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Applying Predict Protocols for Detection of The Potential Presence of Coronaviruses on Bats in Lampung, Indonesia

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Abstract. Emerging Infectious Disease (EID) is one of the potential biological threats that can cause a public health emergency. Bats are potential hosts for zoonotic sylvatic disease reservoirs, including the coronavirus that causes Covid 19. Its presence in residential environments can trigger public health emergency. Data on coronavirus surveillance especially in Lampung, Indonesia is currently not available. The initiation of data collection and detection of the potential presence of EID coronavirus in bats using Predict Protocols was carried out under Higher Education for Technology And Innovation Project. It is in collaboration with Lampung Disease Investigation Center. Bats' oral swab samples were collected by life trapping using mist nets from 4 locations representing urban (Bandar Lampung) and rural areas (Braja Harjosari and Labuhan Ratu VII, East Lampung). Predicting coronavirus molecularly based on Predict Protocols was applied through RNA extraction and amplification, sequencing and molecular analysis. From 30 samples of 5 bat species (*Scotophilus kuhlii, Cynopterus sphinx, Cynopterus horsfieldi, Cynopterus brachyotis, Cynopterus titthaechaeilus*), 1 sample from Braja Harjosari was predicted positive of coronavirus. Following the sequencing result, molecular detection showed negative results for the suspected coronavirus sample. Molecular analysis concluded that bats from both urban and rural areas do not carry coronavirus.

Keywords: Emerging Infectious Disease, bats, predict protocol, zoonosis, Lampung

INTRODUCTION

Infectious diseases account for over 7 million deaths annually, significantly impacting global health and causing economic losses (Morens *et al.*, 2004). Approximately 75% of emerging infectious diseases are zoonotic, originating from animals, particularly wildlife, and transmissible to humans (Jones *et al.*, 2008). Viruses are the primary cause of most emerging zoonotic diseases.

Pandemics, including pH1N1, SARS, and COVID-19, are believed to have originated from wildlife (Wolfe *et al*., 2007). Coronavirus Disease 2019 (COVID-19), caused by the coronavirus SARS-CoV-2, is a recent example. SARS-CoV-2, belonging to the same family as the viruses responsible for SARS and MERS, exhibits a higher transmission rate (Li *et al*., 2020; CDC China, 2020).

Zoonosis, caused by viruses with a long history of adapting to their natural hosts, can spread through activities that interface between wildlife and humans, such as trade. Global trade, including the wildlife trade, is a significant factor in the rise of viral infectious diseases (Piret and Boivin, 2021). The trade of wild animals serves as a gateway for the transmission of zoonotic viral diseases, with potential transmission occurring through the buying and selling of these animals. Bats, in particular, are a commonly traded species.

Bats, the only mammals that have the ability to fly, exhibit high adaptability to residential environments such as houses, barns, agricultural fields, and gardens, which serve as suitable ecosystems for breeding (Santoso, *et al*., 2020; Platto *et al*., 2021). Bats play a crucial role in ecosystems by controlling agricultural pests and pathogen-carrying arthropods, as well as acting as pollinators and seed dispersers, which are ecologically, culturally, and economically significant (Kunz *et al*., 2011; Russo *et al*., 2018). Evolution has endowed bats with unique physiology and immunity, making them resistant to viral pathogens, although these viruses can infect other species, including humans. Species such as short-nosed fruit bats and large flying foxes, commonly found in plantation areas, are potential hosts for the Nipah virus (Morcatty *et al*., 2022). Bats are also primary hosts for aCoV and bCoV. Bats are potential hosts of zoonotic pathogens which are sylvatic *disease reservoirs,* serve as reservoirs for coronaviruses, which can undergo recombination and modification within host cells. The development of CoV is one of the main causes of the COVID-19 pandemic (Platto *et al.,*2021).

Detecting low-prevalence infections, such as certain RNA viruses, is challenging and necessitates appropriate methods (Kuzmin *et al*., 2008). Bats, identified as potential reservoirs for coronaviruses (CoVs), have been detected globally (Hayman *et al*., 2012). COVID-19, an emerging infectious disease (EID), is caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). This virus is closely associated with bats, the natural reservoir. Genome analysis of COVID-19 patients in China revealed a 96% genetic similarity to coronaviruses found in bats (Zhou *et al*., 2020). Genetic modifications in the coronavirus surface protein receptors enable the virus to bind to human cellular receptors in the respiratory and digestive tracts (Lvov and Aikhorky, 2020). Understanding the potential presence of EID in bats can help prevent infection and spread and avoid anthropogenic impacts. The urgency of EID detection in bats is very significant. In Sumatra, Indonesia, there are 73 species of bats from 9

families (Colwel and Payne*et al.,* 2000). Lampung, home to two national parks, provides a natural habitat for both fruit-eating and insect-eating bats.

Detecting the potential presence of EID on bats in Lampung using Predict protocols was carried out with a combination of field and laboratory studies. Predict protocols is the approach taken for sorting priority virus families that is *coronaviruses, filoviruses, flaviviruses, paramyxoviruses, herpesviruses and orthomyxoviruses.* Positive detections from bat oropharynx samples will be further characterized and analyzed molecularly to see disease pathogens, variations and genetic relationships (Goldstein *et.al.,* 2016). Approach Predict protocols widely used in triangulation surveillance to view interactions between humans, wildlife and domestic livestock to detect the presence of EID and to improve understanding of the presence and diversity of viruses and potential human and animal pathogens and their transmission. Detection of the coronavirus virus on bats as one of the viruses that is closely related to SARS CoV-2 as the cause of Covid-19 was done. The research on infectious diseases can support public health by anticipating coronavirus detection on bats that have high interaction with humans/residential areas.

Materials and Methods

Data collection and detection of the potential presence of EID coronavirus in bats using Predict Protocols was carried out under Research Innovation and Collaboration Program - Higher Education for Technology And Innovation Project (HETI). The research was carried out in two stages, (1) bat life trapping and swab sampling, (2) initial EID coronavirus detection on bats by Predict Protocols through RNA extraction, cSNA synthesis, amplification, sequencing and molecular analysis.

Bat life trapping using a mist net was done in an open place with a mist net height of 4 meters from ground level, purposefully focusing on the bat's active path (Ariyanti, 2017) in 4 different locations representing urban (University of Lampung and Lampung Investigation Center, Bandar Lampung) and rural areas (Braja Harjosari and Labuhan Ratu VII, East Lampung). The net was checked from 18.00 to 20.00. Thirty bats caught were identified and oral swabbed using a cotton swab that was gently rubbed into the bat's mouth and then the cotton swab was inserted into the VTM tube.

RNA extraction. Extraction from an oral swab sample was performed based on the protocol of the QIAamp® RNA Mini Kit (250), starting with the lysis phase. It was carried out by adding a 5.6 µl carrier RNA, 560 µl AVL buffer, and adding a sample of 140 µl to the microtube. The solution to which the sample has been added is then homogenized using a vortex, incubated at room temperature for 10 minutes.

Binding. It was done by adding 560 µl of absolute alcohol and homogenizing by vortex for 15 seconds. The solution is transferred to a spin column of 630 µl and centrifuged at 10,000 rpm for 1 minute at 4–8 °C. The supernatant is removed and the collection tubes are replaced. The

remaining solution is transferred to the viral spin column and centrifuged again at 10,000 rpm for 1 minute at 4–8 °C.

Washing. Washing or precipitation was carried out twice using AW1 buffer and AW2 buffer. The first step is done by adding 500 µL of AW1 buffer to the spin column and centrifuging for 1 minute at a speed of 10,000 rpm and a temperature of 4–8°C. The supernatant was discarded and the collection tube was replaced with a new one. The second step was done by adding 500 µL of AW2 buffer to the spin column and centrifuging for 3 minutes at a speed of 14,000 rpm at 4–8 °C. The supernatant was removed and the collection tube was replaced with a new one to be centrifuged for 1 minute at a speed of 14,000 rpm at 4–8 °C. All precipitation processes were completed, the spin column is transferred to the microtube.

Elution. The final stage elution or purification was done by adding 60 µl of AVE buffer to the spin column and centrifuged at a speed of 10000 rpm at 4 – 8ºC for one minute. The spin column was removed and the microtubes containing the extracted RNA were stored in a freezer at -20ºC.

cDNA Synthesis Stage. The RNA extraction obtained was to proceed to the synthesis / manufacture of cDNA through amplification with *the Polymerase Chain Reaction* (PCR) technique. The cDNA synthesis process was carried out using Tetro cDNA synthesis kit (BIO 6201) with two stages, master mix and amplification with PCR. Master mix, mixing reagent materials for the amplification process, is carried out by mixing 2 ml RT buffer, 4.5 ml Depc water, 0.5 ml dNTP, 0.5 RNase, 0.5 Tetro RT, 0.5 Oligo (dt) and 1.5 RNA samples. Amplification with PCR was carried out through three stages, namely denaturation at 48ºC for 20 minutes, annealing at 95ºC for 2 minutes, and extension at 72ºC for 10 minutes. The amplified cDNA was stored in the freezer at -20ºC for the next stage.

Amplification Stage Predict Protocols Coronavirus. The amplification stage of Predict Protocols for coronavirus identification was carried out by *the heminested Polymerase Chain Reaction* (PCR) method using a primer marker *of the RNA Dependent RNA Polymerase* (Rdrp) gene (Watanabe *et al*., 2010). The five stages of DNA amplification include pre denaturation, denaturation, *annealing*, *extension*, and *post extension* (Pestana *et al*., 2010). When predicted positive samples were found, the samples were sent for the next analysis, sequencing of its sequence of nitrogenous bases. Sequencing results were analyzed using the MEGA Version 6.0 application and the BLaST website.

Results and Discussion

Predict protocols is a vital surveillance and virus discovery initiative designed to identify potential zoonotic disease threats at high-risk wildlife-human interfaces (PREDICT Consortium, 2014). By targeting wildlife taxa such as bats, rodents, and nonhuman primate groups most likely to harbor

emerging zoonotic viruses. Predict Protocols) aims to prevent emerging infectious diseases in both human and livestock populations. It uses advanced methods and refined protocols to improve virus detection. By employing sensitive techniques and customized primers, Predict protocols enhance its ability to identify coronaviruses. Predict protocols were applied to detect coronaviruses in bats.

Thirty bats were life trapping representing urban (University of Lampung and Lampung Disease Investigation Center, Bandar Lampung) and rural sites (Braja Harjosari and Labuhan Ratu VII, East Lampung). In the residential area/city of Bandar Lampung, 2 species of fruit-eating bats Megachiroptera, *Cynopterus brachyotis* and *C. sphinx*. In Braja Harjosari obtained 2 species *of Cynopterus brachyotis*, *C. sphinx* and 1 species of Microchiroptera insectivorous bat, *Scotophilus kuhlii.* In Labuhan Ratu VII obtained 3 species *of Cynopterus brachyotis*, *C. sphinx* and *Cynopterus titthaechaeilus*.

From 30 samples analyzed, one bat, *Cynopterus brachyotis,* from Braja Harjosari predicted positive for coronavirus (Figure 2), its cDNA amplification was continued for the sequencing process. Its process showed good sequence quality (Figure 3), and further molecular analysis to identify whether it is a coronavirus family.

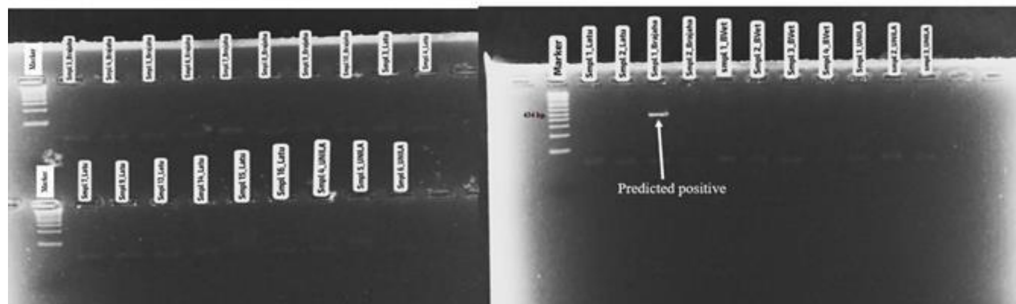

Figure 2. Electrophoresis amplification results Predict protocols for coronavirus using heminested PCR method with primary RNA-Dependent RNA Polymerase (RdRp) gene marker (n = 30)

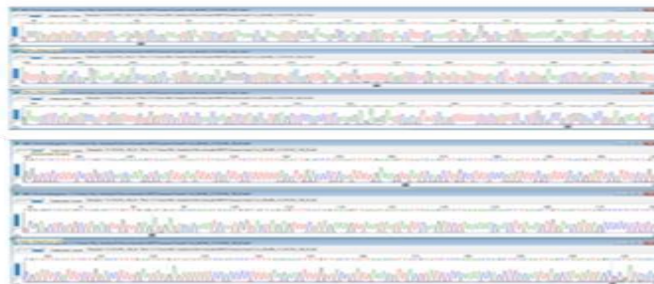

Figure 3. The electropherogram of sequencing result from *Cynopterus brachyotis* predicted positive sample

Sequencing results from the predicted positive bat oral swab sample were analyzed using MEGA (Molecular Evolutionary Genetic Analysis) software version 6.0. This step was conducted to determine the nitrogen base sequence of the predicted positive sample, using a reference gene from the gene bank, specifically the Bat betacoronavirus RNA-dependent RNA polymerase (RdRp) gene (MN312851.1). The sequences were then analyzed with BLAST (The Basic Local Alignment Search Tool) on the NCBI website to identify the similarity between the nitrogen base sequence of the sample and the nitrogen base sequence of the coronavirus in bats, including the Bat betacoronavirus RNA-dependent RNA polymerase (RdRp) gene (MN312851.1) (Latinne et al., 2020). The selection of Bat betacoronavirus RdRp as the reference gene was based on the primer nitrogen base sequence alignment/identification results using BLAST from NCBI, where the Bat betacoronavirus RdRp gene (MN312851.1) showed 100% homology/similarity.

The alignment of the nitrogen base sequence of the predicted positive sample was performed using ClustalW (Figure 4). The alignment results of the forward and reverse nitrogen base sequences from the predicted positive sample were then saved in notepad format (Table 1) for subsequent analysis with BLAST on the NCBI.

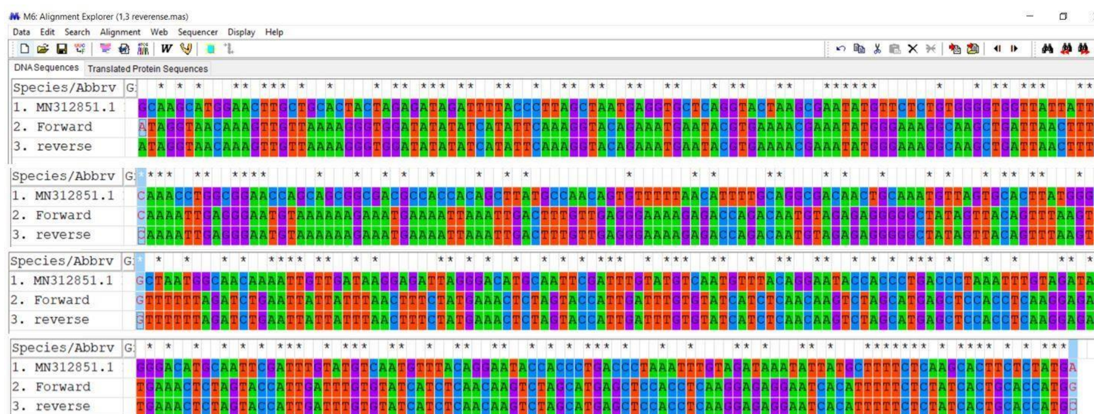

Figure 4. The alignment of the nitrogen base sequence of the predicted positive sample was performed using ClustalW alignment in MEGA software version 6.0.

The nitrogen base sequence analysis using BLAST on the NCBI website indicated that the nitrogen base sequence of the Cynopterus brachyotis sample predicted to be positive for coronavirus from Braja Harjosari was not confirmed as coronavirus. This was demonstrated by the mismatch between the BLAST results of the sample's nitrogen base sequence and the nitrogen base sequence of the Bat betacoronavirus RdRp gene (MN312851.1) in the gene bank (Figure 5).

Table 1. Nitrogen base sequence of the *Cynopterus brachyotis* sample predicted positive for coronavirus from Braja Harjosari, East Lampung

Sample	Nitrogen base sequence
Cynopterus brachyotis predicted positive (Braja Harjosari)	ATAGGTAACAAAGTTGTTAAAAGGGTGGATATATATCATATTCAAAG GTACAGAAATGAATACGTGAAAACGAAATATGGGAAAGGCAAGCTG ATTAACTTTCAAAATTGAGGGAATGTAAAAAAGAAATGAAAATTAAA TTGACTTTGTTGAGGGAAAAGAGACCAGACAATGTAGAGAGGGGGCT ATAGTTACAGTTTAAGTTGTTTTTAGATCTGAATTATTATTTAACTTT CTATGAAACTCTAGTACCATTGATTTGTGTATCATCTCAACAAGTCTA GCATGAGCTCCACCTCAAGGAGAGGAATCACATTTTTTTCTTTCACTG CACCATGG

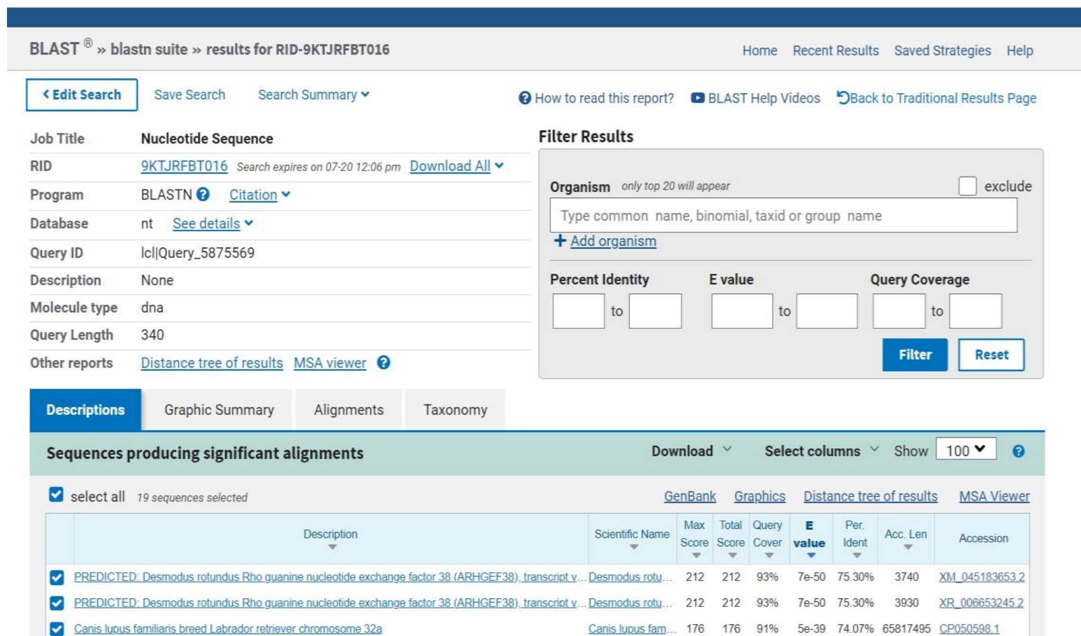

Figure 5. BLAST results of the nitrogen base sequence of the *Cynopterus brachyotis* sample with the nitrogen base sequence of the bat betacoronavirus RdRp gene (MN312851.1).

The suspected positive result for coronavirus was identified based on the detection of DNA bands in the 400-500 bp range during electrophoresis, corresponding to the nitrogen base size of the coronavirus with the RdRp marker gene, which is 434 bp (Latinne *et al*., 2020). The suspected positive result may also be due to successful amplification using coronavirus primers with the RdRp coding gene. The primers used for detecting coronavirus with the RdRp coding gene are markers for the Coronaviridae family, modified with ambiguous/degenerative nitrogen bases, which are bases other than those that make up DNA nucleotides and can be interpreted as derivatives/more than one nucleotide base that makes up DNA (Jamsari, 2013). The use of primers with ambiguous/degenerative nitrogen bases aims to increase test specificity (accuracy in detecting

false negative and positive results) and enhance amplification success, increasing the likelihood of primer binding to the sample DNA due to the similarity of nitrogen base sequences between the primer and the sample. The advantage of degenerative primers is their higher sensitivity in detecting DNA. However, the drawback is that the primers may bind/amplify non-target samples due to the many variations in the nitrogen base sequences of the primers, allowing for similarities/matches with the target/non-target DNA (Santoso et al., 2015).

Sequencing is a crucial step in detecting coronavirus using predictive protocols. Sequencing is key in analyzing the nitrogen base sequence alignment of the amplified predicted positive sample with the identified/registered coronavirus nitrogen base sequences in the gene bank (via NCBI).

Conclusions

Molecular analysis based on the Predict protocols identified one individual *Cynopterus brachyotis* bat from Braja Harjosari Village, East Lampung, as predicted positive for coronavirus, further confirmation results indicated that the sample was not a coronavirus. The Predictive Protocol analysis is effective for long-term EID prediction at the family stratum.

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