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Identification of *Herpesviridae* in *Macaca Fascicularis* Using the Nested PCR Method at PSSP IPB

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ABSTRACT

PCR technique is used to detect the presence of *Herpesviridae* viruses in *Macaca fascicularis* that exhibit no clinical symptoms and identify them using nucleotide base sequences. This study aimed to detect and identify the presence of nucleotide base sequences in the *Herpesviridae* virus family in *Macaca fascicularis* at PSSP IPB. The study utilized 20 nasal swab samples from *Macaca fascicularis*. Extraction of DNA utilizing the QiaAmp™ DNA mini kit and subsequent measurement of DNA concentration. Amplification of target DNA utilizing the nested PCR technique. Sample with positive results from electrophoresis were sequenced to obtain nucleotide sequences. The average measurement value of DNA extraction concentration was 25.07 ng/µl. The DNA purity ratios at wavelengths A260/A280 and A260/A230 averaged 1.95 and 0.27, respectively. The electrophoresis results indicated a band size of 215 bp in sample code 01, corresponding to the positive control band. The sequencing results were analyzed using BLASTn on the NCBI site, revealing similarity with *Macaca fascicularis lymphocryptovirus*. The BLAST sample results demonstrated a Query Cover value of 100% and a percentage of identity of 98.84%.

Keywords: DNA, *Herpesviridae*, *lymphocriptovirus*, *Macaca fascicularis*, Nested PCR

ABSTRAK

Teknik PCR dapat digunakan untuk mendeteksi keberadaan virus *Herpesviridae* pada *Macaca fasicicularis* yang tidak menunjukkan gejala klinis dan identifikasi dilakukan menggunakan sekuen basa nukleotida. Penelitian bertujuan mendeteksi dan mengidentifikasi keberadaan sekuen basa nukleotida pada famili virus *Herpesviridae* pada *Macaca fascicularis* di PSSP IPB. Sampel penelitian menggunakan 20 sampel usap hidung dari *Macaca fascicularis*. Ekstraksi DNA menggunakan *QiaAmp™ DNA minikit* dan dilakukan pengukuran konsentrasi hasil ekstraksi DNA. Amplifikasi DNA target menggunakan metode *nested* PCR. Sampel positif dari hasil elektroforesis kemudian dilakukan penentuan urutan basa nukleotida dalam molekul DNA (sekuensing) untuk mendapatkan sekuen basa nukleotida. Nilai pengukuran konsentrasi ekstraksi DNA diperoleh nilai rataan 25,07ng/µl. Nilai rasio kemurnian DNA pada panjang gelombang A260/A280 dan A260/A230 diperoleh rataan 1,95 dan 0,27. Hasil elektroforesis diperoleh pita ukuran 215 bp pada kode sampel 01 yang sejajar dengan pita kontrol positif. Hasil sekuensing dilakukan BLASTn pada situs NCBI yang menunjukkan kemiripan dengan *Macaca fascicualris lymphocryptovirus*. Kemiripan hasil BLAST sampel diperoleh nilai *Query Cover* sebesar 100% dengan persentase identitas sebesar 98,84%.

Kata kunci: DNA, *Herpesviridae, lymphocriptovirus, Macaca fascicularis, Nested* PCR

INTRODUCTION

The Primate Study Center (PSSP) is a research institute that employs primates as model organisms for scientific investigation. *Macaca fascicularis* is frequently utilized as a model organism in primate research. *Macaca fascicularis* exhibits anatomical and physiological similarities to humans due to its phylogenetic proximity (Sajuthi et al., 2016). Diseases affecting *Macaca fascicularis* may arise from viral, bacterial, parasitic, or fungal infections (Ohta, 2023). Diseases caused by viral infections in *Macaca* include those in the *Herpesviridae* family.

Herpesviridae is a family of double-stranded deoxyribonucleic acid (dsDNA) viruses that can cause diseases in humans, mammals, birds, fish, reptiles, amphibians, and mollusks (Payne, 2017). Viruses within the *Herpesviridae* family are categorized into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Luczkowiak et al., 2019). Infections caused by *Herpesviridae* viruses in primates may present clinical and subclinical symptoms. The clinical symptoms of *Herpesviridae* virus infection in *Macaca fascicularis* may include ulceration and blistering, while subclinical symptoms are indicated by an increase in antibodies or immunity without causing visible clinical symptoms (Eberle and Jones, 2017). *Herpesviridae* virus infection in *Macaca fascicularis* can cause subclinical infections that last a lifetime without visible clinical symptoms. One of the supporting diagnostic techniques that can be used in the diagnosis of *Herpesviridae* virus infections is Polymerase Chain Reaction (PCR). The PCR technique can determine the presence of *Herpesviridae* viruses based on DNA molecule segments. PCR technique provides faster test results and high sensitivity and specificity (Lee et al., 2011).

The PCR technique has several testing methods, one of which is the nested PCR method. The nested PCR method is superior for detecting target DNA, particularly in latent infections or viruses with low copy numbers that conventional PCR methods may fail to identify. This method can detect the presence of *Herpesviridae* viruses exhibiting subclinical symptoms. The nested PCR method employs sequencing to ascertain the percentage of identity or similarity among subfamilies within *Herpesviridae* viruses with unknown nucleotide base sequences. This research aimed to detect and identify the nucleotide bases of the *Herpesviridae* virus in *Macaca fascicularis* at the breeding facility of PSSP IPB in Bogor, Indonesia.

MATERIALS AND METHOD

Materials and Tools

This study utilized 20 nasal swab samples from *Macaca fascicularis*, overseen by the veterinarian in charge of the ethics committee at the Primate Animal Study Center. Extraction reagents used were QiaAmp™ DNA Extraction Blood minikit, GoTaq® Green Master Mix, agarose powder, 1X Tris Acetic EDTA (TAE) buffer solution, ethidium bromide, and DNA marker. The tools were Laminar Air Flow (LAF), Biosafety Cabinet-level 2 (BSC level 2), NanoDrop™ One (Thermo Scientific), PCR machine, electrophoresis device, and *Gel Doc 2000* (Bio-Rad).

DNA Extraction

The extraction of DNA from nasal swab samples of *Macaca fascicularis* was performed using the QiaAmp™ DNA Extraction Blood mini kit (Qiagen, Hilden, Germany). A total of 20 µL of proteinase K, 200 µL of AL buffer, and 200 µL of sample were placed in a 1.5 ml microtube and homogenized. The solution mixture was incubated at 56°C for 10 minutes. Following incubation, 200 µL of absolute ethanol (96-100%) was added and homogenized. The solution mixture was subsequently transferred to a spin column and centrifuged at 8000 rpm for 1 minute. The supernatant was discarded, and 500 µL of Buffer AW1 was added to the spin column, followed by centrifugation at 8000 rpm for 1 minute. The resulting supernatant was discarded again, and 500 µL of Buffer AW2 was added to the spin column, then centrifuged at 8000 rpm for 1 minute. Then, 50 µL of elution buffer (AE buffer) was added and centrifuged at 8000 rpm for 1 minute (Ariyanti and Sianturi, 2019). The supernatant result was centrifuged using the elution buffer and stored as extracted DNA, ready for use in the next stage.

DNA Concentration Measurement

The concentration of DNA extracted was measured using the NanoDrop™ One (Thermo Scientific). A total of 1 µL of the sample was placed on the NanoDrop sensor and measured. The tested parameters were DNA concentration and purity against proteins, carbohydrates, and other impurities.

Formulation of PCR Reagents

The PCR reagent formulation was divided into a first and second round with a volume of 25 µL each. The first round formulation consisted of 1 µL of forward and reverse primers (see Table 1), a 12.5 µL

GoTaq® Green Master Mix, 5 µL of DNA template, and adjusted with nuclease-free water to reach a final total volume of 25 µL. The second round utilized the same PCR reagent formulation as the first, with the sole difference being the inclusion of 1 µL of the first round PCR product. The primer sequence and amplicon sizes are available in Table 1. The primers used have been adjusted to the PCR Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP), which has a very similar amino acid sequence and has been proven to be very effective in identifying and characterizing a single family. The CODEHOP strategy is used to identify new viruses and obtain amino acid sequence information for phylogenetic characterization, gene structure determination, and genomic analysis. These primers are commonly used to identify herpesvirus DNA virus families (Rose, 2005).

PCR amplification

The PCR amplification process in each first and second round began with predenaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 46°C for 1 minute, and extension at 72°C for 1 minute with a cycle used for 45 cycles. The amplification ended with a final extension at 72°C for 10 minutes and cooling at 25°C for 2 minutes (Vandevanter et al., 1996).

Visualization of the PCR Product

PCR result was visualized by electrophoresis on a 2% agarose gel. The electrophoresis process was run at a voltage of 100 V and an electric current of 400 milliamps for 40 minutes. The agarose gel electrophoresis results were observed under UV light (Gel Doc 2000) with the Quantity One (Bio-Rad) program.

Sequencing

The detected positive sample was further analyzed using sequencing. Sequencing was conducted at 1st Base Laboratories Sdn. Bhd. in Malaysia to acquire a nucleotide sequence.

Data Analysis

The DNA sequencing results were manually verified and interpreted using the chromatogram image with BioEdit software. The nucleotide sequence results were validated using data from GenBank. The NCBI search engine was utilized to identify and analyze the nucleotide sequence results, which were compared to the reference nucleotide sequence for similarity.

RESULTS

DNA Extraction Results

The success of DNA extraction was quantified by measuring the values of DNA concentration and purity against contaminants using the NanoDrop™ One tool (Thermo Scientific). Table 2 presents the results of the concentration and purity measurements of the sample DNA.

The measurement results of the extracted DNA indicated DNA concentration values ranging from 1.3 to 84.3 ng/µL, with an average concentration of 25.07 ng/µL. The purity ratio of DNA samples at wavelengths A260/A280 varied from 1.56 to 3.60, with a mean of 1.95. The DNA purity ratio at wavelength A260/A230 in the range of 0.01-1.06 was 0.27. The measurement results for three samples (codes 06, 19, and 20) indicated that the DNA purity was compromised by contaminants, as evidenced by the A260/A280 ratio. Additionally, all samples exhibited A260/A230 ratio values below the acceptable range of 1.8-2.00 (Utami et al., 2023).

Herpesviridae Virus Detection

The *Herpesviridae* virus was qualitatively detected by assessing the thickness of the resulting band. The result of the amplification process in Figure 1 was indicated by the presence of a distinct DNA ladder band, a band at 215 base pairs in the positive control, and the lack of a band in the negative control. The presence of a band in sample 01, aligned with the positive control band and measuring 215 base pairs,

Table 1 *Sequence of primary amplicons*

Process	Primer Name	Nucleotide Sequence	Amplification Size	Annealing Temperature
1st	DFA	5'GAYTTYGCNAGYYTNTAYCC '3	750 *basepair*	46°C
	KG1	5'GTCTTGCTCACCAGNTCNACNCCYTT'3		
2nd	TGV	5'TGTAACTCGGTGTAYGGNTTYACNGGNGT'3	215-315 basepair	46 °C
	IYG	5'CACAGAGTCCGTRTCNCCRTADAT'3		

Source: Vandevanter *et al.,* 1996

Table 2 Concentration and purity of extracted DNA

Sample Code	Concentration (ng/µL)	Concentration (A260/A280)	Purity (A260/A230)
01	6.9	1.95	1.06
02	3.4	1.57	0.08
03	38.9	1.97	0.07
04	11.1	1.84	0.09
05	30.8	1.56	0.09
06	13.3	2.19	0.11
07	10.5	2.09	0.12
08	1.3	1.94	0.01
09	34.5	1.61	0.32
10	34.7	1.77	0.09
11	59.8	1.89	0.19
12	13.7	1.69	0.07
13	21.5	1.76	0.14
14	31.6	1.79	0.21
15	37.4	1.84	1.08
16	19.4	2.05	0.21
17	84.3	1.94	0.86
18	16.9	1.99	0.10
19	24.7	2.14	0.35
20	6.8	3.60	0.15
Average	25.07	1.95	0.27

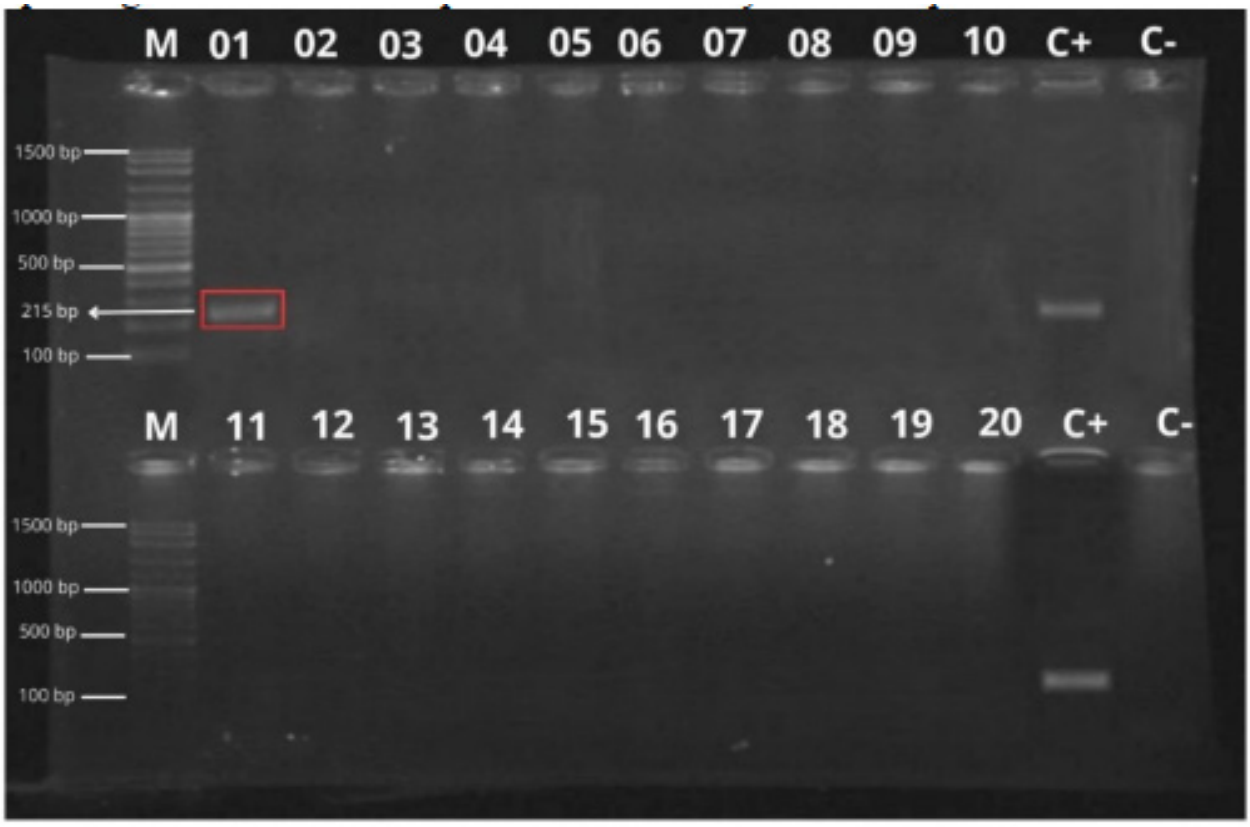

Figure 1 PCR amplification results of *Macaca fascicularis* nasal swab samples against DFA/TGV primers visualized by 2% agarose gel electrophoresis. (Notes: M; 100 bp DNA ladder, code 1-20; test sample, C+; positive control, and C-; negative control).

signifies the detection of the *Herpesviridae* virus.

Herpesviridae Virus Identification

Samples identified as containing *Herpesviridae* viruses underwent sequencing to ascertain the specific species of *Herpesviridae* virus infecting *Macaca fascicularis*. The sequencing process was performed by a third party at 1st Base Laboratories Sdh. Bhd., Malaysia. The sequencing results obtained on the sample with code 01 are as follows:

> Sample of MF Dramaga herpes

CATCGCCGAGACCGTGACGCTACAGGGGCGCAC-
GATGCTGGAGCGGGCCAAGGCCTTCGTGGAGGCCCT-
GAGACCGGCCGACCTGCAGGCCCTGGCCCCCAC-
CCCCGACGCCTGGGCGCCCCTCAACCCCGAGGGCAG-
GCTGCGCGTCATCTACGGCGACACGGACTCTGTGA

DISCUSSION

Measurement of DNA Extraction Concentration

The minimum concentration of DNA that can be amplified is 10 ng/µL (Sophian and Yustina, 2023). The measurement results indicate sample codes 01, 02, 08, and 20 exhibit concentration values below 10 ng/µL. Concentration values that fall below the standard may result from inaccuracies in blank solutions. Blank solutions should maintain identical ionic strength and pH levels to the sample solution (Sophian and Yustina, 2023). The high and low concentration values can be affected by various factors in the modified extraction method, including temperature and the duration of the incubation process (Emilia and Anhar, 2021).

The standard value of DNA purity ranges from 1.8-2.00 (Utami et al., 2023). The A260/A280 ratio measurement indicated that samples coded 06, 19, and 20 exhibited purity values below the acceptable threshold, while all samples also recorded A260/A230 ratios that fell short of the standard value. An A260/A280 ratio value that falls below the standard may result from the introduction of reagents like phenol, alcohol, and chloroform during extraction into the DNA solution, as well as the presence of proteins, RNA, and other impurities. The A260/A230 ratio that causes all samples to be below the standard value can be caused by the entry of phenol residues during extraction or guanidine residues on the spin column membrane (Widayat et al., 2019). Contamination in the A260/A230 ratio may also result from the presence of carbohydrates and other organic materials (Emilia and Anhar, 2021).

Herpesviridae Virus Detection

The *Herpesviridae* virus was identified in sample 01, as evidenced by a band parallel to the positive control band, with an amplicon size of 215 base pairs. This detection utilizes the advantages of the Nested PCR method. This DNA amplification method uses two stages of amplification with a pair of inner primers and a pair of outer primers (Naully and Septriliyana, 2022). The outer primers are utilized in the initial amplification process. Inner primers are utilized in the second amplification process, with the results of the initial reaction product serving as the amplification target (Yusuf, 2010). The outer primer will bind outside the target region by flanking the target DNA sequence, resulting in greater amplification of the target DNA. The inner primer will bind to the inner sequence of the first-round reaction and amplify smaller target DNA (Alemayehu, 2023). Nested PCR exhibits high specificity and sensitivity, making it a widely utilized method for identifying *Herpesviridae* viruses that possess latent properties in host cells with tiny amounts of viral DNA. This is why the nested PCR method is more widely used compared to other PCR methods, such as conventional PCR or Real-time PCR (Machado et al., 2016). However, a drawback of nested PCR is the potential for contamination during the transfer of the initial PCR product into the medium for the second amplification (Trasia, 2020).

Herpesviridae Virus Identification

The nucleotide base sequences obtained from sequencing were processed using bioinformatics techniques by aligning them with the sequence data available in GenBank. The nucleotide sequences of samples aligned with GenBank data are available at the National Center for Biotechnology Information (NCBI). The Nucleotide BLAST feature was selected from NCBI, and the nucleotide sequences were uploaded to the Basic Local Alignment Search Tool-nucleotide (BLASTn). The BLAST results were used to confirm the accuracy and alignment of the sequences with the species of interest. Figure 2 presents the results of the BLAST analysis.

BLAST results reveal similarities to the nasal swab sample from *Macaca fascicularis*, which identified the *Herpesviridae* virus, specifically the *Macaca fascicularis lymphocryptovirus*. BLAST results for the samples indicated similarities with the species *Macacine herpesvirus* 4, *Cercopithecus kandti lymphocryptovirus*, *Macaca arcotoides gammaherpesvirus*, *Macaca mulatta lymphocryptovirus*, and *Pongo pygmaeus lymphocryptovirus*. The similarity of BLAST results obtained a query cover value of 100% with the highest percentage of identities of 98.84%. The query cover

Figure 2 Nucleotide base sequence derived from BLAST results of samples with corresponding data available on GenBank.

value indicates the duration of the match between the query sequence and the target sequence in the database. The percentage identity value indicates the similarity between a query sequence and a target sequence in the database, reflecting the number of identical nucleotide bases in both sequences (Gaffar and Sumarlin, 2020). The minimum acceptable percentage value is 95% (Narita et al., 2012). The results indicate that a value exceeding the standard suggests the sample resembles the GenBank data.

The e-Value of the sample was 8e-80. The E-Value is an estimated metric that offers a substantial statistical assessment of the two sequences (Murtafi'ah and Aeni, 2023). An e-Value below 1e-50 is typically regarded as highly significant. A smaller E-value indicates stronger evidence that the observed similarity is not attributable to chance, but rather to an evolutionary or functional relationship (González-Pech et al., 2019; Choudhuri, 2014). An e-value of 8e-80 indicates that the sequences being compared are very similar, almost certainly not by chance, and are likely to have a strong biological relationship. The BLAST results showed a nucleotide base pair length of 173 pairs. The comparison of the sample nucleotide bases with the GenBank data revealed that out of 173 pairs, 171 pairs were identical, while 2 pairs were not identical. The identified virus belongs to the genus *Lymphocryptovirus* with the subfamily *Gammaherpesvirinae*.

The findings of this study indicate that the nasal swab results in *Macaca fascicularis* for sample code 01 exhibited a tape size of 215 base pairs. The BLAST results indicate a similarity of 171 out of 173 pairs of sample nucleotide base pairs when compared to GenBank data. The sample is identical to the species *Macaca fascicularis lymphocryptovirus*, genus *lymphocryptovirus*, subfamily *Gammaherpesvirinae*. The BLAST sample similarity value achieved a query cover of 100% and an identity percentage of 98.84%, with an E value of 8e-80.

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