

**CORONAVIRUS DETECTION IN BATS IN BRAJA HARJOSARI, EAST
LAMPUNG**

Undergraduate Thesis

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**DEPARTMENT OF BIOLOGY
FACULTY OF MATHEMATICS AND NATURAL SCIENCES
UNIVERSITY OF LAMPUNG**

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LAMPUNG**

Vidyanti Kurniasih

Undergraduate Thesis

**In partial fulfillment of the requirements for the degree on
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ABSTRAK

KAJIAN DETEKSI CORONAVIRUS PADA KELELAWAR DI DESA BRAJA HARJOSARI, LAMPUNG TIMUR

Oleh

VIDYANTI KURNIASIH

Kelelawar merupakan salah satu mamalia yang termasuk dalam Ordo Chiroptera. Keanekaragaman kelelawar di Indonesia terbilang cukup besar yaitu 205 spesies yang mewakili 21% dari seluruh spesies yang tersebar di seluruh dunia. Kelelawar termasuk mamalia reservoir virus zoonosis yang menyebabkan penyakit berbahaya pada manusia, seperti coronavirus. Coronavirus adalah salah satu infeksi virus mewabah dan menjadi pandemi yang termasuk dalam Ordo Nidovirales. Penelitian mengenai keberadaan coronavirus pada kelelawar di Provinsi Lampung belum pernah dilakukan. Penelitian ini bertujuan untuk deteksi dini keberadaan coronavirus pada kelelawar di daerah dekat pemukiman desa. Penangkapan kelelawar dan pengambilan sampel usap oral dilakukan di Desa Braja Harjosari, Lampung Timur, dan dilanjutkan dengan analisis molekuler yang dilakukan di Laboratorium Bioteknologi Balai Veteriner Lampung melalui lima tahapan berdasarkan *Predict Protocol* yaitu ekstraksi, sintesis cDNA, amplifikasi, elektroforesis, sekuensing dan analisis hasil dengan perangkat lunak *Molecular Evolution Genetic Analysis* (MEGA X) dan *Basic Local Alignment Search Tool* (BLAST) pada website NCBI. Hasil penelitian didapatkan tiga spesies kelelawar yang berbeda yaitu *Cynopterus brachyotis*, *Cynopterus horsfieldi*, dan *Scotophilus kuhlii* dari sepuluh individu dengan satu sampel *Predicted Positive* yang memiliki hasil analisis negatif coronavirus.

Kata kunci: coronavirus, Desa Braja Harjosari, kelelawar, Lampung, *Predict Protocol*

ABSTRACT

CORONAVIRUS DETECTION IN BATS IN BRAJA HARJOSARI, EAST LAMPUNG

By

VIDYANTI KURNIASIH

Bats are mammals belonging to the Order Chiroptera. Indonesia has considerable bat diversity with 205 species, representing 21% of all bat species worldwide. Bats serve as reservoir mammals for zoonotic viruses that cause dangerous diseases in humans, such as coronavirus. Coronavirus is an emerging viral infection that has become a pandemic and belongs to the order Nidovirales. No previous research has been conducted on coronavirus presence in bats from Lampung. This study aims to conduct early detection of coronavirus in bats near village settlements. Bat capture and oral swab sampling were performed in Braja Harjosari, East Lampung, followed by molecular analysis at the Biotechnology Laboratory of Lampung Disease Investigation Center through five stages based on Predict Protocol: extraction, cDNA synthesis, amplification, electrophoresis, sequencing, and result analysis using Molecular Evolution Genetic Analysis (MEGA X) software and Basic Local Alignment Search Tool (BLAST) on the NCBI website. The study identified three different bat species (*Cynopterus brachyotis*, *Cynopterus horsfieldii*, and *Scotophilus kuhlii*) from ten individuals, with one Predicted Positive sample showing negative coronavirus analysis results.

Keywords: coronavirus, Braja Harjosari, bats, Lampung, Predict Protocol

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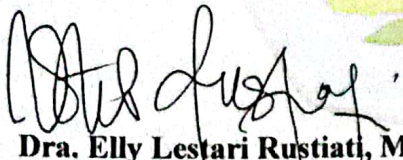
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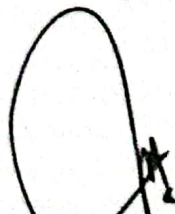


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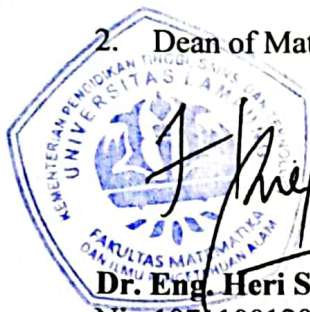
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BIODATA



The author was born in East Lampung, Lampung on February 9, 2002, as the third child of three siblings, from Father Sumadi and Mother Sunarsih.

The author began their early education at LPM Kindergarten from 2007 to 2008. They then attended SDN 2 Raman Aji from 2008 to 2014, continued to SMPN 1 Raman Utara from 2014 to 2017, and pursued further education at SMAN 5 Metro from 2017 to 2020. In 2021, the author was officially accepted as a student in the Bachelor of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung through the National Joint Entrance Selection pathway.

During her studies in the Biology Department, the author served as a laboratory assistant for courses in Ecology, Environmental Impact Assessment, Cell Biology, and Invertebrate Zoology. In 2023, the author and her team successfully secured funding for a Student Creativity Program proposal in Community Service at Liman Benawi, Central Lampung, titled "Teaching Cucumber Cultivation Based on Soilless Culture System for Enriching Eco-Agrotourism in Kampung Liman Benawi, Central Lampung." In 2024, the author conducted Internship Course at the Biotechnology Laboratory of Lampung Disease Investigation Center and published a scientific article titled "**Molecular Detection of Jembrana Disease in Balinese Cattle (*Bos Sondaicus*) Samples at The Lampung Disease Investigation Center**". In the same year, the author presented at the International

Conference On Medical Science And Health (ICOMESH), and is an ongoing process as part of its chapter book with the title of the book “**RNA Isolation on Oral Swab Sample on Bats of Rural Area, Braja Harjosari, East Lampung**”.

The author also conducted a 40-day Field Work Practice (KKN) in Sambirejo, Jabung, East Lampung. Additionally, the author was among the 50 awardees of the Young Women Scholarship Batch 2 program.

DEDICATION WORDS

In the name of Allah, the Most Gracious, the Most Merciful

With gratitude to Allah SWT, I dedicate this work which I have completed with full love and affection to:

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MOTTO

"Whoever treads a path in search of knowledge, Allah will make easy for him the path to Paradise"
(HR. Muslim)

"Whatever is meant for you will find its way to you"
(Ali ibn Abi Talib)

"If you never try, you will never know the result! Take the first step to try, then you can see the results for yourself"
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I. INTRODUCTION

1.1 Background

Indonesia possesses high biodiversity encompassing diverse flora and fauna. This diversity is supported by various habitat types in Indonesia as a tropical country. One of Indonesia's faunal diversities is the Class Mammalia, particularly bats (Saputra *et al.*, 2016). Bats have the ability to fly using their wings. They are classified in the Order Chiroptera, meaning "hand-wing". This name derives from their distinctive feature: forelimbs modified into wings. This makes bats the only mammals capable of true flight (Fitria *et al.*, 2021).

Indonesia is home to 229 bat species, representing 17% of all bat species distributed worldwide (Qaanitah *et al.*, 2018). Bats inhabit diverse habitats ranging from forests, plantations, residential areas, and most frequently in caves. Bats are divided into two categories: insectivorous bats and frugivorous bats. Insectivorous bats are commonly found in caves and settlements, roosting on cave surfaces and in bamboo crevices. Frugivorous bats are more frequently found in forests and large trees as their habitat (Fitria *et al.*, 2021).

According to Marlinda *et al.* (2021), bats play important roles in ecosystems ecologically, economically, and medically. Bats have a significant role

important in seed pollination. There are 186 bat species that play roles in bat-mediated seed pollination, flower pollination, biological malaria control, as well as being predators for agricultural pest insects (Wibowo, 2017). Bat meat is consumed and traded in East Java, West Java, and Yogyakarta, as people believe bat meat can cure diseases, making it a common product in traditional markets and supermarkets (Wibowo, 2017).

The public holds diverse perceptions of bats, considering them to be frightening mammals and agricultural pests that need to be eradicated (Wijayanti *et al.*, 2016). Bats serve as reservoir mammals for zoonotic viruses that infect humans, livestock, and wildlife (Calisher *et al.*, 2006). Bats transmit viruses to humans through intermediate hosts by having close contact with humans (Han *et al.*, 2015).

Intermediate hosts can become infected through various mechanisms, including bats ingesting partially digested food. Physiologically, fruit bats are unable to swallow large quantities of food due to their flight aerodynamics. Food that falls to the ground can be consumed by other animals, potentially becoming a source of infection. Bats can also directly infect humans by consuming meat from infected bats (Han *et al.*, 2015). Pathogen transmission through wild animal meat occurs through direct contact with bodily fluids and feces of wildlife (Nurjanah & Dharmayanti, 2022).

Bats are known to be reservoirs for viruses that cause dangerous diseases in humans, including rabies (RABV), Hepatitis (HeV), Nipah (NiV), Ebola hemorrhagic fever (EBOV), and Marburg (MARV) (Hayman *et al.*, 2013). Valitutto *et al.* (2020) stated that bats also serve as reservoirs for viruses causing Emerging Infectious Diseases, coronaviruses (CoVs), which are known to originate from Wuhan, China. The coronavirus disease 2019 (COVID-19) is one of the viral infections that has caused outbreaks and become a pandemic (Zheng, 2020). Coronaviruses (CoVs) belong to the

Family Coronaviridae and Order Nidovirales, which are groups of viruses infectious to humans (Wong *et al.*, 2019).

Calisher *et al.* (2006) stated that the genome of SARS-CoV isolates found in civets and humans during the 2002-2003 SARS epidemic phylogenetically belongs to the large group of SARS-CoV-like viruses in bats. Antibodies against SARS-CoV-like viruses in bats have also been detected in fruit bats living in caves, indicating that bats can also support the transmission of SARS-CoV-like viruses. Zhou *et al.* (2020) identified bats as carriers of SARS coronavirus (SARSr-CoV) by sequencing the SARS-CoV-2 genome and comparing it with genomes of SARS-related coronaviruses (SARSr-CoV) isolated from bats. The SARS-CoV-2 isolated from patients in Wuhan showed very high genomic similarity (96.2%) with Bat CoV RaTG13, suggesting SARS-CoV-2 likely originated from bats.

Research on coronavirus detection in bats in Lampung has never been conducted before. The detection of coronavirus presence in bats was carried out using the Predict Protocol method, a method used for dangerous infectious viruses that pose a threat to humans. Samples used for coronavirus detection can be oral swab samples or blood samples.

1.2 Objective

This study aims to conduct early detection of coronavirus in oral swab samples from bats in Braja Harjosari, East Lampung.

1.3 Use of Research

The benefit of this research is to provide early information and confirmation regarding the presence of coronavirus in bat oral swab samples in Braja Harjosari, East Lampung.

1.4 Theoretical Framework

Bats belong to the Class Mammalia, distinguished into fruit-eating bats and insect-eating bats. Fruit-eating bats are commonly found in homogeneous and heterogeneous forests where large trees serve as their resting places, and they are believed to aid in seed dispersal. Insect-eating bats are typically found in caves, settlements, or roosting in bamboo crevices. Insect-eating bats play a role in maintaining the balance of insects that disturb crops.

Bats are one of the animals traded in Indonesia as they are believed to be an alternative treatment for asthma. Some people hold different perceptions about bats, considering them frightening as they are believed to be virus carriers. Previous research on bats as virus vectors has been conducted in Indonesia, specifically in Gorontalo, and the detected virus was confirmed to be a coronavirus.

Coronavirus detection needs to be conducted as an effort to record and perform early detection of coronavirus presence in bats. In this study, the selected location for collecting bat oral swab samples is in Braja Harjosari, East Lampung. The oral swab sampling begins with live trapping of bats using mist nets. The collected samples will then be analyzed based on the Predict Protocol at the Biotechnology Laboratory, Lampung Disease Investigation Center. This research aims to perform early detection of coronavirus in bat oral swab samples from Braja Harjosari, East Lampung.

II. LITERATURE REVIEW

2.1 Biology of Bats

Bats are the only flying mammal species belonging to the Order Chiroptera. Chiroptera originates from the Greek words "cheir" meaning hand and "pteros" meaning membrane, and can also be interpreted as "hand wing" because the hand is modified into wings (Santoso *et al.*, 2020). According to Fithria *et al.* (2014), unlike birds, bat wings consist of an elastic membrane called the patagium that extends between the palms and fingers or forelimb bones, and along the sides of the body and hind legs, functioning for flight and covering their bodies.

In the Class Mammalia, bats are the second most numerous species after Rodentia, with 229 species distributed across Indonesia (Fithria *et al.*, 2014). Bats are nocturnal animals. This is due to the adaptation of their wings, which are highly sensitive to solar radiation and absorb more heat than they emit. Additionally, bats possess specialized sensory adaptations that support their nocturnal activities, enabling them to reduce competition with diurnal animals such as birds (Prasetyo *et al.*, 2011). Generally, bats belong to the Order Chiroptera, which is divided into two groups: fruit-eating bats and insect-eating bats.

According to Suyanto (2001), approximately 20% of fruit bats and over 50% of insectivorous bats choose to roost inside caves. Some bats also live in forests, in large trees, building roofs, hollow trees, felled large trees, and dead

trees. Prasetyo *et al.* (2011) state that the presence of trees is very important for bat life. In addition to serving as habitats, fruit trees are also needed as food sources for bats. Trees that serve as insect dwellings are particularly important for insect-eating bats.

2.2 Bat Classification

The classification of bats according to Kunz (1998) is as follows:

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Mammalia
Order	: Chiroptera
Family	: Pteropodidae
	: Vespertilionidae
Genus	: <i>Cyropterus</i>
	: <i>Scotophilus</i>

There are 229 recorded bat species in Indonesia, representing 17% of bat species worldwide. These species are divided into 78 fruit-eating bat species and 151 insect-eating bat species distributed across Indonesia (Qaanitah *et al.*, 2018). This number is further categorized into one family for fruit bats and eight families for insectivorous bats (Saputra *et al.*, 2017).

Nine families were found in the forests of Sumatra, Pteropodidae for fruit bats and Rhinolophidae, Hipposideridae, Emballonuridae, Megadermatidae, Nycteridae, Vespertilionidae, Rhinopomatidae, and Molossidae for insectivorous bats (Prasetyo *et al.*, 2011). Pteropodidae, Vespertilionidae, and Megadermatidae were bats found in Lampung in the study by Santoso *et al.* (2020).

2.2.1 Fruit bats

Fruit bats generally use vision for object detection in their environment, except for *Rousettus* which employs low-intensity tongue vibrations. Pteropodidae is the sole family of fruit bats. Approximately 22 genera and 75 species live in Indonesia. Pteropodidae is divided into four subfamilies: Pteropodinae, Macroglossinae, Nyctimeninae, and Harpionycterinae (Syukri *et al.*, 2018).

According to Prasetyo *et al.* (2011), fruit-eating bats have bodies weighing between 10-1500 grams. Species in the genera *Balionycteris*, *Chironax*, and *Aethalops* weigh approximately 10 grams, while the largest flying fox (*Pteropus vampyrus*) weighs over 1500 grams, has a wingspan of up to 1700 mm, and a forearm length of 36-228 mm. A distinctive characteristic of fruit bats is their dog-like facial structure (Figure 1).



Figure 1. Facial morphology of a fruit bat (*Cynopterus horsfieldi*).
Source: Personal document

2.2.2 Insectivorous Bats

According to Syukri *et al.* (2018), insectivorous bats generally have relatively small bodies. Their body size is small, weighing 2196 grams with a forearm length of 22–115 mm (Prasetyo *et al.*, 2011).

Insectivorous bats typically use their ears and nostrils for directional control of movement (echolocation) when searching for food (Syukri *et al.*, 2018). The distinctive features of insectivorous bats include their flat facial structure and small eyes (Figure 2).



Figure 2. Facial morphology of an insectivorous bat (*Pipistrellus stenopterus*). Source: Prasetyo *et al.*, 2011

2.3 Role of Bats

Bats are the best animals for seed dispersal. Seeds dispersed by bats have a higher germination rate compared to those that germinate naturally. This is supported by their ability to fly long distances, enabling them to spread seeds across vast areas. Plant species that can be dispersed by bats include eggplants, sandalwood, banyan trees, rubber trees, guava, java plum, sapodilla, sugar apple, and candlenut (Prasetyo *et al.*, 2011).

For insectivorous bats, they serve as pollinators for various plants. There are approximately 300 species of tropical plants pollinated and dispersed by bats, including economically valuable plants such as durian, petai, sugar palm, calliandra, banana, mangrove, and kapuk (Fithria *et al.*, 2014). Insectivorous bats also play a role in controlling insect populations, particularly nocturnal insects like mosquitoes, beetles, and moths. Suyanto (2001) states that bats can consume half their body weight, or 600 mosquito-sized insects, in one hour.

2.4 Bats as Reservoirs

Hayman *et al.* (2013) stated that bats harbor viruses potentially dangerous to humans, including Rabies virus (RABV), Hendra virus (HeV), Nipah virus (NiV), Ebola virus (EBOV), Marburg virus (MARV), and Coronaviruses (CoVs). According to Akbar *et al.* (2020), CoV is believed to be transmitted from bats and snakes obtained from a multispecies market in Wuhan, China. SARS-CoV-2 is thought to originate from bats, with transmission likely occurring indirectly. Allocati *et al.* (2016) reported that the transmission pattern of some pathogenic CoVs involves intermediate hosts, believed to be transmitted from bats to humans through masked palm civets (*Paguma larvata*).

CoV transmission through intermediate hosts can also occur via partially digested fruits consumed by bats. Physiologically, fruit bats cannot swallow large quantities of food due to their flight aerodynamics. Consequently, partially eaten fruits dropped by bats may be consumed by other animals, potentially leading to CoV transmission to humans if they consume the meat of these animals (Han *et al.*, 2015). According to Nugroho *et al.* (2020), SARS-CoV-2 can be transmitted from bats to humans using angiotensin-converting enzyme 2 (ACE2), the same receptor as SARS-CoV.

2.5 Coronavirus

In early 2020, China reported to the World Health Organization (WHO) that there were 44 cases of severe pneumonia in one region, namely Wuhan, and a severe infectious disease of unknown cause began spreading worldwide. Particularly in Hubei, China at the end of 2019 (Handayani *et al.*, 2020). According to Tamara (2021), on February 11, 2020, the World Health Organization (WHO) announced that this disease outbreak was named Coronavirus Disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), which was declared a pandemic on March 12, 2020.

Coronavirus is transmitted from person to person and has spread widely in China and over 190 other countries and regions (Susilo *et al.*, 2020). The first two cases of novel coronavirus infection were reported in Indonesia on March 2, 2020. Based on data as of March 31, 2020, there were 1,528 infected people and 136 deaths. The COVID-19 mortality rate in Indonesia was 8.9%, the highest in Southeast Asia (Susilo *et al.*, 2020). According to Yunus and Rezki (2020), coronavirus is a group of viruses belonging to the Family Coronaviridae and Subfamily Orthocoronavirinae of the Order Nidovirales. Viruses in this group can cause diseases in mammals, including birds and humans.

There are six types of coronaviruses that can infect humans:

alphacoronavirus 229E, *alphacoronavirus* NL63, *betacoronavirus* OC43, *betacoronavirus* HKU1, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV), and Middle East Respiratory Syndrome Coronavirus-2 (MERS-CoV). These viruses can cross mucous membranes, particularly the nasal and laryngeal mucosa, and enter the lungs through the respiratory tract. The viruses then attack target organs that express the Converting Enzyme 2 (ACE2), such as the lungs, heart, renal system, and digestive tract (Tamara, 2021).

Coronavirus is an encapsulated, non-segmented positive-sense single-stranded RNA virus (Yuliana, 2020) (Figure 3a). The genetic structure of coronavirus contains 6 main Open Reading Frames (ORFs) which are nucleotide triplets read as codons that determine amino acids. These six ORFs include regions encoding replication enzymes (ORF 1a and 1b), E gene (envelope protein), M gene (membrane protein), S gene (spike protein) and N gene (nucleocapsid protein) (Wang *et al.*, 2020) (Figure 3b). This virus belongs to the biological group superdomain, specifically the Virus kingdom which represents the largest group of viruses in the Order Nidovirales. All viruses from the Order Nidovirales are non-segmented positive-sense RNA viruses (Santoso, 2022). This RNA virus has particle sizes ranging from 120 to 160 nm and primarily infects animals such as bats and camels.

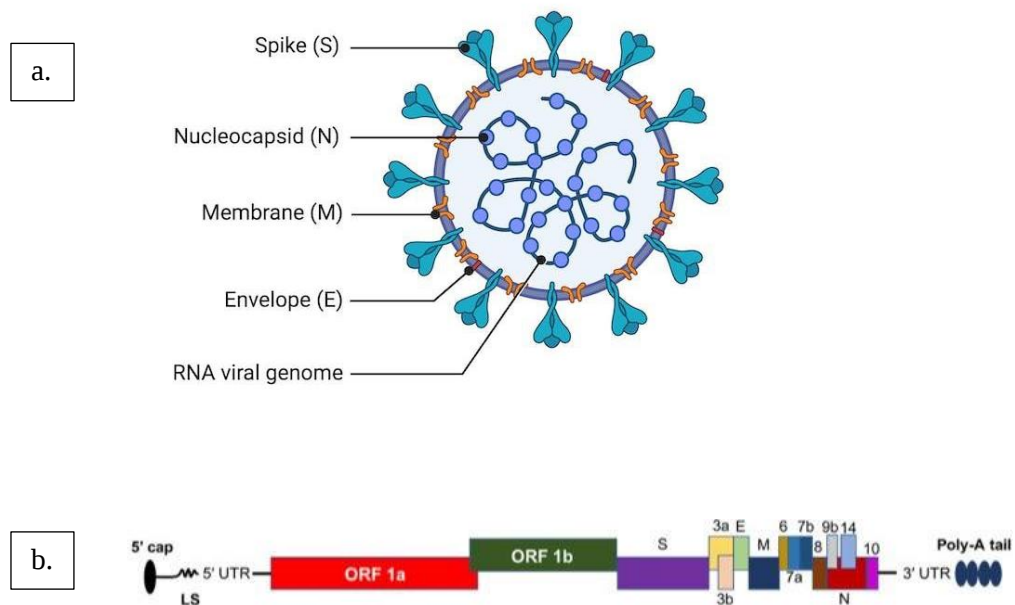


Figure 3. a. Coronavirus structure; b. Genetic structure diagram of coronavirus components coronavirus. Source: Lee *et al.*, 2020

2.6 Detection of Coronavirus in Bats

The presence of coronavirus in bats in Indonesia has been previously studied. Febriani *et al.* (2018) reported that 95 anal swab samples were collected from *Pteropus alecto* bats in the Olibuu mangrove forest of Gorontalo, Indonesia,

with 24 out of 95 samples (23.3%) suspected to be CoV-positive. Eight of these suspected positive samples underwent further nucleotide sequencing analysis and were confirmed as CoV-positive. The coronavirus (CoV) found in bat samples from Olibuu-Gorontalo was identified to have nucleotide sequences identical to virus samples previously detected in Indonesia by Anindita *et al.* (2015).

Zhou *et al.* (2020) identified bats as carriers of SARS coronavirus (SARSr-CoV) by sequencing the SARS-CoV-2 genome and comparing it with SARS-related coronaviruses (SARSr-CoV) isolated from bats. The results showed that SARS-CoV-2 isolated from patients in Wuhan had a very high genomic similarity (96.2%) with Bat CoV RaTG13.

2.7 Predict Protocol

The Predict protocol is a method used to identify responses to emerging infectious diseases before they spread to humans. The U.S. Agency for International Development (USAID) has established the Emerging Pandemic Threats (EPT) program consisting of four projects: Predict, Respond, Identify, and Prevent. Predict serves as a step to identify dangerous infectious diseases that may pose threats to humans (Muflihanah *et al.*, 2015). Bats, rodents, and non-human primates are the focus of Predict research as animals capable of causing zoonotic diseases.

PREDICT has developed virus detection protocols at the family level for animals that cause diseases in humans, including alphavirus, arenavirus, astrovirus, bunyavirus, coronavirus, filovirus, flavivirus, herpesvirus, orthomyxovirus, paramyxovirus, poxvirus, reovirus, retrovirus, and rhabdovirus (USAID PREDICT, 2019). A total of 812 new virus types and 147 known types in wildlife, including those in Indonesia, were successfully

identified by PREDICT. Therefore, FAO and PREDICT collaborated with the Ministry of Agriculture to conduct virus analysis at the family level in mammals and poultry to assess the potential presence of Emerging Diseases (Muflihanah *et al.*, 2015).

III. RESEARCH METHODS

3.1 Time and Location

"Molecular Detection Study of Coronavirus in Bats in Braja Harjosari, East Lampung" is part of the research by Dra. Elly Lestari Rustiati, M.Sc titled "Detection of Potential Emerging Infectious Disease (EID) in Bats in Lampung Based on Predict Protocols," funded by the Research Innovation and Collaboration Program of the Higher Education for Technology and Innovation Project (HETI) in the year 2024. This research was conducted in two phases: bat capture and sampling in February 2024, and molecular analysis in January-February 2025. Bat capture and sampling were carried out in Braja Harjosari, East Lampung, while molecular analysis was performed at the Biotechnology Laboratory, Lampung Disease Investigation Center.

3.2 Tools and Materials

The equipment and materials used for bat capture included mist nets, mist net poles, masks, gloves, and calico bags. A Pesola scale and calipers were used for bat identification. Sample collection was conducted using tools and materials such as cotton swabs, VTM tubes, and an ice box.

The tools and materials used in the molecular analysis include: PCR WorkStation, class II biosafety cabinet, laminar air flow, vortex, collection tube with spin column, micropipette with filter tips, Thermo Cyclor, centrifuge, chamber, agarose gel electrophoresis equipment, oral swab samples, absolute alcohol, agarose gel powder, SYBR Safe DNA Gel Stain Lot No. 2452353, Tris-acetate-EDTA (TAE) buffer Lot No. 2086438, QIAGEN RNA isolation kit (QIAamp® RNA Mini Kit (250) cat. no. 51306) containing AVL buffer, Carrier RNA Lot No. 1720172220, AW1 buffer, AW2 buffer, AVE buffer, amplification kit with Nuclease Free Water, forward primer, Tetro cDNA synthesis kit (BIO 6201), primers encoding the Rdrp gene (forward and reverse) and reverse primer (Table 1).

Table 1. Primer set for RNA-Dependent RNA Polymerase gene marker (Rdrp) for Predict Protocols coronavirus (Watanabe *et al.*, 2010)

Primer	Primer Sequence
Round 1: Forward	5'GGTTGGGAYTAYCCHAARTGTGA'3
Round 1: Reverse	5'CCATCATCASWYRAATCATCATA '3
Round 2: Forward	5'GAYTAYCCHAARTGTGAYAGAGC'3

3.3 Procedures

3.3.1 Bat capture

A site survey was conducted to determine bat capture locations in Braja Harjosari, East Lampung. Information about bat presence was obtained from local residents. Mist net installation sites were selected based on the availability of food sources, water sources, natural roosting habitats for bats, and visible fruit remnants or bat droppings. Mist nets were set up at two points: near residential areas (Location 1) and at the border between rubber plantations and settlements (Location 2). Fruit-bearing trees around the mist net locations included calabur (*Muntingia calabura*), mango (*Mangifera indica*), papaya (*Carica papaya*), and

nearby oil palm plantations (*Elaeis guineensis*). Water sources were also available near the mist net installation sites adjacent to rubber plantations.

The mist net was set up at a height of approximately 4 meters to trap bats in flight. Installation was carried out from 17:00 to 17:25 WIB at the residence of Waryoko and from 17:25 to 17:50 at the rubber plantation border location. From 18:00 to 22:00 WIB, the mist net was fully and slowly deployed. Bats caught in the mist net were then retrieved by lowering the net poles to facilitate handling. The captured bats were placed into cloth bags and numbered/tagged. The numbering of cloth bags was done to facilitate species identification according to the location and time of bat capture.

3.3.2 Bat identification

Bat identification was conducted to determine bat species by referring to Suyanto (2001). Morphological observation and morphometric measurements were performed by removing the bat from the muslin bag and handling it using the pinch grip method to facilitate observation and measurement while making the bat feel more comfortable.

Morphological observation was conducted by examining the body shape and fur color of bats. Each bat species has distinctive external body characteristics that differentiate it from other species. Fruit-eating bats have more prominent snouts, possess thumbs, and lack tails. Whereas insect-eating bats have short and flat snouts, lack thumbs, and have long tails. Morphological observation is also performed to determine sex by examining neck fur color and reproductive organs. Males tend to have darker neck fur compared to females. Reproductive organ identification is determined by the presence of a penis in males and mammary nipples in females, which become clearly visible when

female bats are nursing or have recently stopped nursing (Suyanto, 2001).

Morphometric measurements were conducted by measuring the forearm length (FA), tibia length (Tib), and ear length (Ear) using calipers, while body weight (WT) was measured using a Pesola scale and a cloth bag. Body weight measurements were taken twice: first while the bat was still in the cloth bag (W1), and after releasing the bat by weighing only the empty cloth bag (W2). The final weight was calculated as (W1-W2). After completing morphological observations and morphometric measurements, oral swab samples were collected from the bats for the detection of coronavirus.

3.3.3 Sample collection

Oral swab sampling of bats was conducted to test for the presence of coronavirus. Samples were collected by gently swabbing the bat's mouth with a cotton swab and stored in sample storage tubes or VTM tubes. The VTM tubes containing samples were then labeled N_BH, where "N" represents the sample number and "BH" stands for Braja Harjosari (the sampling location) corresponding to the captured bats. The VTM tubes containing samples were stored in an ice box to maintain sample quality before being transported to the Biotechnology Laboratory at the Lampung Disease Investigation Center. After sampling, the bats were given water by dripping clean water onto cotton. The bats were then released at the capture site by placing them on the hand and allowing them to fly away.

3.3.4 Extraction

The molecular analysis based on the four-step Predict Protocol, consisting of RNA extraction, cDNA synthesis, coronavirus amplification using Predict protocol, and electrophoresis, was conducted at the Biotechnology Laboratory of Lampung Disease Investigation Center. The first stage, the lysis phase, was performed by adding 5.6 µl of carrier RNA, 560 µl of AVL buffer, and 140 µl of sample into a microtube. The solution containing the sample was then homogenized using a vortex mixer followed by incubation at room temperature for 10 minutes.

The second stage is the binding of RNA compounds to the silica gel in the spin column. This step is performed by adding 560 µl of absolute alcohol and homogenizing with a vortex for 15 seconds. The solution is transferred to the spin column (630 µl) and centrifuged at 10,000 rpm for 1 minute at 4–8° C.

The supernatant was discarded and the collection tubes were replaced, then the remaining solution was transferred to the viral spin column and centrifuged again at 10,000 rpm for 1 minute at 4–8° C. The function of absolute alcohol is to dissolve all compounds except RNA. These compounds bind to the silica membrane present in the spin column. The third stage involves washing or precipitation, which is performed twice using buffer AW1 and buffer AW2. The first step is carried out by adding 500 µL of buffer AW1 into the spin column, followed by centrifugation at 10,000 rpm for 1 minute at 4–8° C. The supernatant is discarded and the collection tube is replaced with a new one. The second step is performed by adding 500 µL of buffer AW2 into the spin column and centrifuging at 14,000 rpm for 3 minutes at 4–8° C. The supernatant is discarded, and the collection tube is replaced with a new one before proceeding with centrifugation at 14,000 rpm for 1 minute at

4–8° C. Once all precipitation processes are completed, the spin column is transferred to a microtube.

The fourth stage is elution or purification. This stage is carried out by adding 60 µl of AVE buffer into the spin column and centrifuging at 10,000 rpm at 4–8° C for one minute. The spin column is discarded and the microtube containing the extracted RNA is stored in a refrigerator at -20° C.

3.3.5 cDNA synthesis

The cDNA synthesis process was carried out using the Tetro cDNA synthesis kit (BIO 6201). The master mix or reagent was prepared by mixing 2 ml RT buffer, 4.5 ml DEPC water, 0.5 ml dNTPs, 0.5 RNase, 0.5 Tetro RT, 0.5 oligo (dT), and 1.5 RNA sample. cDNA synthesis was performed in three stages: denaturation at 48°C for 20 minutes, annealing at 95° C for 2 minutes, and extension at 72°C for 10 minutes. The obtained cDNA was then stored in a -20°C freezer.

3.3.6 Predict Protocol for Coronavirus Detection at the Amplification Stage

The five steps of DNA amplification include pre-denaturation or preparation for separating double-stranded DNA into single-stranded DNA, denaturation or separation of the two DNA strands, annealing or the process of primer attachment to single-stranded DNA, extension or DNA multiplication, and post-extension as the refinement stage of amplification. The coronavirus detection protocol using heminested polymerase chain reaction is conducted in two PCR stages (PCR round

1 and round 2). The first step (round 1) of PCR is performed by mixing 13 μL of Red Mix, 1.4 μL of nuclease-free water, 0.8 μL each of forward and reverse RdRp gene-coded primers with a primer concentration of 20 pmol, and 5 μL of cDNA synthesis template. The sample consists of 21 μL of DNA amplified in the first round. In the second round of amplification, the same materials are used as in the first round except for the cDNA synthesis template. A 5 μL sample of DNA amplified in the first round is used to replace the 5 μL cDNA synthesis template.

The amplification steps in rounds 1 and 2 follow the same five stages: pre-denaturation at 94° C for 2 minutes, denaturation at 94° C for 20 seconds, annealing at 50° C for 30 seconds, extension at 72° C for 30 seconds, and post-extension at 72° C for 7 minutes. The denaturation, annealing, and extension processes are repeated 35 times in a thermal cycler.

3.3.7 Preparation of Agarose Gel

Agarose gel is used in molecular research to visualize DNA through electrophoresis. The preparation of agarose gel begins by adding 1 gram of agarose gel powder to 100 ml of TAE solution, then heating it in a microwave for 3 minutes. 10 μL of SYBR Safe DNA Gel Stain (dye solution) is added. The agar solution is poured into a mold containing a comb, left to solidify for 30 minutes, after which the comb is removed and the gel is placed into the chamber.

3.3.8 Electrophoresis

Electrophoresis is performed by running DNA through the agarose gel in the chamber. The electrophoresis process begins by loading the

marker into the first well. A 5 µl sample is taken and loaded into the well. The chamber is connected to the power supply with the black terminal as the cathode and the red as the anode. Electrophoresis is conducted for 15 minutes at 100 volts and 400 amperes. Visualization is performed as the final step of electrophoresis to observe the presence of bands in the sample wells. Predicted positive samples show DNA bands in the 400-500 bp range, corresponding to the nitrogenous base size of coronavirus with a marker gene of 434 bp (Latinne *et al.*, 2020). Samples identified as predicted positive then proceed to the next step of nitrogenous base sequence sequencing.

3.3.9 Sequencing

The sequencing stage is conducted to determine the nitrogen base arrangement in the sample by sending it to PT. Genetika Science Indonesia. The samples are shipped in boxes containing silica gel along with the primers used. The shipment includes the identities of both sender and recipient. The sequencing process is performed using a device called a sequencer, equipped with a laser and detector to identify fluorescent markers present in the amplified DNA strands.

3.3.10 Data Analysis

The bat identification results through morphological observation and morphometric measurements for species determination were analyzed descriptively and presented in tabular form. The sequencing results were analyzed using Molecular Evolutionary Genetics Analysis software version ten (MEGA X) with nitrogen base sequencing (alignment), followed by Basic Local Alignment Search Tool (BLAST)

testing on the NCBI website to ensure the sequencing results matched the desired target.

The analysis using MEGA software begins by opening the MEGA X application, then accessing the Align menu and selecting the "Edit/Build Alignment" option. Next, the Alignment Editor interface will appear, where "Create a new alignment" is selected to establish a new alignment. The "DNA" option is then chosen in the Data Type for Alignment display. The Alignment Explorer interface will appear, where reverse and forward sequencing results are imported by selecting "Insert Sequence From File" from the Edit menu. The "Reverse Complement" option is selected for the reverse sequence. All sequences are then selected by choosing "Select All" from the Edit menu. The "Align by ClustalW" option is selected from the Alignment menu, and the "OK" button is clicked in the ClustalW Options interface to proceed with the multiple alignment process. The resulting nitrogen base sequences will subsequently be displayed.

The obtained nitrogen base sequences were then analyzed using BLAST software (The Basic Local Alignment Search Tool) on the NCBI website. BLAST analysis was performed to compare the similarity between a query sequence and database sequences in GenBank (subject sequence) (Sjafaraenan et al., 2018). "Nucleotide BLAST" was selected to conduct the BLAST test. The sequencing results were entered in the "Enter Query Sequence" section under the column "Enter Accession number(s), gi(s), or FASTA sequence(s)". The search was executed by clicking the "BLAST" button. Database sequences showing similarity to the input sequence were displayed in the "Descriptions" section. The sequence appearing at the top position represents the most similar sequence to the input, with the highest similarity value visible in the "Percent Identity" column.

3.4 Research Flowchart

The research was conducted based on the following flow diagram (Figure 4):

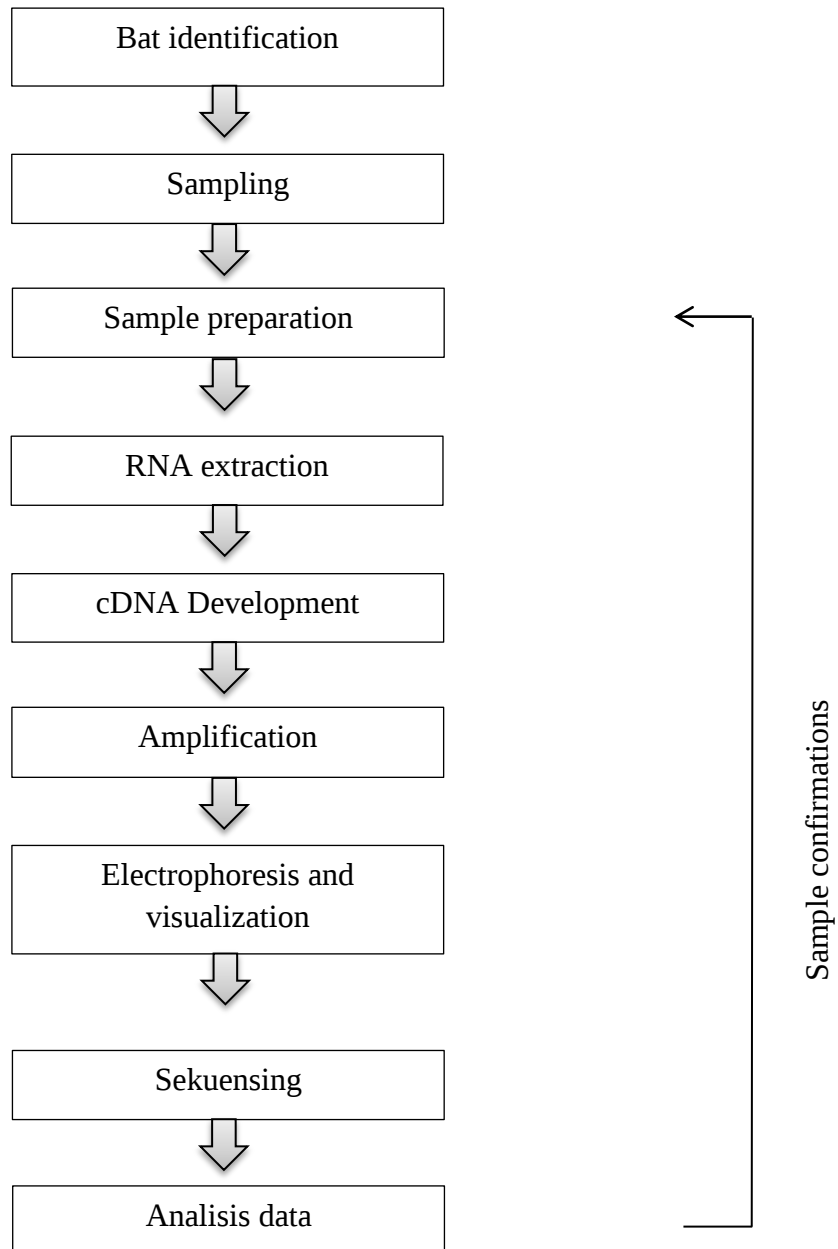


Figure 4. Flowchart of the study on coronaavirus detection in bats in Braja Harjosari, East Lampung

V. CONCLUSION AND SUGGESTIONS

5.1 Conclusion

The conclusions obtained from the research "Study on Coronavirus Detection in Bats in Braja Harjosari, East Lampung" are as follows:

1. Of ten bats, three species have been identified: *Cynopterus brachyotis*, *Cynopterus horsfieldii*, and *Scotophilus kuhlii*.
2. One bat individual shows predicted positive in *Cynopterus brachyotis* bats with negative coronavirus sequencing test results.

5.2 Suggestions

The Predict Protocol method implemented can be utilized as a coronavirus detection method for bats in wider area.

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