# Thesis progression

**Thesis title:** Association of human herpesvirus 6 (HHV-6) with depression and its predictive biomarkers

**Student:** Areeya Suktas Student ID: 647070031-5

**Advisor:** Asst. prof Sirinart Aromseree

**Co-advisor:** Prof. Chamsai Pientong, Asst. Prof. Tipaya Ekalaksananan, and Asst. prof Sureewan Bumrungthai

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## 1. Introduction

Depression is a major common mental disorder in all ages. According to the World Health Organization (WHO), depression affects up to 3.8% of the worldwide population, of those 5.0% are adults and 5.7% are over 60 years old. In Thailand, it is estimated that 1.5 million Thai people suffer from depression. Females had a higher prevalence compared with males at 2.9% and 1.7%, respectively [1]. Symptoms of depression are divided into psychological symptoms (such as continuous low mood or sadness, feeling hopeless and helpless, having suicidal thoughts, feeling tearful, feeling anxious or worried, not getting any enjoyment out of life, having no motivation or interest in thing, feeling guilt-ridden, finding it difficult to make decision) and physical symptoms (such as unexplained aches and pains, moving or speaking more slowly than usual, changes in appetite or weight, constipation, changes to your menstrual cycle, insomnia or lack of sleep, low sex drive, lack of energy) Various factors have been determined to influence the development of depression such as genetics, brain chemistry, certain medical conditions, substance use, stress, poor nutrition, viral infection, and environment. The theories about depression symptoms are, mostly believed to cause by chemical imbalance in the brain like serotonin imbalance that is associated with various factors. However, antidepressants to increase serotonin levels are still ineffective to relieve symptoms. Recently, animal models showed that depression is associated with the decreased generation of new cells in the hippocampus -a region of the adult brain known to regulate emotion, mood, cognition, and stress [2]. Mitochondria are the main source of energy and are associated with the decreased generation of new cells in the hippocampus. Therefore, the dysfunction of mitochondria can lead to a cascade of effects that result in depression [3].

The infection of several viruses is reported as the risk factor for depression including Cytomegalovirus (CMV), and found that higher CMV antibodies are associated with a higher incidence of depression, but it is yet unknown how CMV infection induces depression [4]. Patients with herpes simplex virus 2 (HSV-2) infection are at a significantly higher risk for depression [5]. In addition, the prevalence of

depression in patients with Hepatitis C virus infection (HCV) is 1.5 to 4.0 times higher than in the general population [6]. These viruses are not yet clear about the mechanism of virus-induced depression.

Recently human herpesvirus 6 (HHV-6) has been reported as a risk factor of depression. The infection of HHV-6 through saliva via nasopharynx and olfactory entry routes causes roseola in the initial infection and then the virus is persistent in monocytes and bone marrow progenitor [7]. HHV-6 is reactivated in response to immunosuppression in transplant patients and use of some drugs. HHV-6 reactivation certainly leads to neurological diseases such as multiple sclerosis, encephalitis, epilepsy, and depression. Furthermore, HHV-6 can integrate the genomes into chromosomal telomeres in somatic and germ cells, around 1% of the population the HHV-6 genome is present in all nucleated cells of the body [8].

Additionally, high infection rate of Purkinje neurons with the human herpesvirus (HHV-6) was found in patients with bipolar disorder and/or severe depression. Purkinje cells are a central part of the human cerebellum [9], the part of the brain that plays an important role in motor learning, fine motor control of the muscle, equilibrium, and posture but also influences emotions, perception, memory, and language. Therefore, HHV-6 are capable of infecting neurons and possibly causing cognitive disturbances leading to mood disorder.

The molecular mechanisms behind HHV-6 mediated cellular damage of Purkinje neurons are reported. Transcript of HHV-6-1 (SITH-1), or “small protein encoded by intermediate state transcript,” is produced via expression of an HHV-6 latency-associated gene. Additionally, anti-SITH-1 antibody is found in high concentration in depression, psychiatric disorder, chronic fatigue syndrome. It has been reported that inserting the SITH-1 gene into adenoviral vector and introducing it into mice, can induce depression symptoms. Corticotropin releasing hormone (CRH), urocortin, and REDD1 (an acronym for “regulated in development DNA responses-1) are proteins activated by HHV-6B latency in the hypothalamus. REDD-1 is increased in the prefrontal cortex in autopsy samples from patients with major depression, and urocortins play a role in regulating anxiety and social behavior. SITH-1 production also results in increased intracellular calcium levels, a common finding in depression and psychiatric disorders [10].

The genetic family history of depression is another significant potential cause that may increase risk of developing the depression condition. It has been reported that the heritability rate for depression is approximately 40%. Many studies have searched for the candidate genes involved in progression of depression. Various methodological approaches (analysis of candidate genes, genome-wide association analysis, genome wide sequencing) have been used, and a large number of associations between genes and different clinical depression have been published. In most studies, the association of these genes have been proven to be depression development. However, important role of intergenic interactions and the underlying mechanism has not been studied [11, 12]

Currently, the diagnosis of major depressive disorder (MDD) mainly relies on clinical examination and subjective evaluation of depressive symptoms. At present, there is no approved biomarker as part of the diagnostic criteria for any psychiatric disorder [13]. Treatment and diagnosis choices can be influenced by biomarkers. Many studies are looking for biomarkers, but there are still problems. Some of the currently

discovered biomarkers such as BDNF (Brain Derived Neurotrophic Factor), 5-hydroxy tryptamine (5-HT) can be used for diagnosis and treatment that effect on some patients due to their low sensitivity and specificity [13, 14]. Further studies and investigations are needed for biomarkers of depression.

To understand the association between risk factors, HHV-6 infection, and genetic factors that give rise to depression development. This study aims to determine an association of risk factors, HHV-6infection and genetic landscape with depressive symptoms using for construction of panel biomarkers and evaluate for its prediction.

## 2. Objective

**2.1.** To determine the risk factors and common gene polymorphism of depression.

**2.2.** To determine the association of HHV-6 infection with depression.

**2.3.** To determine the association of depression with their genetic landscape.

**2.4.** To investigate the interaction between risk factors and construct the panel biomarkers for prediction of depression.

## 3. Materials and methods

### 3.1. DNA extraction from a saliva sample

Saliva samples were collected in the morning before eating and drinking for up to 1 hour. The saliva sample was stored at -20°C. A saliva sample of 300 ul were used to extract DNA. Add 300 ul of TES buffer and 5 ul of RNase, and incubate at room temperature for 30 min. Add 200 ul of protein precipitation buffer, mix by inverting, and centrifuge at 13,000 rpm at 4 °C for 5 min. Transfer the supernatant to a new tube, add 1 volume of isopropanol, and incubate at -80°C for at least one hour. Centrifuge at 13,000 rpm at 4 °C for 15 min, discard the supernatant, and wash the pelleted cell with 1 ml of 70% ethanol. centrifuged at 13,000 rpm at 4 °C for 5 min (repeat this step again). Dry DNA and add 30 ul of nuclease-free water, stored at -20°C.

### 3.2. Human herpesvirus detection by nested PCR and real-time PCR

Human herpesvirus DNA from healthy were detected by nested PCR. Amplification reaction was performed in total volume 25 µl. The first reaction consists of two mixtures, including sense-1 and antisense-1. The reaction mixture contains 10X PCR buffer minus Mg, 10 mM dNTP mixture, 50 mM Mg2Cl2, 10 uM primer forward, 10 uM primer reverse, DNA template, Taq DNA polymerase, and distilled water, as shown in Table 2. The cycling conditions were started with 40 cycles of Pre denaturation at 94°C for 3 min, denaturation cycles at 94°C for 30 s, annealing at 47°C (first amplification) for 1 min and extension at 72°C for 30 s, followed by a cycle of final extension at 72°C for 10 min as show in Table

3. 2 ul of each amplified product were analyzed by electrophoresis on 3% agarose run at 100 volts for 50 min. The samples that were positive from the first amplification are used to determine the types of herpesviruses, including HSV-1 & HSV-2, EBV (EBNA1), EBV (LMP1), and HHV-6, by using real-time PCR. The reaction contains 5X FIREPol Master Mix Ready to Load, 10 uM Primer forward, 10 uM Primer Reverse, DNA template, and distilled water as shown in Table 4-5. The cycling conditions started with 40 cycles of Initial activation at 95 °C for 12 min, denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec, and melting at 65-95 °C for 5 sec.

### 3.3 DNA and RNA extraction by TRIzol method

Saliva samples were subjected to simultaneous DNA and RNA extraction using the TRIzol reagent method. Briefly, 250 µl of saliva was mixed with 750 µl of TRIzol reagent and incubated at room temperature for 5 min. Subsequently, 200 µl of chloroform was added, and the mixture was incubated for 2–3 min before centrifugation at 12,000 × g at 4 °C for 15 min. Following phase separation, the aqueous phase was collected for RNA extraction, while the interphase was reserved for DNA extraction. Subsequently, 500 µl of isopropanol was added to the aqueous phase, followed by incubation at −80 °C for 1 h. The samples were then centrifuged at 12,000 × g at 4 °C for 10 min. After discarding the supernatant, the RNA pellet was washed twice with 75% ethanol by centrifugation at 7,500 × g at 4 °C for 5 min. Finally, the RNA was eluted in 30 µl of TE buffer and incubated at 55–60 °C for 10 min to ensure complete dissolution. DNA was precipitated from the interphase by adding 300 µl of ethanol, followed by incubation at −20 °C for 15 min and centrifugation at 2,000 × g at 4 °C for 5 min. The resulting DNA pellet was washed with 0.1 M sodium citrate in 10% ethanol and incubated for 30 min. The samples were then centrifuged at 2,000 × g at 4 °C for 5 min, and this wash step was repeated three times. Subsequently, the DNA pellet was washed three times with 75% ethanol, each followed by centrifugation at 2,000 × g at 4 °C for 5 min. The pellet was air-dried for 5 min and resuspended in 30 µl of 10 mM Tris-HCl. The concentrations and purity of both DNA and RNA were assessed using a NanoDrop spectrophotometer by measuring absorbance ratios at 260/280 and 260/230 nm.

### 3.4 16s rRNA detection by PCR

DNA extracted from saliva samples was used to determine the presence of 16S rRNA by polymerase chain reaction (PCR). The reaction mixture contains 10X PCR buffer minus Mg, 10 mM dNTP mixture, 50 mM Mg2Cl2, 10 uM primer forward (27F 5'-AGAGTTTGATCCTGGCTCAG-3'), 10 uM primer reverse (1492R 5' TACGGTTACCTTGTTACTT-3'), DNA template, Taq DNA polymerase, and distilled water. The cycling conditions were started with 40 cycles of pre-denaturation at 94°C for 3 min,

denaturation cycles at 94°C for 30 s, annealing at 52°C for 30 sec and extension at 72°C for 30 s, followed by a cycle of final extension at 72°C for 10 min. 5 ul of each amplified product were analyzed by electrophoresis on 1.5 % agarose run at 100 volts for 50 min.

### 3.5. Statistical analysis

The data were analyzed using IBM SPSS software version 28. A univariate analysis using Pearson's chi-squared test was used to compare the categorical variables between groups. An independent Student's t-test was used to compare separate mean ± standard deviation (SD) sets. Factors that were found significant association with depression in univariate analysis ($p < 0.05$), and without association in the univariate analysis but found to play important roles as factors for CCA risk from literature reviews were included in the multivariate analysis using logistic regression to determine the association between risk factors and depression. The interaction between viral infection, smoking, and alcohol consumption by logistic regression $p < 0.05$ was considered statistically significant.

**Table 1 Primer sequence for PCR**

| Reaction | Primer | | Sequence (5'-3') | PCR product size |
|---|---|---|---|---|
| **First** | Sense-1 | HSV1 & HSV-2 | 5'-CGCATCATCTACGGGGACACGGA -3' | 194 bp |
| | | EBV | 5'-CGAGTCATCTACGGGGACACGGA -3' | |
| | | HHV-6 | 5'-GAGGTAATTTATGGTGATACGGA -3' | |
| | Antisense-1 | HSV-1 & HSV-2 | 5'-ATGACGCCGATGTACTTTTTCTT-3' | |
| | | EBV | 5'-AGCACCCCCACATATCTCTTCTT-3' | |
| | | HHV-6 | 5'- TGTCTACCAATGTATCTTTTTTT-3' | |

**Table 2 PCR component for PCR**

| Reagent | Final Conc. | Volume for 25 ul reaction |
|---|---|---|
| 10X PCR Buffer, minus Mg | 1X | 2.5 ul |
| 10mM dNTP mixture | 0.2 mM | 0.5 ul |
| 50 mM $MgCl_2$ | 1.5 mM | 0.75 ul |
| 10 uM Primer forward | 0.2 uM | 0.5 ul/each |
| 10 uM Primer Reverse | 0.2 uM | 0.5 ul/each |
| Template DNA 30 ng/ul | 60 ng | 2 ul |
| Taq DNA polymorease | 1-2.5 unit | 0.125 ul |
| Distilled water | - | 13.125 |

**Table 3 Thermocycling conditions for PCR**

| Cycle step | Temp. | Time | Cycles |
|---|---|---|---|
| Pre denaturation | 94 °C | 3 min | |
| Denaturation | 94 °C | 30 sec | 40 cycles |
| Annealing | 47 °C (first round) | 30 sec | |
| Extension | 72 °C | 30 sec | |
| Final extension | 72 °C | 10 min | |

**Table 4 Primer sequence for real-time PCR**

| Reaction | Primer | | Sequence (5'-3') | PCR product size |
|---|---|---|---|---|
| **First** | Forward | HSV1 & HSV-2 | 5'-CGCATCATCTACGGGGACACGGA -3' | 120 bp |
| | | EBV (EBNA1) | 5'-CAAAACCTCAGCAAATATATGAG -3' | 99 bp |
| | | HHV-6 | 5′-GCTAGAACGTATTTGCTGCAGAACG-3′ | 258 bp |
| | Reverse | HSV-1 & HSV-2 | 5'-ATGACGCCGATGTACTTTTTCTT-3' | 120 bp |
| | | EBV (EBNA1) | 5'-CCACAATGTCGTCTTACACC -3' | 99 bp |
| | | HHV-6 | 5′-ATCCGAAACAACTGTCTGACTGGCA-3′ | 258 bp |

**Table 5 PCR component for real-time PCR**

| Reagent | Final Conc. | Volume for 10 ul reaction |
|---|---|---|
| 5X FIREPol Master Mix Ready to Load | 1X | 2 ul |
| 10 uM Primer forward | 0.1-0.3 uM | 0.2 ul |
| 10 uM Primer Reverse | 0.1-0.3 uM | 0.2 ul |
| Template DNA 30 ng/ul | 60 ng | 2 ul |
| Distilled water | - | 5.6 ul |

**Table 6 Thermocycling conditions for real-time PCR**

| Cycle step | Temp. | Time | Cycles |
|---|---|---|---|
| Initial activation | 95 °C | 12 min | |
| Denaturation | 95 °C | 15 sec | |
| Annealing | 60 °C | 30 sec | 40 cycles |
| Melting | 65-95 °C | 5 sec | |

## 4. Results

To investigate the presence of herpesviruses in saliva samples from individuals with major depressive disorder (MDD) and healthy controls (HC), polymerase chain reaction (PCR) and real-time PCR were performed. The pattern of herpesvirus releasing in saliva samples was significantly lower for overall herpesviruses (p = 0.009, CI: 0.13–0.75) and Epstein–Barr virus (EBV) (p = 0.009, CI: 0.18–22.06) in individuals with major depressive disorder (MDD) compared with healthy controls within the young adult group (18–30 years), as shown in Figure 1. In addition, the pattern of all herpesvirus types showed an increasing trend among individuals aged 31–43 years.

Multivariate analysis was conducted to estimate adjusted odds ratios (ORs) and 95% confidence intervals (CIs). Several factors were significantly associated with an increased risk of MDD, including familial relationship conflicts (OR = 12.93, 95% CI: 21.25–134.17), alcohol consumption (OR = 3.44, 95% CI: 1.05–11.33), smoking status (OR = 4.93, 95% CI: 1.26–19.33), and stress levels (OR = 71.89, 95% CI: 19.90–259.73), as shown in **Figure 2, Table 7.** In addition, a significant interaction between saliva herpesviruses releasing factor (HSV, EBV, and HHV-6) with smoking and alcohol, was associated with an increased risk of major depressive disorder, as shown in **Table 8**.

DNA extracted from saliva samples was used for microbiome analysis. Salivary DNA samples were screened for bacterial presence by amplification of the 16S rRNA gene to confirm suitability for microbiome profiling, as shown in **Figure 4**. Microbiome analysis was stratified into six groups to facilitate comparison of microbial composition across groups.

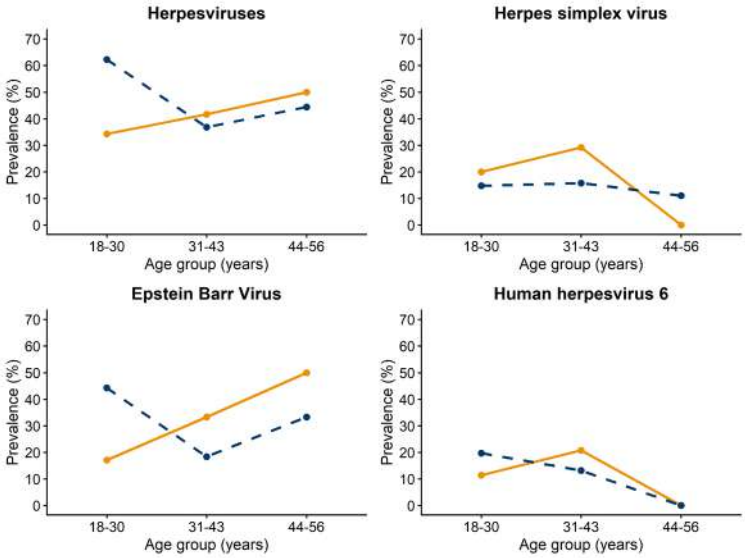



**Figure 1**: Salivary herpesvirus detection among individuals with depression and healthy controls, categorized into three age groups: young adults (18–30 years), adults (31–43 years), and middle-aged adults (44–56 years), in the Thai population. A: Herpesvirus, B: Herpes simplex virus, C: Epstein-Barr virus (EBNA1, LMP1), D: Human herpesvirus 6.

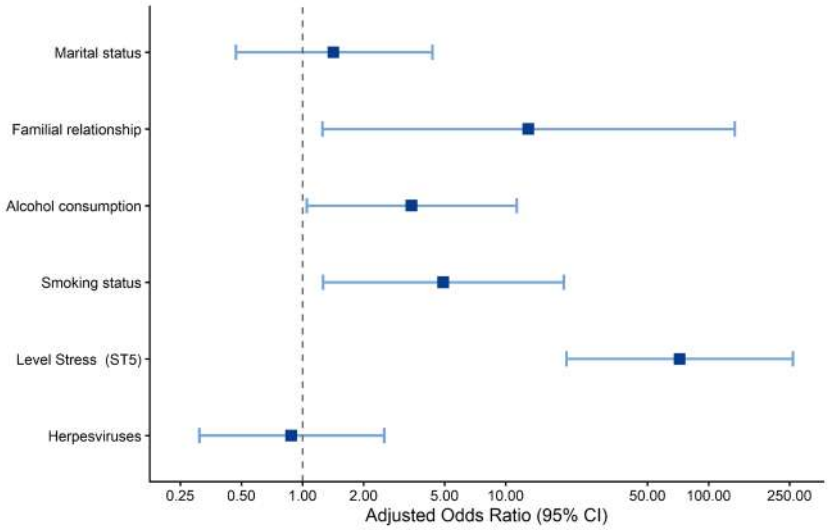



**Figure 2:** Forest plot of multivariate analyses determining the associations between environmental factors, saliva herpesviruses releasing factors and risk of major depressive disorder (MDD) in the Thai population.

**Table 7** Multivariate analysis of environmental factors and viral infection factors with the risk of Major depressive disorder (MDD) in the Thai population

| Environmental factors | | Cases | | Controls | | OR | OR* | 95% CI* | P-value |
|---|---|---|---|---|---|---|---|---|---|
| | | n | Percent | n | Percent | | | | |
| Marital status | Single | 49 | 77.8 | 62 | 57.4 | 2.60 | 1.42 | 0.47-4.36 | 0.535 |
| | Married | 14 | 22.2 | 46 | 42.6 | | | | |
| Familial relationship | Quarrel | 13 | 20.6 | 2 | 1.9 | 14.06 | 12.93 | 1.25-134.165 | 0.032 |
| | No Quarrel | 50 | 79.4 | 106 | 98.1 | | | | |
| Alcohol consumption | Yes | 40 | 63.5 | 40 | 37 | 2.96 | 3.44 | 1.05-11.33 | 0.042 |
| | No | 23 | 36.5 | 68 | 63 | | | | |
| Smoking status | Yes | 29 | 46 | 16 | 14.8 | 4.90 | 4.93 | 1.26-19.33 | 0.022 |
| | No | 34 | 54 | 92 | 85.2 | | | | |
| Level Stress (ST5) | Moderate severe-Severe stress | 58 | 92.1 | 17 | 15.7 | 62.09 | 71.89 | 19.90-259.73 | <0.001 |
| | Mild-Moderate stress | 5 | 7.9 | 91 | 84.3 | | | | |
| Herpesviruses | Pos | 24 | 38.1 | 56 | 51.9 | 0.57 | 0.88 | 0.31-2.52 | 0.808 |
| | Neg | 39 | 61.9 | 51 | 48.1 | | | | |

**Table 8** Interaction between saliva herpesviruses releasing factor with smoking and alcohol on the risk of major depressive disorder (MDD) in the Thai population.

| Viral infection | Smoking/ Alcohol consumption | Cases | | Controls | | OR* | 95% CI* | P-value |
|---|---|---|---|---|---|---|---|---|
| | | n | Percent | n | Percent | | | |
| Herpesviruses | Smoking | 11 | 17.5 | 10 | 9.3 | 1.48 | 0.78-2.84 | 0.233 |
| | Non smoking | 13 | 20.63 | 46 | 42.6 | 0.15 | 0.06-0.40 | < 0.001 |
| HHV-6 | Smoking | 7 | 11.1 | 3 | 2.8 | 2.41 | 1.26-4.59 | 0.008 |
| | Non smoking | 2 | 3.2 | 15 | 13.9 | 0.25 | 0.12-0.56 | < 0.001 |
| HSV | Smoking | 6 | 9.5 | 4 | 3.7 | 4.07 | 2.10-7.87 | < 0.001 |
| | Non smoking | 8 | 12.7 | 12 | 11.2 | 0.22 | 0.10-0.48 | < 0.001 |
| EBV | Smoking | 6 | 9.5 | 3 | 2.8 | 1.89 | 1.00-3.55 | 0.050 |
| | Non smoking | 10 | 15.9 | 34 | 31.5 | 0.24 | 0.11-0.52 | < 0.001 |
| Herpesviruses | Alcohol consumption | 15 | 23.8 | 17 | 15.7 | 1.28 | 0.62-2.67 | 0.501 |
| | Non-alcohol consumption | 9 | 14.3 | 39 | 36.1 | 0.41 | 0.21-0.81 | 0.011 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HHV-6 | Alcohol consumption | 7 | 11.1 | 4 | 3.7 | 2.00 | 1.05-3.81 | 0.035 |
| | Non-alcohol consumption | 2 | 3.2 | 14 | 13 | 0.45 | 0.24-0.86 | 0.015 |
| HSV | Alcohol consumption | 8 | 12.7 | 5 | 4.6 | 3.02 | 1.54-5.92 | 0.001 |
| | Non-alcohol consumption | 6 | 9.5 | 11 | 10.2 | 0.46 | 0.25-0.88 | 0.018 |
| EBV | Alcohol consumption | 8 | 12.7 | 11 | 10.2 | 2.04 | 1.01-4.09 | 0.046 |
| | Non-alcohol consumption | 8 | 12.7 | 26 | 24 | 0.36 | 0.18-0.68 | 0.002 |

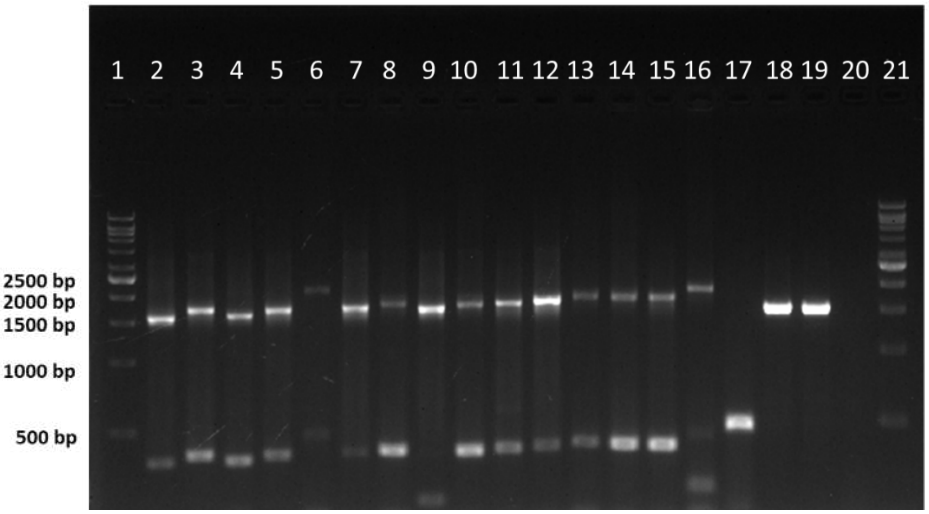


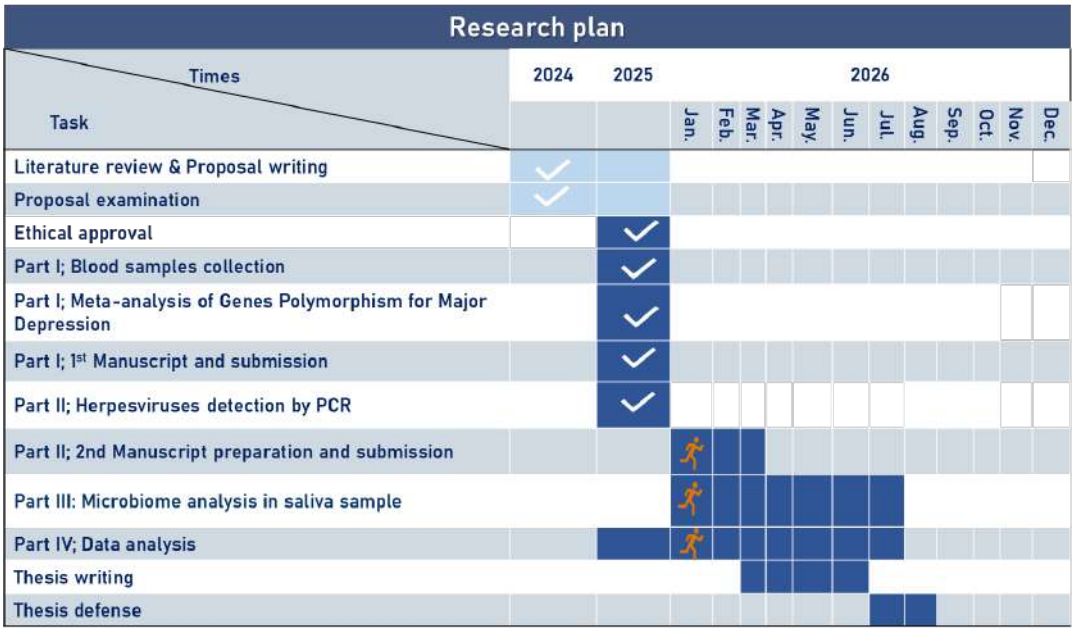

**Figure 3**: Gel electrophoresis of 16s rRNA product size approximately 1500 bp: Lane 1 marker 1 kb, Lane 2-17 DNA saliva sample, Lane 18-19 positive controls, Lane 20 negative controls, Lane 21 marker 1 kb.

## 5. Conclusion

In this study, there are two patterns of herpesvirus releasing in the saliva sample. Herpesviruses and Epstein–Barr virus (EBV; EBNA1 and LMP1) were significantly lower in individuals with major depressive disorder (MDD) than in healthy controls within the young adult group. While all herpesvirus types showed an increasing trend among individuals aged 31–43 years. Multivariate analysis revealed that familial relationship conflicts (OR = 12.93, 95% CI: 21.25–134.17), alcohol consumption (OR = 3.44, 95% CI: 1.05–11.33), smoking status (OR = 4.93, 95% CI: 1.26–19.33), and higher stress levels (OR = 71.89, 95% CI: 19.90–259.73) were significantly associated with an increased risk of MDD. Although herpesvirus releasing alone was not significantly associated with depression, significant interactions were observed

between herpesvirus releasing and alcohol consumption and smoking status, which were associated with an increased risk of MDD. These findings suggest that the risk of MDD may be influenced by multiple factors, including smoking, alcohol, and viral releasing in saliva-related interactions.

## 6. Research plan



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