrences varied from less than 1 recurrence up to 15 recurrences per year. In this study recurrence rate is divided into 3 categories: ≤1-2 (very low), 3-5 (low) and ≥6 (high) recurrences per year.

Poly(G_n)1 region (119340-119350 nucleotide positions of HSV-2 reference strain HG52, position 237 in the HSV-2 alignment (Figure 19)) showed 3 types of sequences: „CCCGCGTGCCG“, „CCCGCGGGCCG“ and „TTG_n“. The sequence „CCCGCGTGCCG“ was dominant and almost equally distributed between Lithuanian and Swedish samples. Homopolymer „G“ sequences were detected only in 17/71 (23,9%) samples. Poly(G) stretches showed 4 variants of „G“ residues ranging from 9 to 12 nucleotides with G_9 and G_{10} variants being most prevalent (Table 20).

Table 20. Characteristics of HSV-2 LAT promoter region sequences containing homopolymer **Poly(G_n)1** region

HSV-2 Poly(G_n)1	Lithuanian	Swedish	Total
CCCGCGTGCCG	26	27	53
CCCGCGGGCCG	0	1	1
TTG_9	4	1	5
TTG_{10}	2	4	6
TTG_{11}	1	1	2
TTG_{12}	1	3	4

Recurrence rate and Poly(G_n)1. As shown in the table 14, the majority of the patients with the sequence CCCGCGTGCCG had up to 6 recurrences of genital herpes per year. TTG_{9-11} variants have not been not found in the group of very low rate of recurrences (Table 21).

Table 21. Variation in HSV-2 **Poly(G_n)1** region and rate of recurrences

Sequence HSV-2 Poly(G_n)1	**Recurrences/year**			**Total**
	≤1-2	**3-5**	**≥6**	
CCCGCGTGCCG	10	4	7	21
TTG_9	0	1	1	2
TTG_{10}	0	1	0	1
TTG_{11}	0	0	1	1
Total	**10**	**6**	**9**	**25**

Poly(C_n) and the downstream **Poly(G_n)2** regions (similar to **Poly(T_n)** and **Poly(C_n)2** regions in HSV-1) are located 75 nucleotides apart from each other. As in HSV-1, 7 Lithuanian samples and 13 Swedish had to be cloned together due to failure to get reads from both ends.

Poly(C_n) tract (120065-120079 nucleotide position in strain HG52, position 962 in the HSV-2 alignment (Figure 19)) showed a variable number of „C“ ranging from 9 to 14. Among Lithuanian samples the variant with 12 „C“ residues was most prevalent, while in Swedish samples 13 „C“ predominated (Table 22).

Table 22. Characteristics of HSV-2 LAT promoter region sequences containing homopolymer **Poly(C_n)** region

HSV-2 Poly(C_n)	**Lithuanian**	**Swedish**	**Total**
C_9	0	2	2
C_{10}	2	4	6
C_{11}	8	19	27
C_{12}	20	21	41
C_{13}	14	23	37
C_{14}	0	4	4

Recurrence rate and Poly(C_n). The sequence variants with 11, 12 and 13 „C“ residues were distributed equally in all 3 recurrence rate categories except sequences C_{10} which were found only in the group of very low

recurrences (≤1-2/year). Variant C_{14} was absent in the group of Lithuanian samples (Table 23).

Table 23. Variation in HSV-2 **Poly(C_n)** region and rate of recurrences

Sequence HSV-2 Poly(C_n)	**Recurrences/year**			**Total**
	≤1-2	**3-5**	**≥6**	
C_{10}	2	0	0	2
C_{11}	2	2	2	6
C_{12}	6	5	4	15
C_{13}	3	2	2	14
Total	**13**	**9**	**8**	**30**

Poly(G_n)2 region (120154-120166 nucleotide positions in strain HG52, position 1051 in the HSV-2 alignment (Figure 19)) contained homopolymer tracts of „G“ residues ranging from 8 to 17. The number of 9 „G“ residues was the most prevalent in Lithuanian samples, G_{12} – in Swedish samples (Table 24).

Table 24. Variation in HSV-2 **Poly(G_n)2** region and rate of recurrences

HSV-2 Poly(G_n)2	**Lithuanian**	**Swedish**	**Total**
G_8	3	4	7
G_9	12	14	26
G_{10}	7	15	22
G_{11}	9	14	23
G_{12}	9	16	25
G_{13}	2	8	10
G_{14}	0	2	2
G_{16}	1	0	1
G_{17}	1	0	1

Recurrence rate and Poly(G_n)2. G_9 was distributed almost equally in all categories of recurrences. G_9 was dominant in the category of low recurrences, G_{10} – very low, and G_{11} in high rate category, respectively. The patient with the longest HSV2 HPT composed of 17 nucleotides suffered from recurrences twice per year.

Table 25. Variation in HSV-2 **Poly(G_n)2** region and rate of recurrences

Sequence HSV-2 Poly(G_n)2	**Recurrences/year**			**Total**
	≤1-2	**3-5**	**≥6**	
G_8	0	0	1	1
G_9	2	5	2	9
G_{10}	5	1	0	6
G_{11}	4	2	3	9
G_{12}	0	1	2	3
G_{16}	1	0	0	1
G_{17}	1	0	0	1
Total	**13**	**9**	**8**	**30**

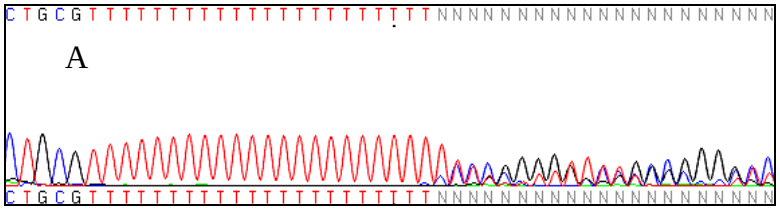
In HSV-2 samples, as in HSV-1 samples, variable number of „C“ and „G“ residues in **Poly(C_n)** and **Poly(G_n)2** regions both in different clones from the same patient and in samples/strains from different patients (see below) were detected.

4.2.4. Inter- and intraindividual HSV variability

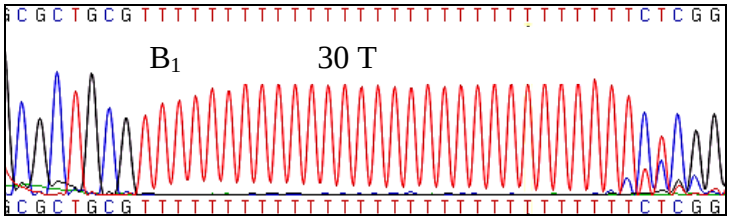
Sequencing of the PCR products/plasmid DNA revealed a variable number of nucleotide residues in HPTs in different samples and also in clones from the same sample in HSV-1 (**Poly(T)** and **Poly(C_n)2**) and HSV-2 **(Poly(C_n)** and **Poly(G_n)2)** regions.

In most cases the sequence (especially with longer HPT) immediately after HPT was not clearly readable. In order to eliminate artefacts caused by Taq polymerase slippage occuring during PCR amplification in

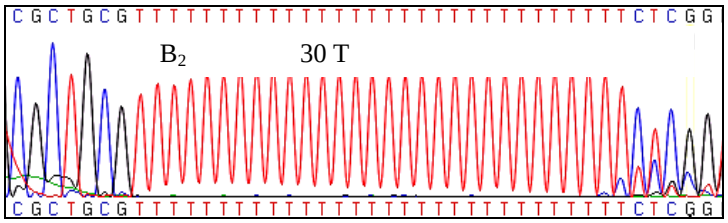
vitro, 5 samples (2 of HSV-1 and 3 of HSV-2) were analyzed in several ways: 1) the amplified DNA was sequenced directly; 2) the plasmid with cloned DNA was sequenced directly or 3) the plasmid with cloned DNA was amplified in a PCR reaction prior to sequencing. As mentioned above, in most samples we got different number of variable nucleotides in homopolymer stretches in different clones from the same patients. But the same cloned DNA used in direct sequencing and amplified in PCR reaction prio sequencing showed the same number of residues even if the plasmid sequence after PCR amplification was less clear. Sequences amplified in PCR from directly from the sample were not always readable in the nucleotides following a homopolymer tract, in contrast to directly sequenced clones. Therefore we consider that variation of mononucleotide stretches is not due to artefacts of PCR amplification. Our observation is consistent with data of Rocha et al (Rocha et al, 2002) (see chromatograms in Figure 15-16).



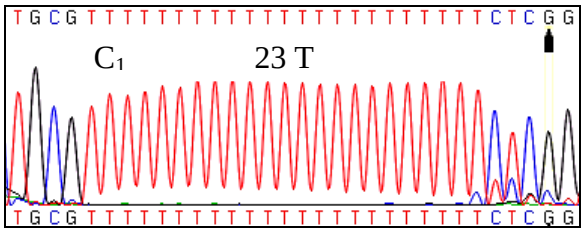
Chromatogram of sequenced directly DNA amplicon. After homopolymer „C“ tract sequence is not readable



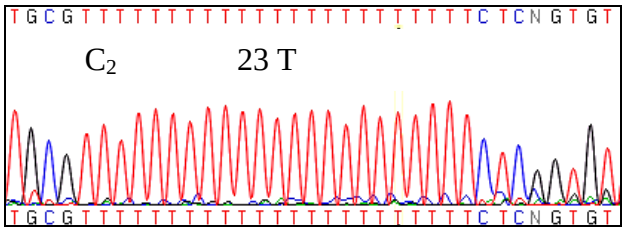
HSV-1 sequencing after cloning: plasmid DNA sequenced directly



HSV-1 sequencing after cloning: plasmid DNA sequenced after amplification in PCR reaction

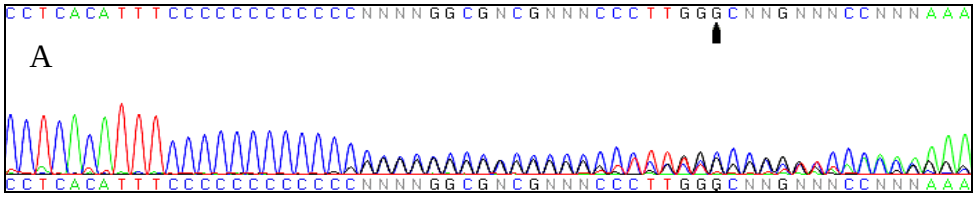


HSV-1 sequencing after cloning: plasmid DNA sequenced directly

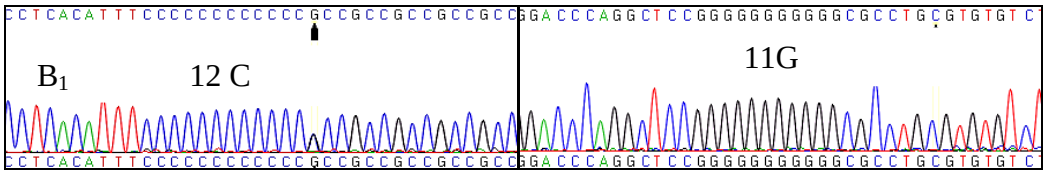


HSV-1 sequencing after cloning: plasmid DNA sequenced after amplification in PCR reaction

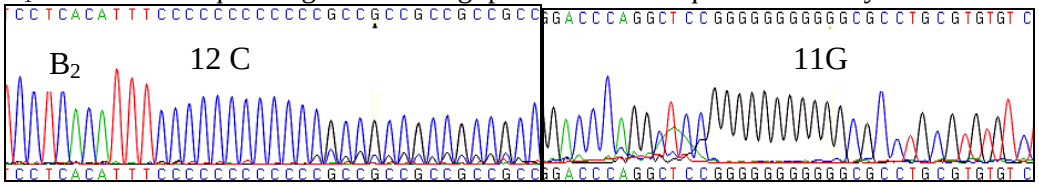
Figure 15. Chromatograms of HSV-1 **Poly(T)** region sequences (A-C) from the same patient



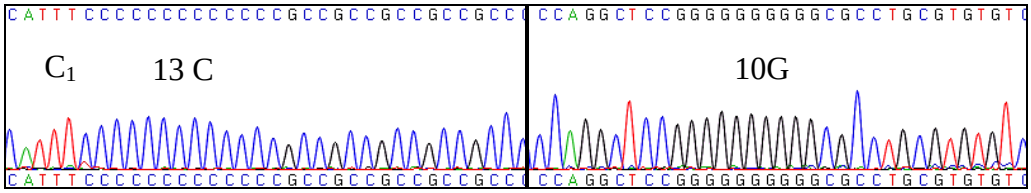
Chromatogram of sequenced directly DNA amplicon. After homopolymer C tract sequence is not readable



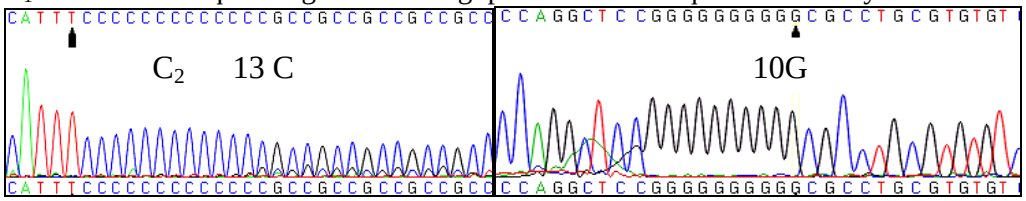
B_1. Plasmid 1. Sequencing after cloning: plasmid DNA sequenced directly



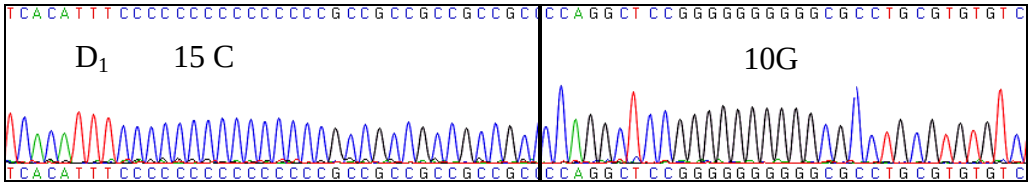
B_2. Plasmid 1. Sequencing after cloning: plasmid DNA sequenced after amplification in PCR reaction



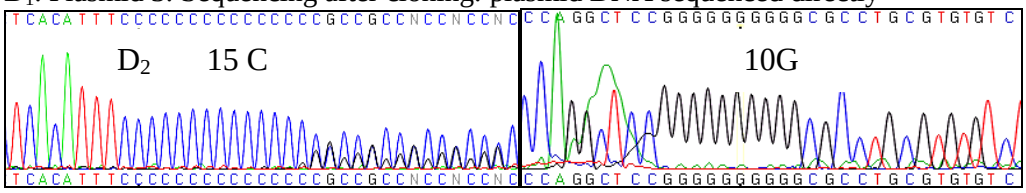
C_1. Plasmid 2. Sequencing after cloning: plasmid DNA sequenced directly



C_2. Plasmid 2. Sequencing after cloning: plasmid DNA sequenced after amplification in PCR reaction



D_1. Plasmid 3. Sequencing after cloning: plasmid DNA sequenced directly



D_2. Plasmid 3. Sequencing after cloning: plasmid DNA sequenced after amplification in PCR reaction

Figure 16. Chromatograms of HSV-2 **Poly(C_n)** and **Poly(G_n)2** sequences (A-D) from the same patient

4.2.5. Simplots of the LAT promoter sequences

To display the degree of similarity of aligned nucleotide sequences, simplots were created.

The simplots of the HSV1 LAT promoter region show that the sequence variation is confined to a a small number of sites. Some of these sites reside within the coding regions of the UOL, AL and AL2 proteins, and will influence the amino acid sequence of these proteins. One of the proteins, AL, also overlaps the transcriptional start site which is close to the TATA promoter signal. There is no conspicuous difference in variability pattern between orofacial and genital HSV-1. Neither is there a difference between HSV-1 from Vilnius and Uppsala, nor between high and low recurrence rate among Vilnius samples. This indicates that the variation in the HSV1 LAT promoter region does not confer clear pathobiological variation (Figure 17-18).

The simplots of the HSV2 LAT promoter region also show that the sequence variation is confined to a small number of sites. As there are no known proteins encoded from the LAT promoter sequence this variation cannot be placed in a protein context. Also for HSV2, the variation is independent of geography (the Vilnius-Uppsala comparison) and rate of recurrence (Figure 19).

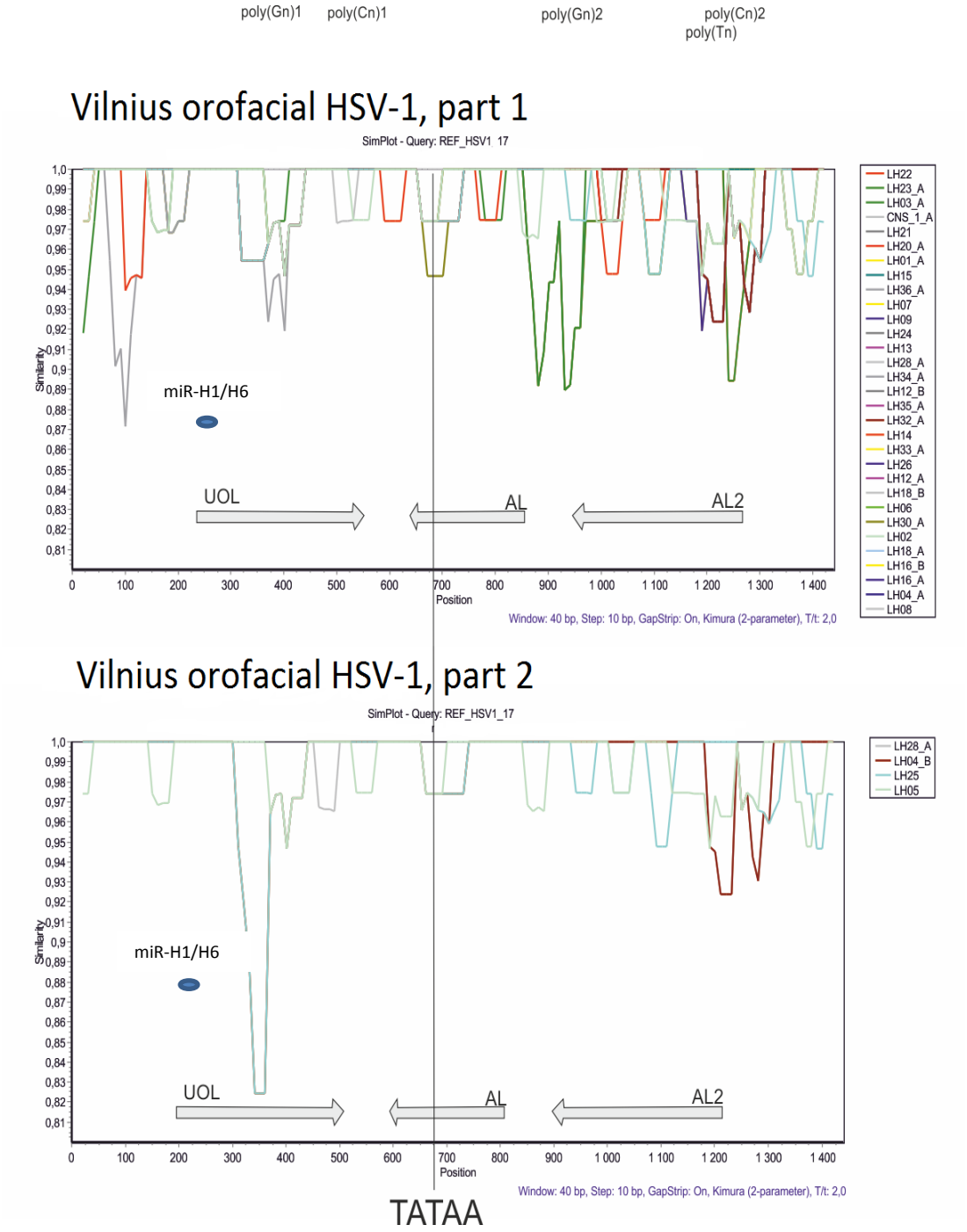


Figure 17. Similarity plots of the HSV-1 LAT promoter region (orofacial strains from Vilnius) compared to strain 17. The y-axis gives the percentage of identity within a sliding window of 40 bp wide centered on the position plotted, with a step size between plots of 10 bp. On the top HPTs are indicated.
Sense to LAT (UOL) and antisense to LAT (AL, AL2) transcripts, micro-RNAs (miR-H1 and miR-H6) encoded in LAT sequences are shown.

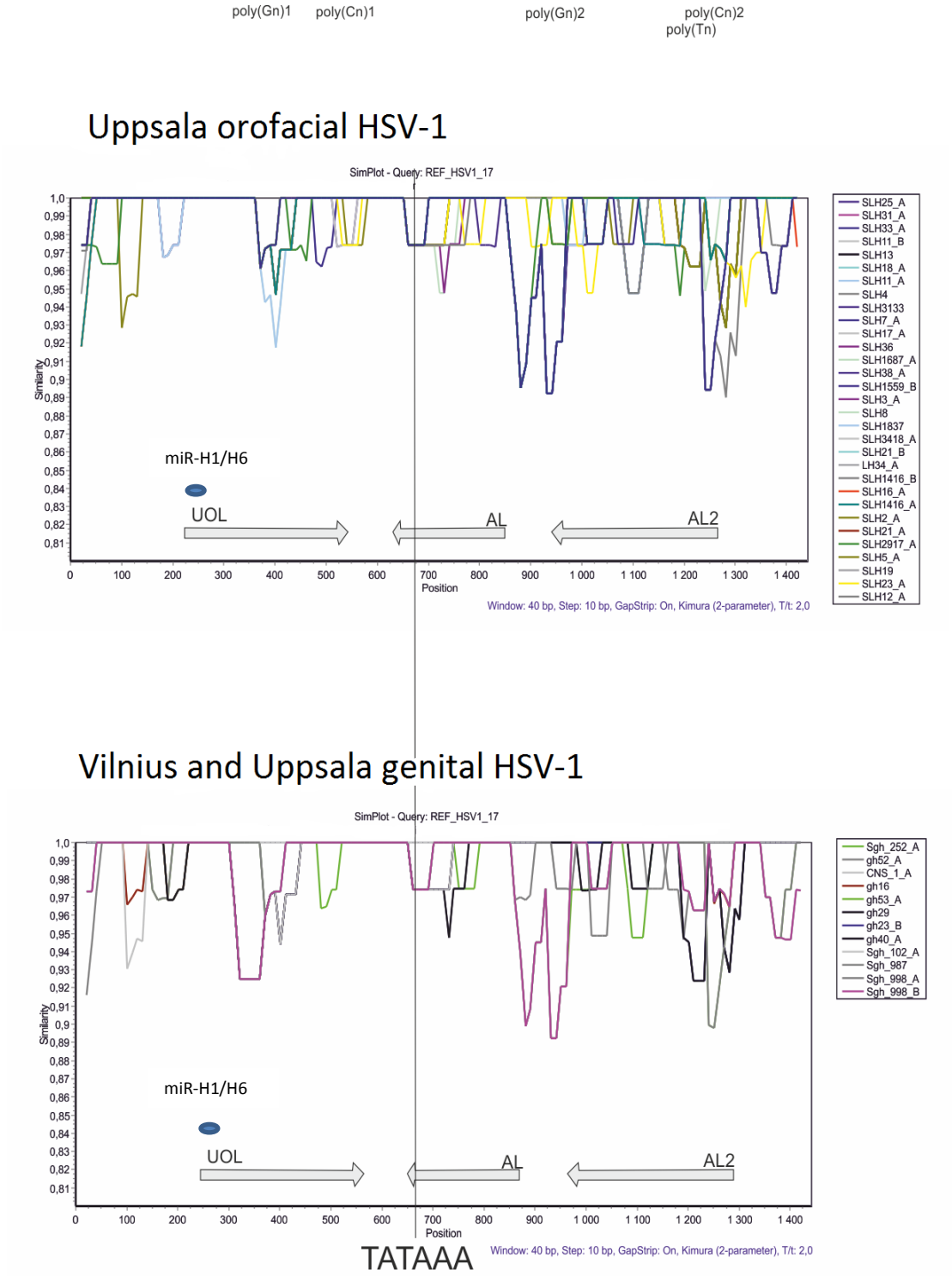


Figure 18. Similarity plots of the HSV-1 LAT promoter region (orofacial strains from Uppsala and genital strains from both Uppsala and Vilnius) compared to strain 17. The y-axis gives the percentage of identity within a sliding window of 40 bp wide centered on the position plotted, with a step size between plots of 10 bp. On the top HPTs are indicated.
Sense to LAT (UOL) and antisense to LAT (AL, AL2) transcripts, micro-RNAs (miR-H1 and miR-H6) encoded in LAT sequences are shown

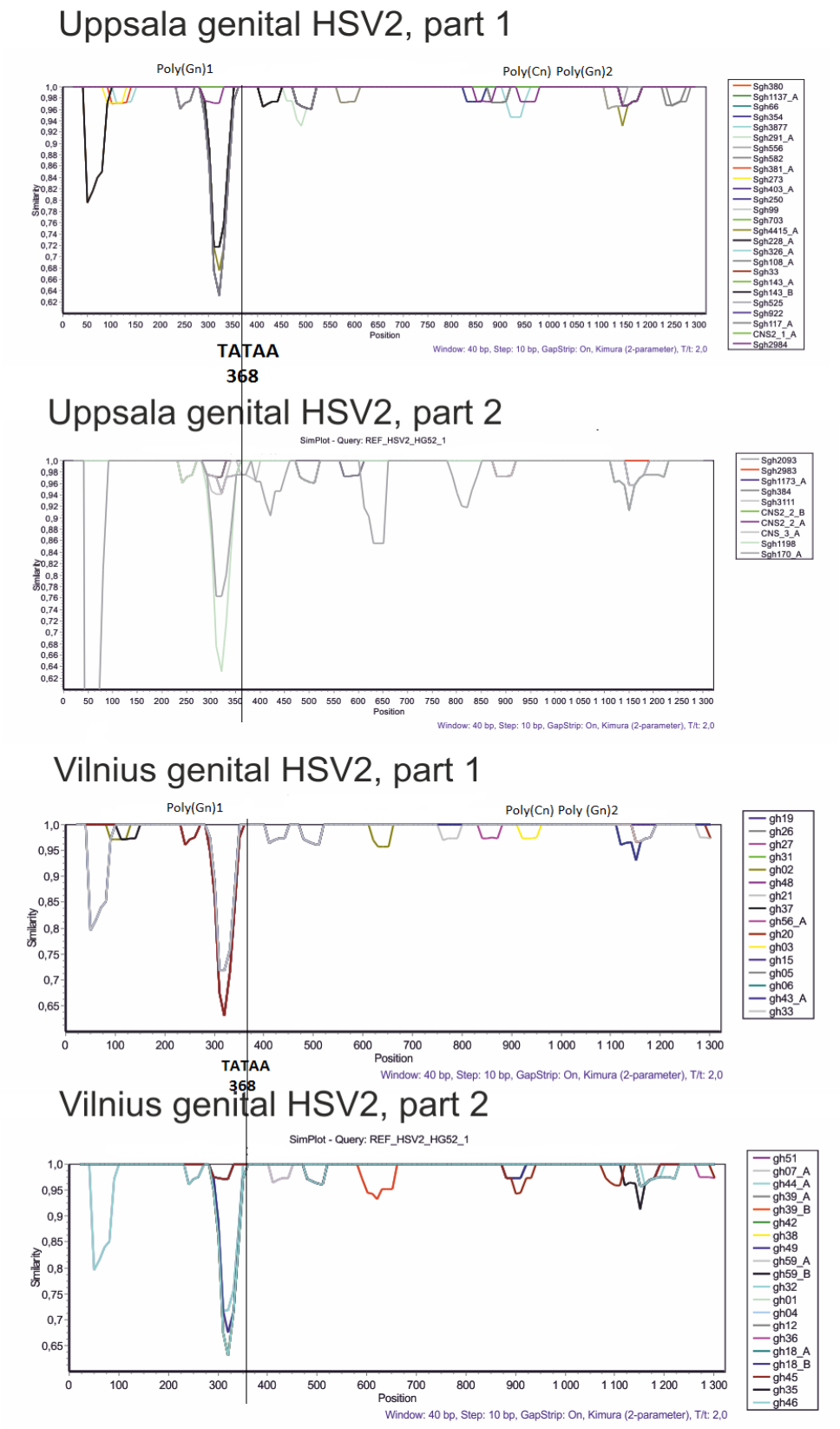


Figure 19. Similarity plots of the HSV-2 LAT promoter region (genital strains from both Vilnius and Uppsala) compared to strain HG52. The y-axis gives the percentage of identity within a sliding window of 40 bp wide centered on the position plotted, with a step size between plots of 10 bp. On the top HPTs are indicated

4.2.5. Phylogenetic analysis of the LAT promoter sequences

After sequencing of the LAT promoter region from HSV-1 and HSV-2 clinical samples, other HSV-1 strains (17, F, H129, KOS, CJ311, CJ360, CJ394, CJ970, CR38, OD4, 134, E25, E03, E07, E08, E10, E11, E12, E13, E14, E19, E22, E23, E35, F06, R11, R62, S23, S25, TFT401) and HSV-2 strains (HG52, 333) were taken from the NCBI Reference Database (www.ncbi.nlm.nih.gov) for phylogenetic analysis. According to A.J. Davison, the E HSV-1 strains were of East African origin (personal communication).The sequences were aligned and phylogenetic trees were constructed as described in methods.

Phylogenetic analysis did not reveal significant differences between Lithuanian and Swedish strains, nevertheless we can distinguish clusters where Swedish strains dominate upon Lithuanian and vice versa.

Both the cladograms and phylogenetic trees (phylograms) showed that the LAT promoter region had a geographically dependent variation, with East African HSV-1 strains occurring in two clusters, East Asian – in one cluster (encircled in HSV-1 phylogenetic tree). The Uppsala and Vilnius HSV-1 and HSV-2 strains were not clearly separated. Neither were the labial and genital HSV-1 strains separated from each other. Sequences from the same sample clustered with each other, with few exceptions. Although the number of HSV-1 and HSV-2 strains with known neurotropism was small, there could be a tendency to clustering of them. The variation bar at the bottom of the phylograms depicts that branch lengths represent rather few nucleotide differences. This can also be inferred from the low bootstrap values for some branches in the tree. The HSV strains with indicated recurrence rate were not clustered (Figure 20-23).

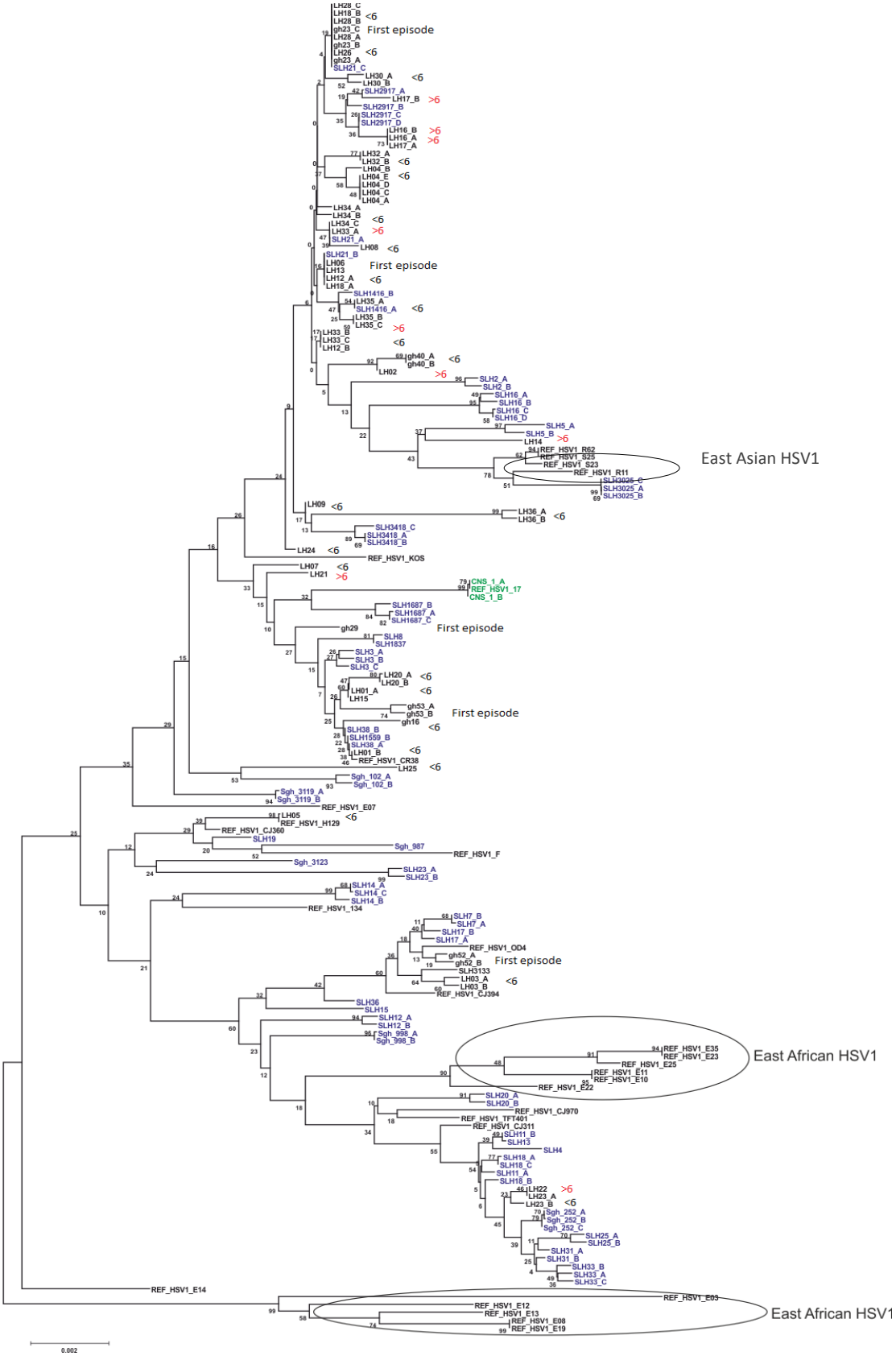


Figure 20. The phylogenetic tree of HSV-1

LH – orofacial Vilnius strains (black), SLH – orofacial Uppsala strains (blue), gh – genital Vilnius strains (black), Sgh – genital Uppsala strains (blue). Recurrence rate <6 or >6 is also indicated. CNS – central nervous system (encephalitis) Uppsala strains (green),

REF – reference strains taken from GenBank database. A, B, C, D indicate clones from the same patient

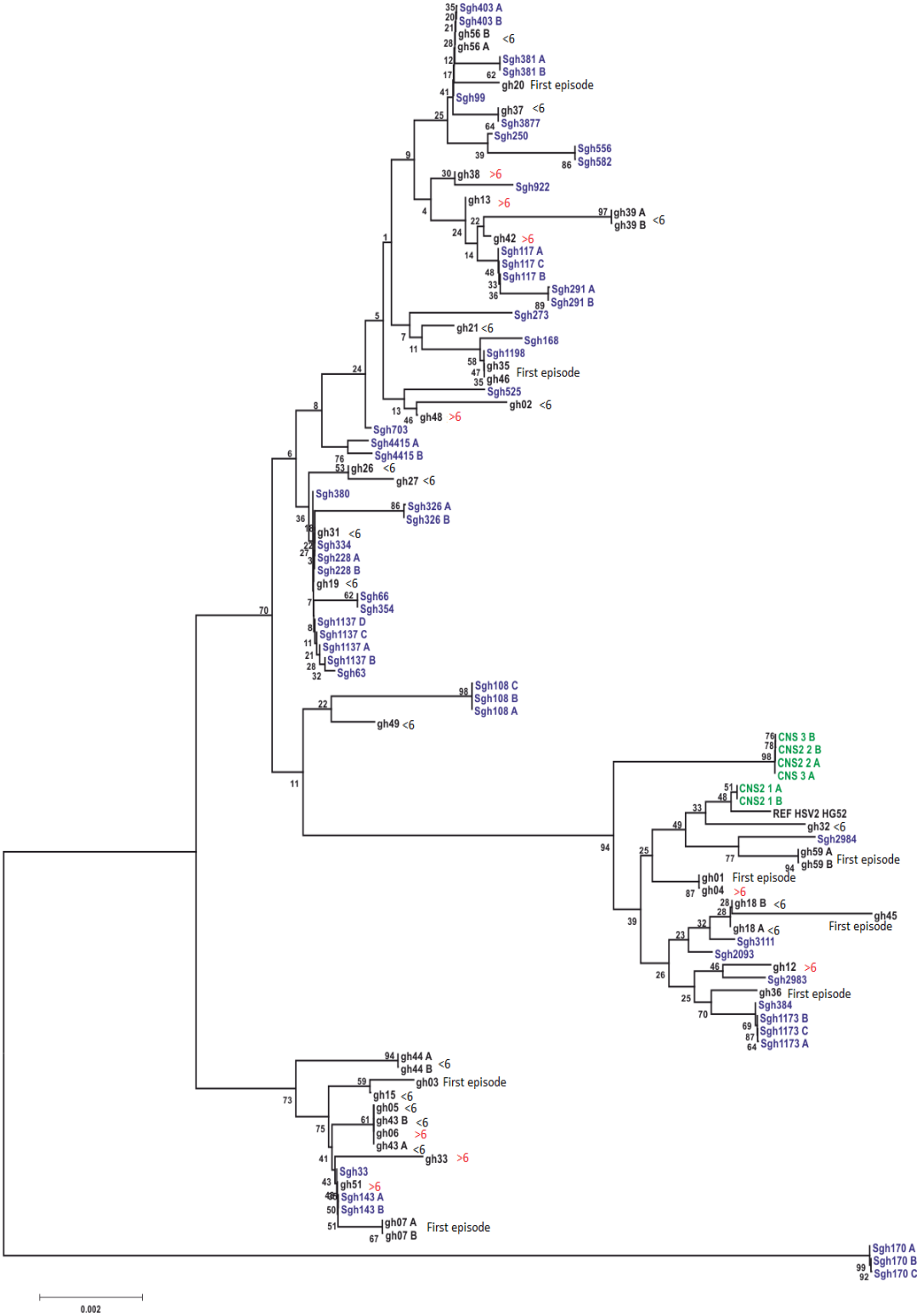


Figure 21. The phylogenetic tree of HSV-2
gh – genital Vilnius strains marked in black, Sgh – genital Uppsala strains marked in blue. Recurrence rate <6 or >6 is also indicated.
CNS – central nervous system (meningitis) Uppsala strains marked in green
REF – reference strains taken from the GenBank database. A, B, C, D indicate clones from the same patient

Figure 22. The cladogram of HSV-1
LH – orofacial Vilnius strains (violet), SLH – orofacial Uppsala strains (blue), gh – genital Vilnius strains (pink), Sgh – genital Uppsala strains (light blue). CNS – central nervous system (encephalitis) Uppsala strains (black). REF – reference strains taken from GenBank database; seq – sequences
A, B, C, D indicate clones from the same patient

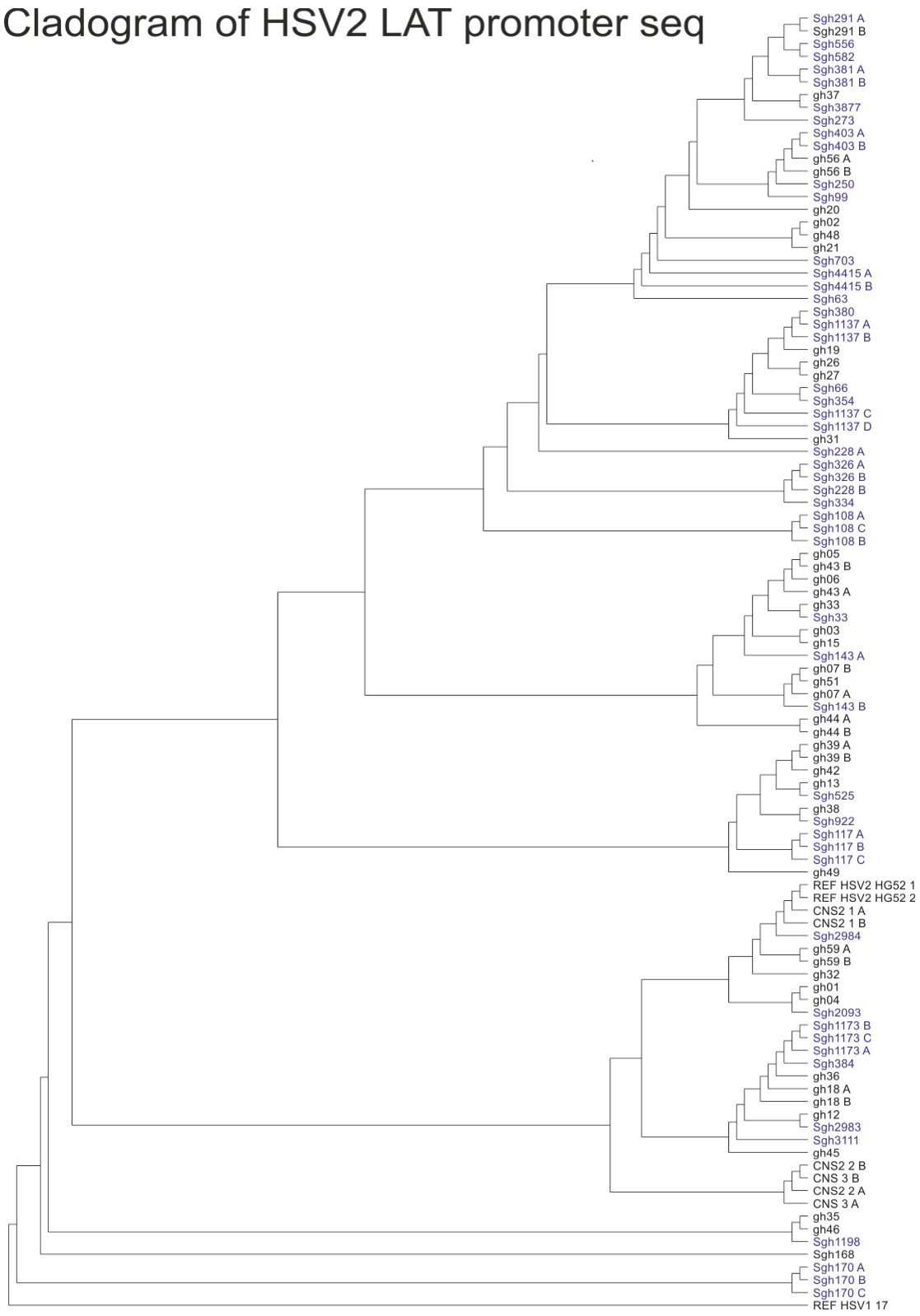


Figure 23. The cladogram of HSV-2
gh – genital Vilnius strains (black), Sgh – genital Uppsala strains (blue). CNS – central nervous system (meningitis) Uppsala strains (black). REF – reference strains taken from the GenBank database; seq - sequences
A, B, C, D indicate clones from the same patient

4.2.6. Stop codon variability in the HSV LAT promoter

The potential of variable HPTs and single nucleotide polymorphisms to influence the function of the HSV LAT promoter was studied by translating all six reading frames and noting the number of resulting stop codons (data not shown). The HSV1 LAT promoter translations contained an average of 6.31, sd 0.23 (n=176), stop codons. The corresponding average for the HSV2 LAT promoter translations was 3.28, sd 0.11 (n=101). The difference is significant, $p<0.001$, Wilcoxon rank sum test. A likely explanation could be the lower GC content of the HSV1 LAT promoter (average 69%) compared that of the HSV2 LAT promoter (average 77%). A high GC content decreases the likelihood of stop codon creation due to random mutation. The three stop codons (TAG, TAA, and TGA are less likely to develop from a GC-rich sequence because G occurs only in 2 of 9 stop codon positions, and C in none.

5. DISCUSSION

5.1. Clinical part of the study

5.1.1. Risk factors and genital herpes

Most studies on HSV-2 showed female gender (Lafferty, 2002; Jonsson et al,. 2006), age (Pebody et al., 2004), higher number of lifetime sexual partners, early age at first intercourse, sexual contact with commercial sex workers and history of STI (Faber et al., 2011; Pereira et al, 2012) to be the risk factors for acquisition of genital herpes (Wald, 2004). Condom use is not atributed to risk factors as it reflects sexual activity (Wald, 2004). Condom use does not give an absolute protection from acquiring HSV-2 infection but the risk of transmission is usually reduced (Wald et al., 2005).

Our results showed that patients, as expected, had more lifeftime sexual partners, reported more cases of positive history of other STIs, oral sex,

earlier age at sexual debut and more rare use of condoms than controls. In this study there was not significant difference between gender and age between study groups.

5.1.2. Herpes simplex virus typing

Genital HSV infection. According to the published data, HSV-2 still remains the main cause of genital herpes worldwide (Lafferty, 2002; Page et al., 2003; Janier et al., 2006) but many studies report an increasing role of HSV-1 in causing genital herpes infection (Pebody et al., 2004; Lowhagen et al., 2000; Ashley and Wald, 1999; Coyle et al., 2003; Filén et al., 2004; Thompson, 2000; Lafferty et al., 2000; Lowhagen et al., 2002), especially in first-episode herpes (Ryder et al., 2009; Lafferty, 2002; Lowhagen et al., 2000; Nieuwenhuis et al., 2006). It is considered to be associated with oro-genital contact (Thompson, 2000; Lafferty et al., 2000) and less frequent previous exposure to HSV-1 during childhood (Ryder et al., 2009; Lafferty, 2002; Lowhagen et al., 2000, Malkin, 2004; Wald, 2006; Pereira et al., 2012), improvements in living conditions and better hygiene (Xu et al., 2006).

In this study HSV-1 was detected in 6 (age range 19-39) female patients, 4 of them declared first episode of genital herpes. Data of other investigators showed that HSV-1 cases were especially associated with female gender (Lafferty, 2002; Janier et al., 2006; Jonsson et al., 2006; Lafferty et al., 2000; Kortekangas-Savolainen and Vuorinen, 2007), younger age (Thompson, 2000) and, as mentioned above, with the first episode of genital herpes.

We detected HSV-2 in 85% of the patients with positive PCR. The predominance of HSV-2 in our study could be explained by the fact that more than half of PCR-positive patients (65%) reported recurrent episode of the disease and according to the data obtained from other investigatots most cases of recurrent genital herpes are caused by HSV type 2 (Janier et al., 2006; Slomka et al., 1998; Filén et al., 2004, Pereira et al, 2012). Our results correspond to the data obtained by Ramaswamy et al. (Ramaswamy et al.,

2004) who reported HSV-2 detection in the majority of both first and recurrent episodes of genital herpes (91% of HSV-2, 9% of HSV-1**)** (Ramaswamy). Besides, it is well known that previous oral HSV-1 infection protects against genital HSV disease (Xu et al., 2006; Lowhagen et al., 2000). In this study most of the patients had anti-HSV-1 IgG antibodies and maybe most probably this is the reason why very few of them had genital herpes caused by HSV-1 despite of rather common practice of oral sex.

Orofacial HSV infection usually is caused by HSV-1 although cases of HSV-2 are recorded (Wald et al., 2004). In our study no HSV-2 virus was detected in orofacial lesions, only HSV-1, and these data are consistent with studies of other investigators (Boivin et al., 2006).

5.1.3. Clinical picture, time of sampling and DNA quantification

A very important factor in detecting herpes simplex virus in genital lesions is **clinical picture and time of sampling** (Ramaswamy et al., 2005, Corey and Holmes, 1983; Scoular et al., 2002). Vesicles and wet erosions give a higher yield of virus than crusted lesions (Goldman, 2000). With time the amount of virus in lesions decreases (Filén et al., 2004). Viral shedding from onset of symptoms in primary genital herpes has been described to be present for 11-12 days, in recurrent lesions for 3-4 days (Corey and Wald, 1999; Crumpacker, 1999). In a study conducted by Filén et al. (Filén et al., 2004), HSV was detected up to 14 days from onset of symptoms. In this study, HSV was detected in genital lesions up to 3 weeks in first episodes and up to 14 days in recurrent episodes.

Boivin et al. (Boivin et al., 2006) investigated HSV-1 DNA by qPCR in orofacial lesions and found HSV-1 was detected in lesions lasting up to 96 hours. No virus DNA has been detected in longer standing lesions. Our data show that HSV was detected on the fifth day of the infection although it is necessary to keep in mind that the majority of the patients had lesions up to 5

days (except 1 patient) and information about longer duration in our study is lacking.

Ramaswamy et al. (Ramaswamy et al., 2005) reported a statistically significant association between visible ulceration, time of sampling during the first 4 days from the onset of symptoms and positive PCR result and these results are consistent with our data. The majority of our PCR-positive patients with HSV infection (orofacial and genital) presented with visible ulceration and had symptoms which lasted for less than 5 days.

Two patients with initial negative PCR but signs suspected for HSV referred to our clinic after 2 months with a new recurrent episode and this time HSV-1 (in one patient) and HSV-2 (in another patient) have been detected in genital lesions. In the group of patients with negative PCR 2 study participants presented with atypical symptoms (erythema, burning sensation, fissures), three other patients with ulceration were on a self treatment with local antiviral medication and antiseptic solutions before visiting the doctor.

It is also important to keep in mind potential causes of genital lesions other than HSV: among the patients with not confirmed genital herpes one patient developed psoriasis, meantime yeast infection was detected in other two patients.

Quantification is an additional measurable factor (Boivin et al., 2006) to compare viral load during first episodes and recurrences. Our results support the previously described data by Filén et al. (Filén et al., 2004) that the amount of virus in genital lesions, as detected by qPCR, was higher during first episodes of genital herpes infection compared with recurrences, although in our study this difference did not reach statistical significance.

It was not possible to compare viral load during first episodes and recurrences in orofacial herpes because the majority of cases (31/32 (98.9%)) were recurrent infection.

„Wet“ swabs versus „dry“ swabs. As storing samples till analysis as „wet“ or „dry“ swabs enables HSV DNA detection, they both can be used in routine practice for HSV DNA detection.

5.1.4. Herpes simplex virus serology

According to the published data, more than 50% of adults have antibodies to HSV-1 and only 20%-40% have experienced labial herpes outbreaks at some time (Boivin, 2006). In a study from the United States where 16.2 % of patients were HSV-2 seropositive only 18.9% knew of being infected (Wald, 2004). In the present study the majority of the patients and controls (86.7% - genital, 97.1% - orofacial infection, 90% - controls) had anti-HSV-1 IgG antibodies This is mostly due to the labial HSV infection in the past. Our data show that less than half of patients (48.9%) with genital HSV infection reported episodes of labial herpes in the past. Five patients with negative PCR had antibodies to HSV-2. Seven subjects from the control group were HSV-2 seropositive but genital herpes episodes in the past were reported by only 2 of them.Therefore serological testing results are sometimes important for establishing a diagnosis.

Our data are consistent with other studies of HSV infection that some persons are unaware of having HSV infection because many cases are undiagnosed or asymptomatic (Goldman, 2000).

5.2. Experimental part of the study

This work presents for the first time an extensive analysis of HSV-1 and HSV-2 LAT promoter sequences in clinical samples. It was our aim to test the hypothesis that variation in the LAT promoter region influenced clinical and pathobiological variables of HSV-human interaction, e.g. frequency of recurrence or the site of infection.

HSV LAT is located in the long repeats and are present in two copies per viral genome (Perng et al., 2002). The repeated regions of DNA are the essential element in HSV-1 genome (Ouyang et al, 2012) found mostly in non coding regions with characteristic high level of polymorphism (Deback et al, 2009). The main focus was on HPTs as such mononucleotide repeats were

reported to be dominant in microsatellites of the HSV-1 genome (Deback et al, 2009) as well as in others organisms (Kashi and King, 2006).

As already mentioned, HPTs are prone to a high level of variability. Functionally significant mutations in homopolymer stretches due to their ability to expand and shrink were detected to play a role in HSV (Griffiths, 2011; Sasadeusz et al, 1997; Piret and Boivin, 2011) and other viruses (Duke et al, 1990) as well as in eukaryotes and prokaryotes (Vinces et al, 2009; Li et al, 2004; Coenye and Vandamme, 2005; Grimwood et al, 2001). SSRs also have been detected in promoter regions and were shown to influence gene expression (Vinces et al., 2009;).

We analyzed 145 clinical samples and found that sequences of HSV LAT promoter contained variable HMP tracts. As the HSV genome, including the LAT promoter, is G+C rich (the base composition of G+C is 68% for the entire HSV-1 and 69% for the entire HSV-2 sequence), the majority of homopolymer sequences in our study, as expected, were composed of „G“ or „C“ nucleotide tracts. As mentioned in results, we analyzed mononucleotide sequences composed of at least 7 nucleotides.

In general, alignment of LAT promoter sequences showed a variable number of nucleotide residues in all homopolymer tracts in HSV LAT promoter region. The samples with DNA amplicons sequenced directly showed variability between strains, while we detected different number of nucleotides in different clones from the same sample in many cases, i.e. variation within an individual.

We found 5 homopolymer tracts in HSV-1. Deback et al (Deback et al, 2009) (due to a higher cutoff of 9 nucleotides in HPT) detected 3 variable homopolymer tracts (C_{10} 118568-118577 nucleotide position, T_9 119301-119309 and C_{10} 119356-119365) which corresponded to **Poly(C_n)1**, **Poly(T_n)** and **Poly(C_n)2** in our study. Deback et al found several variants of HMT (5 HMT variants for 12 strains) and concluded that individual strains have their own profile of variable HPT which could be used in strain identification (Deback et al, 2009).

Three variable HPTs (2 poly(G) and 1 poly(C)) were detected in HSV-2 LAT promoter in our study. As HSV-2 has been studied less extensively compared with HSV-1, data regarding HSV-2 homopolymer variability are obscure, mostly regarding resistance to thymidine kinase.

5.2.1. Polymerase slippage

Homopolymer stretches are extremely difficult to analyze (Kieleczawa, 2006) due to polymerase slippage occuring during DNA amplification (Linnertz et al, 2012). Polymerase slippage is caused by slipped strand mispairing (van Belkum et al, 1998), therefore less or more nucleotides (−1, −2, +1, +2, and so on) can occur in a newly polymerized strand compared with the template strand (Figure 10). Sequences that contain mononucleotide repeats are particularly vulnerable to +1 or −1 insertion/deletion errors when copied by DNA polymerase. Gragg et al. showed that polymerase slippage occurs more often in poly(C)/poly(G) than in poly(T)/poly(A) runs; at the same time poly(T)/poly(A) tracts are repaired more efficiently than poly(C)/poly(G) HMTs (Gragg et al., 2002).

Nevertheless PCR is one of the most widely used techniques in diagnostics and research Clarke at al. described a high error rate occuring in PCR amplification of mononucleotide and dinucleotide repeats from genomic DNA (Clarke et al, 2001). They analyzed poly(T) stretches with different number of „T“ residues and found that sequencing of individual cloned PCR products showed correct amplification of short poly(T) stretches and 11 „T“ nucleotides were the limit of correct amplification. Longer stretches (T_{13}, T_{23}) showed an incorrect number of residues with a tendency of HPTs to contract. Thus, the frequency of errors on a homopolymeric sequence during DNA amplification increases with the length of the homopolymeric tract (Clarke et al, 2001).

Slippage is not completely avoidable (Linnertz et al, 2012) and also occurs in vivo, which probably the reason why homopolymer tracts are

hypervariable and are particularly susceptible to frameshift mutations (Sasadeusz et al, 1997) which can be restored by the DNA mismatch/mutation repair system. Other sequences located near HPTs also could play a role in frameshifting (Pan and Coen, 2012). In humans, there is a connection between several cancers, notably colon cancer, microsatellite instability and a deficient mismatch system (Ramel, 1997; Duval et al., 2002; Li et al., 2004). If the DNA mismatch system is inadequate, deletions or insertions of repetitive sequences can occur (van Belkum et al, 1998).

Alternatively, variation in repeat numbers can be explained by DNA recombination between multiple loci consisting of homologous repeat motifs (Gemayel et al, 2010; Richard et al, 2000).

5.2.2. Variable homopolymer „C“ and „G“ tracts

In our study 3 out of 4 HSV-1 and 3 HSV-2 cloned homopolymer regions showed different number of nucleotides in both PCR amplicons and clones. First we interpreted that this was due to polymerase slippage during DNA amplification (Linnertz et al, 2012). However, the sequences of HPTs showed the same number of HPT mononucleotide residues in both directly sequenced cloned DNA and in the same cloned DNA which has undergone PCR amplification prior to sequencing, in all tested samples (Figure 8-9). In our opinion, these results could indicate that variable number of mononucleotides were due to HPTs variability and not to PCR artefacts as reported by Rocha et al. (Rocha et al, 2002).

To confirm this fact, the **Poly(G_n)2** region reaching up to 12 „G“ residues in HSV-1 showed variability only between strains: the nucleotide number was stable and fixed in both directly sequenced PCR amplicons and cloned DNA.

5.2.3. Variable homopolymer „T“ tract in HSV-1

We found different number of „T“ residues in HSV-1. Short and long poly(T) patterns were patient specific. Each strain was characterized by its own „long“ or „short“ profile of poly(T) streches. Moreover, 2 samples were obtained from the same patient at two different times and sequencing showed a long poly(T) profile in both cases. Deback et al. (Deback et al, 2009) demonstrated that HSV-1 microsatellites remained stable for any given strain over time in cell culture in vitro and in vivo and sequential HSV-1 isolates from the same patient showed the same microsatellite pattern.

5.2.4. Role of simple sequence repeats in promoters

Vinces et al. (Vinces et al, 2009) showed that mutations of SRRs promoters alter gene expression in different species of yeasts. Variable lengh of microsatellites in promoter may down- or up regulate gene transcriptional activity by several mechanisms: 1) increasing or decreasing the number of binding sites for binding factors; 2) affecting the distance/spacing between regulatory elements/important domains; 3) altering the nucleosome positioning, i.e. the structure of chromatin by affecting histone binding to DNA and allowing a gene to be silenced or activated depending on how tightly the region was bound (Vinces et al, 2009).

HSV DNA is not associated with histones inside the virion. Upon entry into the host cell nucleus, host cell mechanisms assemble chromatin on HSV DNA. During latency the viral genome is circular and is associated with nucleosomes. It was hypothesized that HSV uses the LAT gene to promote the formation of heterochromatin, to decrease the amount of active euchromatin on viral lytic-gene promoters and shut down the expression of these genes. Therefore, during latent infection the LAT gene is associated with “active” euchromatin whereas the lytic genes are associated with “inactive”, i.e., compact heterochromatin (Knipe and Cliffe, 2008).

Several classes of repetitive DNA sequences can form an unusual left handed form of DNA, called Z-DNA, which may block binding of regulatory proteins to the promoter or weakly enhance gene transcription (Gemayel et al, 2010).

Poly(A)/Poly(T) tracts are often found upstream of genes in eukaryotes (Shimizu et al, 2000). Many yeast promoters contain homopolymeric poly(T) and poly(A) sequences, so called T-tracts. Such sequences are important promoter regulatory elements which were detected to play a functional role for wild-type levels of gene transcription in yeasts. T-tracts form unusual rigid DNA structure which is different from typical B-DNA and destabilize nucleosome formation by keeping an open chromatin structure, i.e regions of free DNA (Iyer and Struhl, 1995). Such homopolymer sequences are supposed to influence transcriptional activity via their own intrinsic DNA structure (Iyer and Struhl, 1995; Suter et al, 2000) by increasing accessibility of DNA to transcription factors (Iyer and Struhl, 1995). The unusual structure of poly(T) tracts is length dependent (Jansen and Verstrepen, 2011): the function of such homopolymer runs increases with their length (Iyer and Struhl, 1995; Shimizu et al, 2000). Several studies suggested that variable poly(T)/Poly(A) stretches play a significant role in establishing nucleosome free regions and therefore affect gene expression in nearby regions (Jansen and Verstrepen, 2011; Yuan et al, 2005; Shimizu et al, 2000; Segal and Widom, 2009) .

Iyer et al. (Iyer and Struhl, 1995) also tested the transcriptional effects of poly(G) and poly(C) stretches which also formed unusual rigid DNA structure affected nucleosome formation. In spite of that Poly(T)/Poly(A) and Poly(G)/Poly(C) form completely different structures, they both showed similar transcriptional effects (Iyer and Struhl, 1995).

In contrast to yeast, the HSV genome, including the LAT promoter, is G+C rich. Human promoters typically have a high G+C content and high intrinsic nucleosome occupancy whis is supposed to restrict access to

regulatory information that will ultimately be utilized in only a subset of differentiated cells (Tillo et al, 2010).

Our data show that the LAT promoter region is G+C rich and contains variable HPTs but their function has not yet been determined. The paucity of stop codons appearing by random mutation in G+C sequences creates unique opportunities to maintain several open reading frames in overlapping frames, and obtain alternative coding sequences from variable HPTs. Thus, HPTs can cause much more amino acid diversity than their length variation indicates, because the amino acid sequences of overlapping reading frames can be very different. One nucleotide difference in an HPT can cause a rather drastic difference in the downstream amino acids.

5.2.5. Variability of homopolymer tracts in HSV and other organisms

The genetic mechanism of bacterial adaptation to host enviroment (phase variation) in many cases is expansion and contraction of homopolymer sequences. Variation in poly(G) streches of polymorphic membrane proteins genes pmp10 within and between strains of *Chlamydia pneumoniae* is supposed to play a role in chlamydial virulence and pathogenesis (Grimwood et al, 2001), changes in outer membrane proteins (Ppp) influence pathogenesis of *C. Pneumonie* (Rocha et al., 2002). A truncated poly(C) region dramatically attenuates the pathogenicity of the Mengo virus in mice (Duke et al, 1990).

Functionally significant mutations as a hot spot in homopolymer satellites were detected also in other DNA regions of HSV-1 and HSV-2. High G+C content and „G“, „C“ homopolymer tracts are related to mutations in the thymidine kinase (TK) gene which is important in resistance to antiviral drugs and plays a role in HSV reactivation from latency. Almost half of the mutations which cause HSV resistance to acyclovir are due to insertions or deletions in homopolymeric stretches of guanines (G_8, G_9, G_6 TK mutants) and cytosines (C_5 TK mutants). Such mutations can lead to a frameshifting which cause synthesis of a nonfunctional protein (Piret and Boivin, 2011;

Sasadeusz et al., 1997). In spite of lower levels of TK activity in mutants, HSV was able to reactivate from latency. It was hyphothesised that this could be due to HSV mutants with reversion to the active TK phenotype occurring as the result of genetic instability (Griffiths, 2011; Pan and Cohen, 2012). Such reversion enables virus to be both resistant and stay pathogenic at the same time (Griffiths and Coen, 2003).

Significant variability in homopolymer „T“ regions plays a role in development of Alzheimer disease in humans. Roses et al discovered that poly(T) polymorphism within intron 6 of the *TOMM40* gene in chromosome 19 influenced the onset and progression of Alzheimer disease. Three polyT distribution profiles were distinguished: short (T≤19), long (20≤T≤29) and very long (T≥30) while the number of „T“ residues in the human reference sequence was 35 „T“. Long and very long alleles were significantly associated with earlier age of disease in *APOE* gene adjacent to *TOMM40* ε3/4 and ε3/3 carriers (Roses et al, Lutz et al). The short, long and very long polyT alleles also had different distribution among different ethnic groups (Linnertz et al, 2012) .

Frameshift mutations in **HPT coding regions** can affect the structure and function of encoded protein, especially in trinucleotide repeats where they are closely related to amino acid sequences (Ouyang et al., 2012). **Poly(C_n)1** in HSV-1 is overlapping the UOL region where this transcript is transcribed in the sense direction of the LAT LAP1 promoter. Mutations in this region will affect UOL protein synthesis. However, the function of this protein in HSV latency and reactivation still remains unclear.

5.2.6. Simplots

Although the sequence variation within LAT is limited, the length polymorphisms observed in the HPTs have the potential to generate diversity both at the RNA and protein levels. The original HG52 sequence (NC_001798, Dolan et al., 1998) was done with Sanger sequencing, the other sequence

(JN561323, Davison AL, 2011) with Illumina sequencing (where the sequence is assembled from overlapping short reads of oligonucleotides). The two HG52 sequences are independent of each other. It is interesting that these HSV-2 sequences (HG52_1 and HG52_2), from the same sample, showed the same kind of ORF polymorphism as some Vilnius-Uppsala sequences, within the same sample.

Our studies on the HSV LAT promoter region both demonstrated a strong conservation and a paradoxical variety. The variety is generated by single nucleotide polymorphisms (SNPs) and by length variation in the HPTs. Hypothetically this diversity may give rise to both coding and noncoding RNAs with a variety of effects on the host cell. Two microRNAs were detected in conserved portions of the HSV-1 LAT promoter region (miR-6 and miR-1) (Umbach et al., 2008). Thus, a population of HSV virions in a blister is not homogeneous. What good does this LAT promoter diversity do to the virus? One could be that the virus becomes flexible and able to interact with different host cells, and to vary its response to transcription factors and reactivation signals.

5.2.7. Phylogenetic analysis

The cluster analysis demonstrated both that sequences from the same sample were closely similar, and that large geographic distances gave rise to differential clustering in the tree. Sakaoka et al (Sakaoka et al., 1994) studied quantitative evaluation of genomic polymorphism of HSV-1 strains from six countries (Japan, Korea, China, Sweden, USA and Kenya) and showed that HSV-1 variability was higher among non-Asian countries compared to Asia. The distance between Uppsala and Vilnius was obviously too small to give a clear sequence difference in this conserved region. Herpes simplex virus type 1 and 2 have been with humans for millions of years (Davison AJ, 2011). The few thousands of years which separate many Swedes

and Lithuanians were not enough to give clear differences in the LAT promoter sequence.

This study did not reveal a relation between LAT promoter variation and anatomical site of HSV infection. Other studies of the HSV-1 glycoprotein genes also did not show an association with the sequence variation and anatomical site of infection, including the brain (Norberg et al., 2004; Harishankar et al., 2012; Rozenberg and Lebon, 1996).

6. CONCLUSIONS

1. HSV-2 was found to be the main cause of genital herpes in both first clinical (71%) and recurrent episodes (92%). All observed cases of orofacial herpes simplex virus infection were caused by HSV-1.

2. Higher number of lifetime sexual partners, early age at first intercourse, history of other STIs, practice of oral sex were the risk factors for acquisition of genital herpes.

3. The structure of the LAT promoter region was studied applying new PCR method in 145 HSV-1 and HSV-2 clinical samples. HSV LAT promoter is G+C rich and contains variable homopolymer tracts. We found an inter- and intrastrain variability of HPTs in the promoter region, potentially giving rise to a large variation at the protein level, leading to phenotypic variation.

4. HSV LAT promoter analysis did not reveal clinically important differences between HSV strains of persons with different rates of HSV recurrence, or between HSV samples obtained from different body sites. The HSV LAT promoter sequences of the samples obtained in Lithuania and Sweden were close to each other regarding the geography.

7. PRACTICAL RECOMMENDATIONS

1. The diagnosis of genital herpes is significantly dependent on the time of sampling, even when PCR methodology is applied. PCR is usually positive if there are untreated visible ulcerated lesions but negative initial diagnostic tests in swabs from such lesions do not exclude HSV infection and subsequent repeated tests on a number of occasions should be performed for a definitive diagnosis in additional cases.

2. Storing samples before analysis as „dry“ swabs in Eppendorf tubes at room temperature enables HSV DNA detection and is convenient and effective for HSV DNA detection by PCR.

3. We analyzed 145 HSV LAT promoter sequences. When these sequences will be deposited in GenBank database they could serve as a long lasting resource for further HSV research and a basis for future HSV therapies.

8. PUBLICATION LIST

List of original papers:

- **Aukštuolienė E**, Ambrozaitis A, Friman G, Barkevičienė D, Blomberg J. Detection and quantification of herpes simplex virus DNA in genital lesions. Medicinos teorija ir praktika (Theory and Practice in Medicine). 2012;18(4.2): 349-355 (Index Copernicus).
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- **Aukštuolienė E**, Ambrozaitis A, Friman G, Barkevičienė D, Blomberg J. Role of the latency associated gene in herpes simplex virus latency reactivation cycle. Medicinos teorija ir praktika (Theory and Practice in Medicine). 2012;18(4.2): 614-620 (Index Copernicus).

Poster presentation:

- **Aukštuolienė E**, Ambrozaitis A, Friman G, Blomberg J. Detection and quantification of herpes simplex virus DNA in genital lesions. Electronic poster presentation in the International Conference „Evolutionary medicine: new solutions for the old problems“. Vilnius, Lithuania, June 12^{th}-15^{th}, 2012.

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Appendix 1.

Patient Informed Consent

Project title: Herpes simplex control of latency: Sequence variation in the promoter of the Latency Associated Transcript (LAT). Its correlation with clinical features of HSV infection.

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Introduction

The principal goal of this document is to provide a comprehensive information to a potential participant in order to help him/her in passing a decision on participation in the clinical study. In the document, information on scientific reasons of the study, the expected effect of the study and its benefit for the involved persons is provided in an understandable form. In addition, the

document informs the potential participants on their rights and duties while participating in the study.

In the Form of consent, you may find unknown words. Please, ask the researcher or the medical staff to explain you any unknown terms or phrases.

Why the study is carried out?

Herpes simplex virus (HSV) type 1 (HSV-1) and 2 (HSV-2) are common viral agents causing labial or genital herpes. They establish latency in neurons and persist for the lifetime of the host. From this site they can reactivate and be transported to the skin where virus-laden vesicles occur. The clinical picture of herpes simplex virus infection is diverse, ranging from asymptomic forms, typical vesicular lesions on the skin or mucosa to severe conditions such as neonatal herpes, meningitis, encephalitis. Some patients get more frequent and intense reactivation than others. Herpes simplex infection is an incurable disease. HSV-1 infection has a more favourable outcome than HSV-2. Because of the wide clinical spectrum of genital herpes, clinical picture, alone, usually is not sufficient to diagnose this infection and laboratory confirmation is very important.

Confirmation

Lithuanian Bioethics Committee had examined the protocol of the study and assented to arranging the study.

Participation

You'll be entitled to be involved in this study, if:

- skin or mucosal herpes infection will be suspected in you;
- your eligibility for the study will be confirmed;
- you'll provide a consent for participation in writing.

During the visit:

- your consent for participation in the study shall be obtained;
- a medical history shall be taken;
- a swab from skin lesions shall be taken for HSV nucleic acid detection;
- a blood sample shall be drawn for detection of antiviral antibodies;
- you'll be provided a questionnaire about herpes infection;

The taken samples will be explored at the Laboratory of Virology of Upsala University Hospital (Sweden).

The risk bound with the study

Blood sampling may cause a short-time discomfort. The volume of blood sample shall cause no symptoms or anemia.

The benefit of the study

Participation in the study is beneficial because the herpes virus DNA and antibodies found in the laboratory examination will show whether you've been ever infected by herpes virus of the types 1 and 2. At your request, you'll be informed on the result of the research by phone.

Voluntariness of participation

Your participation shall be voluntary. A refusal to participate in the research shall cause no consequences and shall provide no impact upon your health care. You'll be provided a signed copy of this Form.

Confidentiality

Your participation in the study shall be confidential. This means that the researchers shall follow the rules of strict confidentiality in storage and processing of any person's identification data. Only the relevant staff of the researcher or the competent institutions shall be provided an access to such data. Your name shall not be mentioned in any report on the study. Your identification data shall not be provided to any person, except of the above-mentioned circumstances.

If you wish to be provided your data, you'll apply to the doctor involved in carrying out the study.

The right to put questions

You'll be entitled to put questions about the study. If you have any questions, please, contact with the Principal researcher:

Full name of the researcher:
The address of the researcher:
The phone No. of the researcher:
The fax No. of the researcher:

THE FORM OF CONSENT OF THE INFORMED PERSON

The goals and the procedures of the study were explained to me in an understandable way. I have read the above-provided information sheet and I have understood the provided information. I hereby provide my consent for participation in the study. I understand that I am entitled to refuse participation in the study for any reason without any consequences for care of my health and attention for the side of my provider of health care services both at present and in future. I confirm that I was provided a copy of this Consent that is usable as a base in future.

I (*the full name*) _____

voluntarily consent to be involved in this clinical study.

The signature of the Participant:_____

Date: _____

Confirmation by the physician having contacted with the informed person on providing the consent:

I have carefully explained the essence, goals, expected risks and benefits of the study to the above-mentioned person, answered all questions before signing the Form of Consent and certified signing the Form of Consent by the Informed Person.

Full name _____

Signature:_____

Position:_____

Date: _____

Appendix 2.

Questionnaire about herpes simplex infection

1. Age _____
2. Sex _____
3. Marital status: single ☐, married ☐, divorced ☐, widowed ☐
4. Education: university ☐, college ☐, school ☐
5. Social status: employed ☐, unemployed ☐, student ☐, pensioner ☐
6. Smoking? yes ☐, no ☐
7. Height_____, weight _____

8. Your complaints:

pain ☐ duration ____days ,
vesicles, ulcers ☐ duration ____days,
erythema ☐ duration ____days,
fever ☐ duration ____days,
enlarged lymphnodes ☐ duration ____days,
headache ☐ duration ____days,
photophobia ☐ duration ____days

9. Is this the first episode of herpes infection in your life? yes ☐, no ☐.
10. Have you ever sufferred from such herpes episodes in the past? yes ☐, no ☐
11. Rate of recurrences per year _____
12. Have you ever used antiviral drugs (aciclovir, valaciclovir, famciclovir)?

Additional questions for the patients with genital herpes :

13. Age at first intercourse: _____
14. Lifetime number of sexual partners: _____
15. History of STI: yes ☐, no ☐. If yes, please specify (clamidial infection, urethritis, colpitis, syphilis, gonorrhea, bacterial vaginosis, trichomoniasis, genital warts, HIV, etc.)
16. Oral sex: yes ☐, no ☐
17. Sex workers or being paid for sex: yes ☐, no ☐
18. Condom use: always ☐, occasionally ☐, never ☐
19. Have you ever sufferred form labial herpes? yes ☐, no ☐. If yes, rate of recurrences of labial herpes per year: ____