Novel Characterization of SARS-CoV-2 Isolates Originated from Stray Cats (*Felis catus domestica*) in Indonesia using Serological and Molecular Methods as Vaccine Candidates

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Abstract

This study aimed to analyze SARS-CoV-2 isolates originating from stray cats (Felis catus domestica) in Indonesia. In this study, we collected the samples from the various region of Indonesia. All the samples analyzed in Molecular Laboratory, Professor Nidom Foundation, Surabaya, Indonesia by using molecular and serological methods. This study approved by the Institutional Animal Care and Use Committee of the Professor Nidom Foundation (IACUC-PNF), Indonesia. We found that SARS-CoV-2 was successfully isolated from stray cats (Felis catus domestica). Six of the 45 serum samples (13.3%) contained antibodies to the SARS-CoV-2 and rapid antigen test as many as 15.9% was reactive. Through real-time PCR test, two swab samples were obtained that showed positive results for the SARS-CoV-2 Virus. One sample processed to the genome sequencing analysis. The virus obtained showed high kinship (more than 98%) with the reference virus, namely the Wuhan variant (Wuhan-Hu-1). In summary, we revealed the SARS-CoV-2 isolates originating from stray cats (Felis catus domestica) in Indonesia. However, a genomic surveilance of SARS-CoV-2 in mammals, especially domestic cats (Felis catus domestica), stray and companion cats should be improved to unlock an epidemiology of COVID-19 in animals.

Keywords: *Felis catus domestica, COVID-19, SARS-CoV-2, Indonesia, Virology.*

1. Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory coronavirus-2 (SARS-CoV-2) was first reported at the end of 2019, which eventually became a global outbreak. As of early July 2020, it was reported that more than half a million were infected with SARS CoV-2. This case began with information from the World Health Organization (WHO) on December 31, 2019 which stated that there was a case of cluster pneumonia with an unclear etiology in Wuhan City, Hubei Province, China. It continued to develop until it was finally discovered that the cause of cluster pneumonia was the novel coronavirus and resulted in death [1,2,3,4,5].

This zoonotic virus was initially believed to be related to traditional markets (wet markets) selling food derived from living marine animals and wildlife, although the truth is still questionable. SARS-CoV-2 has spread rapidly around the world and was declared a pandemic by the WHO in March 2020. At this time, the first reports of sporadic infection of SARS-CoV-2 have occurred in dogs and cats after human-to-animal transmission was suspected. Diseases caused by coronaviruses are well known in the world of veterinary medicine, because many different species of coronaviruses (CoVs) affect wild animals (especially bats or avian species) as well as domestic species such as cattle, pigs, cats, and dogs [6,7,8,9,10,11,12,13,14].

On the other hand, a previous study concluded from the results of their research that cats can be infected with human influenza viruses and also bird flu viruses. Several findings have provided further evidence that cats should be included as one of the animals responsible for transmission between species of influenza A virus. In addition, these findings also suggest that cats may play the role of intermediate hosts between mutant viruses with pandemic potential. The phenomenon of pets as a species that can be infected with COVID-19 both in nature and in the laboratory raises its own concerns, considering that so far only wild animals have been suspected as sources or intermediaries for COVID-19. The close relationship between humans and companion animals in an ecosystem is the key in the chain of transmission, breaking the chain until it can reappear. Further knowledge of the potential role of pets, particularly cats in the current outbreak and their control will be essential for effective disease prevention and control in a more global review [15,16,17,18,19,20]. Therefore, we aimed to analyze SARS-CoV-2 isolates originating from stray cats (*Felis catus domestica*) in Indonesia.

2. Materials and Methods

Samples and Study Design

Figure 1. Collect swab samples from stray cats.

The ethical feasibility of the research was obtained from the Institutional Animal Care and Use Committee of the Professor Nidom Foundation (IACUC-PNF), Indonesia. This research is a descriptive observational study using an exploratory research design. Stray cat samples were taken from various areas in Indonesia that have a risk of being infected with the SARS CoV-2 virus from humans. The research was conducted at the Molecular Laboratory of Professor Nidom Foundation (PNF), Surabaya, Indonesia. The laboratories at the PNF used include the BSL-2 and Animal BSL-3 laboratory facilities which have obtained a permit to organize a COVID-19 examination and research laboratory from the Ministry of Health of the Republic of Indonesia by Decree of the Minister of Health of the Republic of Indonesia in 2021 (HK.01.07/MENKES/4642/2021).

RNA Extraction

We used QIAamp Viral RNA Kits (Qiagen, USA) for RNA extraction in this study as instructed by Nur *et al*. (2022) [6].

Real-Time PCR

The RNA sample was added to the master mix in the new eppendorf, namely 2x rf buffer 12.5 µL, 2x enzyme 1µL, 1 µL F primer, 1 µL R primer, 1µL probe, 3.5 µL distillated water, and 5 µL template. The RNA sample mixed with the master mix was put into the RT-PCR plate as demonstrated by Nur *et al*. (2022) [6].

Virus Isolation

The positive specimens were targeted for virus isolation. Briefly, samples were diluted 1:2 to 1:3 in minimum essential medium. Vero cells (ATCC CCL-81) were inoculated with 1.5 mL of diluted sample and adsorbed for 1 hour at 37 °C. After adsorption, replacement medium was added and cells were incubated at 37 °C for up to seven days. Cell cultures without cytopathic effects (CPE) were frozen, thawed and carried out blind passage, by inoculation of

fresh cultures with lysate. Viral infection was confirmed via reduced Ct values in cell culture by SARS-CoV-2 specific RT-PCR using primers and CDC probe sets N1 and N2 [6].

Serological Analysis using ELISA

In this study, IgG antibody titers from samples were analyzed by ELISA method. All working procedures for serological analysis with ELISA follow the procedures from Nidom *et al.* (2022) [7] and Afifah *et al.* (2022) [8].

Molecular Analysis of SARS-CoV-2 S Protein Gene

The reverse-transcriptase PCR (RT-PCR) performed for the SARS-CoV-2 virus S gene consisted of a pre-denaturation temperature of 94 °C for 3 minutes, followed by 35 cycles of denaturation of 94 °C for 30 seconds, annealing of 62 °C for 40 seconds, and extension of 72 °C for 1 minute and elongation at 72 °C for 5 minutes [6]. Then, we used the 3500 Genetic Analyzer (Thermo Fisher Scientific, USA) to unlock the S protein gene of SARS-CoV-2. Molecular analysis was performed using Biological Sequence Alignment Editor (BioEdit) version 7.0 and Molecular Evolutionary Genetics Analysis (MEGA) XI software. The alignment process is carried out using the MUSCLE available in MEGA XI [1,2].

3. Results

Samples

Samples were taken from several regions in Indonesia. East Java Province includes Surabaya; Gresik; Sidoarjo and Ponorogo. West Java province originates from the city of Bandung; Banten Province originates from Tangerang and South Sulawesi Province originates from Makassar (Figure 1 and Table 1).

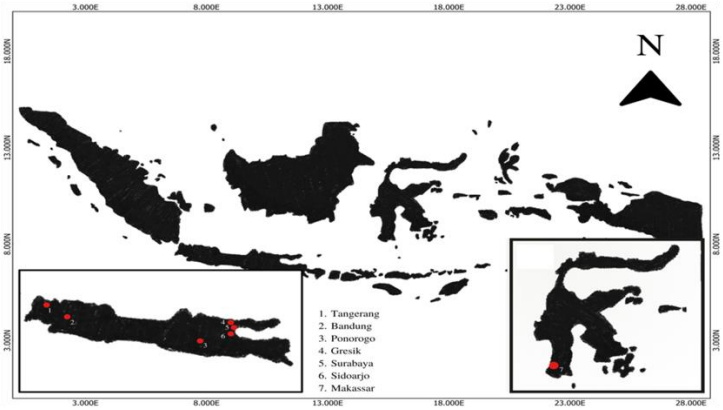

Figure 2. Map of Provinces and Sampling Areas in Indonesia. Location 1: Tangerang; Location 2: Bandung; Location 3: Ponorogo; Location 4: Gresik; Location 5: Surabaya; Location 6: Sidoarjo; and Location 7: Makassar.

Table 1. Stray Cat Swab Samples from Various Areas in Indonesia.

No	Area	Number of Stra Cats	Swabs		
			Nasal	Nasopharyı	Feathers
1	Surabaya	70	70	47	70
2	Gresik	15	15	10	15
3	Sidoarjo	20	20	10	20
4	Ponorogo	40	40	22	40

5	Bandung	10	10	10	10
6	Tangerang	10	10	5	10
7	Makassar	20	20	10	20
Total		195	195	114	195

Serological Analysis of Stray Cats IgG Antibody

Serum is obtained by taking blood from the femoral vein, then centrifuged to separate serum and sediment. Not all cats have succeeded in taking blood or serum. Serum that can be obtained from each area as listed in Table 2 and the results of the antibody titer test are as listed in Table 3.

Table 2. Number of Stray Cat Serum Samples from Various Areas in Indonesia.

No	Area	Number of Samp	Serum
1	Surabaya	70	22
2	Gresik	15	9
3	Sidoarjo	20	4
4	Ponorogo	40	6
5	Bandung	10	0
6	Tangerang	10	0
7	Makassar	20	4
Total		195	45

Table 3. Stray Cat Serum IgG Antibody Titer Results

No	Area	Code	OD	Titer	Interpretatio
1	Surabaya	S4	0.09	0	Negative
		S5	0.31	1:200	Negative
		S6	0.18	0	Negative
		S10	0.10	0	Negative
		S13	0.25	1:200	Negative
		S20	0.35	1:200	Negative
		S21	0.47	1:3200	POSITIVE
		S27	0.17	0	Negative
		S38	0.28	0	Negative
		S40	0.30	1:200	Negative
		S43	0.21	0	Negative
		S44	0.42	1:400	POSITIVE
		S45	0.12	0	Negative
		S53	0.19	0	Negative
		S57	0.27	0	Negative
		S58	0.36	1:200	Negative
		S60	0.28	0	Negative
		S61	0.16	0	Negative
		S64	0.23	0	Negative
		S65	0.51	1:1600	POSITIVE

		S68	0.32	0	Negative
		S69	0.26	0	Negative
2	Gresik	G1	0.19	0	Negative
		G3	0.41	1:300	POSITIVE
		G5	0.37	1:200	Negative
		G7	0.22	1:200	Negative
		G8	0.12	0	Negative
		G9	0.24	0	Negative
		G12	0.26	0	Negative
		G14	0.21	0	Negative
		G15	0.18	0	Negative
3	Sidoarjo	Sd9	0.22	0	Negative
		Sd14	0.16	0	Negative
		Sd17	0.58	1:400	POSITIVE
		Sd18	0.28	0	Negative
4	Ponorogo	P1	0.24	0	Negative
		P3	0.24	0	Negative
		P7	0.26	0	Negative
		P23	0.21	0	Negative
		P32	0.29	0	Negative
		P35	0.28	0	Negative
5	Makassar	M6	0.18	0	Negative
		M9	0.458	1:400	POSITIVE
		M17	0.27	0	Negative
		M20	0.19	0	Negative

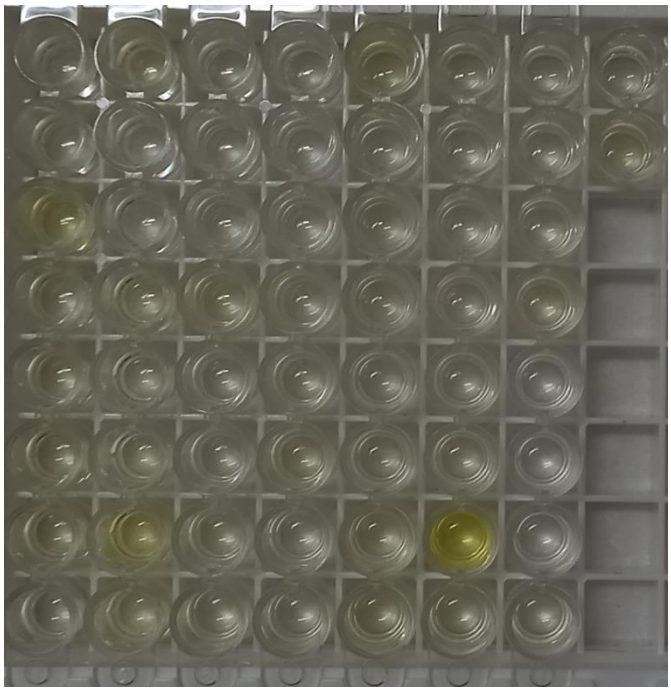

Figure 3. The results of serological analysis (ELISA method).

Molecular Analysis and Phylogenetic Tree

A total of 195 samples originating from stray cats collected from March 2022 to November 2022, originating from various regions were carried out. The screening test is in the form of testing using the Antigen Rapid Test against COVID-19. From 195 samples, 12 samples were obtained which were reactive. Furthermore, positive serum was added, especially from non-reactive samples added in the real-time PCR test, so that the total samples tested by real-time PCR were 31 samples (Table 4). The positive real-time PCR results were S7 and S30, followed by nucleotide sequencing test (Table 5). In this study, only S30 was able to produce nucleotide sequences which were then carried out for kinship analysis with several SARS-CoV-2 worldwide. The results of consanguinity analysis in a phylogenetic tree can be seen in Figure 3.

Table 4. Result of Antigen Rapid Test Against COVID-19

No	Area	Sample	Antigen Rapid Test (COVID-19)	
			Reactive	Non-Reactive
1	Surabaya	70	14 (20%)	56 (80%)
2	Gresik	15	2 (13.33%)	13 (86.67%)
3	Sidoarjo	20	5 (25%)	15 (75%)
4	Ponorogo	40	0 (0%)	40 (100%)
5	Bandung	10	3 (30%)	7 (70%)
6	Tangerang	10	2 (20%)	8 (80%)
7	Makassar	20	5 (25%)	15 (75%)
Total		195	31 (15.9%)	164 (84.1%)

Table 5. Result of Real-Time PCR from Reactive Swabs and Antibody Test

No	Area	Code	E Gene	RdRP Ge	IC	Interpretatic
		S5	-	-	23.91	Negative
		S7	31.64	34.86	23.57	POSITIVE
		S17	-	-	21.95	Negative
		S21	-	34.86	23.57	Inconclusive
		S18	-	-	22.87	Negative
		S26	-	-	23.45	Negative
		S28	-	41.92	29.36	Inconclusive
		S29	-	-	27.96	Negative
1	Surabaya	S30	32,64	36,16	23.31	POSITIVE
		S37	-	-	20.49	Negative
		S39	-	-	26.07	Negative
		S40	-	35.86	21.57	Inconclusive
		S44	-	-	26,36	Negative
		S49	33.77	-	19.36	Inconclusive
		S62	-	-	25.29	Negative
		S65	-	-	30.12	Negative
		S67	-	-	21.11	Negative

2	Gresik	G3	-	-	22.9	Negative
		G6	-	40,42	19.81	Inconclusive
3	Sidoarjo	Sd 17	33.77	-	19.36	Inconclusive
4	Bandung	B2	-	-	24.33	Negative
		B6	-	42.45	21.19	Inconclusive
		B8	-	-	27.96	Negative
5	Tangerang	T4	-	34.09	17.52	Inconclusive
		T10	-	-	25.33	Negative
6	Makassar	M1	-	41.56	20.34	Inconclusive
		M4	-	-	26.88	Negative
		M9	-	-	21.06	Negative
		M11	-	-	25.29	Negative
		M15	-	34.64	17.52	Inconclusive
		M20	-	-	25.33	Negative

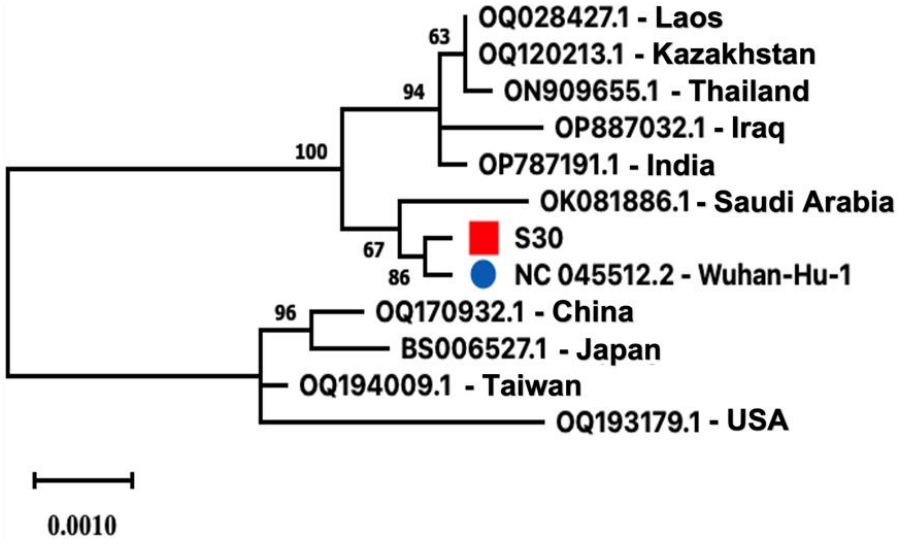

Figure 4. Phylogenetic tree of the SARS-CoV-2 virus isolated from the stray cats in Indonesia (S30).

4. Discussion

This research was conducted considering that the SARS-CoV-2 virus is a zoonotic virus [3,4,5]. Cats are animals that are close to human life both as pets (pet animals) and as street animals (stray animals). Serum antibody testing using indirect ELISA showed that several street cats had antibodies against SARS-CoV-2. It is said to be positive if the dilution is more than 1:200.

Samples from the Surabaya area obtained serum results containing antibodies against SARS-CoV-2 in three samples; while Gresik, Sidoarjo and Makassar each had a cat containing antibodies against SARS-CoV-2 in their serum. The total serum containing antibodies against SARS-CoV-2 was 6 out of a total of 45 cats (13.3%). Not all cats can take the serum. The amount of serum that could be collected was 45 from 195 target cats (23.07%)

Compared to the results of serology conducted on cats in Korea, it showed that 18 cats out of 346 cats (5.20%) showed antibodies to SARS-CoV-2. Even though the serological data obtained from this study is higher than Korea, it is difficult to say that cats are the spreader of the SARS-CoV-2 virus [21]. This conclusion is different from other research results, namely that cats show evidence of being a factor in transmitting the SARS-CoV-2 virus to humans. Although the mode of transmission has yet to be determined [22].

Swab testing of each cat was first carried out with Rapid Antigen testing. The results obtained showed that 20% of reactive swab samples from Surabaya; Gresik 13.33%, Sidoarjo 25%; Ponorogo 0%; London 30%; Tangerang 20%; and Makassar 25%. Of all the swab tests with an average antigen of 15.9%. The results obtained from the study showed greater results than those found in Korea, namely as much as 0.58% (2/346). This is possible due to differences in isolated cat samples. The data from Korea comes from household cats which allows the biosecurity aspect to be of great concern. While the data from this study came from cats that roamed the streets (Stray cats) [21,22,23].

In addition, based on previous research, it has been known that several animals were infected with the SARS-CoV-2 virus. According to Gaudreault *et al*. (2020) who carried out artificial infections on cats as experimental animals, showed that all cats did not show clinical symptoms (asymptomatic) [24]. Viral RNA can be isolated from infected cats and the virus can be transmitted to santinel cats. The results of this study are conditions that demand attention because naturally they can be contracted from infected humans or have the potential to spread to other cats [24,25].

In this study, the RNA of the SARS-CoV-2 virus was isolated from several cities. The results of the relatime PCR from the city of Surabaya showed that two specimens were successfully isolated from the SARS-CoV-2 virus (positive). While the results showed inconclusive as many as four samples.

An inconclusive criterion could be that one of the genes tested does not show its reading results. Positive results were only found in samples isolated from Surabaya. While from other cities other than negative, the results of testing with real-time PCR showed inconclusive results. Inconclusive results can be caused by several factors, including the presence of another beta corona virus; still in the early stages of infection, can also be burdened by the quality and quantity of RNA extracted from the swab samples.

The results obtained are consistent with the findings that stray cats kept at home with people with COVID-19 are very sensitive to transmission of this virus and can be reservoirs [24,25]. Then the positive realtime PCR results were followed by sequencing the isolated RNA. The sequencing sample has code S30 originating from a stray cat in Surabaya, Indonesia.

The results of the phylogentic tree analysis showed that the SARS-CoV-2 virus is close to the reference virus, the Wuhan variant (Wuhan-Hu-1), which is also found in COVID-19 sufferers in Surabaya, Indonesia. The isolate originating from this stray cat has not shown to be from a new variant, such as Delta (B.6.172.2) or Omicron (B.1.1.529).

5. Conclusion

In summary, we revealed the SARS-CoV-2 isolates originating from stray cats (*Felis catus domestica*) in Indonesia. However, a genomic surveilance of SARS-CoV-2 in mammals, especially stray and companion cats (*Felis catus domestica*) should be improved to unlock an epidemiology of COVID-19 in animals.

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Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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