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Molecular Detection of FIPV among Imported Felines through Soekarno Hatta Airport, Indonesia

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Abstract

One of the viruses that can cause disease in cats is feline coronavirus (FCoV). This virus is often divided into type I and type II. Type I is a highly pathogenic strain, feline infectious peritonitis virus (FIPV). Type II is a milder strain, feline enteric coronavirus (FECV). The FIPV variant is said to be a result of a mutation from FECV. FIP disease is responsible for 0.3%-1.4% of cat deaths in veterinary clinics. This study aims to determine if there is an FIPV in imported cats at Soekarno Hatta Airport, Indonesia. Samples were taken from 15 imported cats from Russia and Vietnam. These two countries were chosen based on previously unreported cases. The samples, consisting of blood and rectal swabs, were tested molecularly using RT-PCR. Four samples from rectal swabs showed positive results with a single band at 677 bp. Two positive samples, namely 123v and 682v, were further sequenced. The study results indicate that the FCoV virus can be found in asymptomatic imported cats. Further research is needed to better understand the mechanism causing genetic diversification of FCoV or FIPV and its impact on the pathogenesis of FIP. Furthermore, the application of FIP vaccines from other countries should be tested for compatibility with the FIPV strains present in Indonesia.

Keywords

cats, coronavirus, FCoV, FIP, import

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Introduction

Cats are one of the favorite pets that live close to humans (Turner, 2017). One of the diseases in cats is Feline Infectious Peritonitis (FIP), which is caused by a type of feline coronavirus (FCoV). The FCoV virus is often divided into type I and type II. Type I is a highly pathogenic strain, feline infectious peritonitis virus (FIPV), while type II is a milder strain, feline enteric coronavirus (FECV). Feline coronavirus infections are endemic among cats worldwide. Feline coronavirus generally causes asymptomatic infection or mild diarrhea, but can also cause lethal systemic diseases such as FIP. Feline Infectious Peritonitis is also the most common infectious disease of the cat's central nervous system (CNS) (Lewis *et al.*, 2015; Andre *et al.*, 2019). According to various sources, FIPV originates from mutations of the FECV. The FIPV variant affects approximately 5% of FCoV-infected cats and causes fatal peritonitis (Chawla *et al.*, 2023). In recent years, the number of FIP cases and of FCoV-infected cats has been increasing worldwide. The rate of FCoV RT-PCR-positive and/or seropositive cats in multi-cat environments often approaches 100% (Paltrinieri *et al.*, 2021).

Feline coronavirus belongs to the genus Alphacoronavirus, species Alphacoronavirus 1, within the subfamily Coronavirinae, family Coronaviridae, and order Nidovirales. The genome of FCoV is a single stranded positive RNA, enveloped, and consists of 11 open reading frames (ORF). Two main ORFs encode replication, four ORFs encode structural proteins, and five ORFs encode non-structural proteins. Structural proteins are spike (S), envelope (E), membrane (M), and nucleocapsid (N). Non-structural proteins are 3a, 3b, 3c, 7a, and 7b (Tekes and Thiel, 2016; Haake *et al.*, 2020; Ibrahim *et al.*, 2022). FCoV infections are self-limiting in most cases. Common sources of infection include shared litter boxes and ingestion of virus particles through self-grooming. The deadly variant, FIPV, is associated with systemic inflammation, organ failure, and death (Sweet *et al.*, 2022).

Feline Infectious Peritonitis was first described by Holzworth in 1963 in Boston (Pedersen, 2022). FCoV infection is influenced by host factors such as age and immunity, as well as environmental factors such as rearing patterns and transportation periods. The seroprevalence of FCoV infection is estimated at 25% in pet cats and 75 – 90% in cats living together in groups (Healey *et al.*, 2022). Cats with a weak cell-mediated immune response will develop acute FIP in the form of wet or effusive FIP. Cats with partial cell-mediated immunity will develop chronic FIP in the form of dry or non-effusive FIP. The dry form of FIP may become effusive in the later stages of the disease, especially when the immune system is weakened (Ibrahim *et al.*, 2022). The wet type accounts for about 70-80% of overall cases of this disease and is more malignant than the dry type. FIP disease is responsible for 0.3–1.4% of cat deaths in veterinary clinics (Andre *et al.*, 2019; Hartono *et al.*, 2022; Chawla *et al.*, 2023). Some cat breeds are at higher risk for developing FIP, including Abyssinian, Bengal, Birman, Ragdoll, and Rex cats. This may be due to genetic factors inherited in these breeds that make them more susceptible to FIP. FIP has also been seen in a number of wild cat species infected with coronaviruses (Haake *et al.*, 2020). Cheetahs are particularly susceptible to FIPV and can have symptoms similar to those of house cats (Islam *et al.*, 2021).

The importation of cats in Indonesia has increased, especially during the COVID-19 pandemic. Limited travel access during the

pandemic encouraged people to keep pets at home, such as cats. However, vaccination for FIP itself, both domestically and internationally, has not been widely applied due to its debated effectiveness. Some reasons include inappropriate age recommendations for vaccination, many seropositive cats in the environment, and different circulating virus serotypes from those in the environment. The occurrence of FIP in imported cats from Russia and Vietnam is the subject of this study.

Material and Methods

Blood samples were taken using a needle syringe with a volume of 0.3 cc from the cephalic vein and collected in ethylene diamine tetra acetic acid (EDTA) tubes. Rectal swab samples were taken using sterile swabs, which were then stored in viral media transport (VTM) solution. All types of samples were stored at -20°C before testing. The sample codes are listed in Table 1. Both blood and rectal swab samples were extracted using the Viral Nucleic Acid Extraction Kit II by Geneaid® according to the manufacturer's instructions.

Table 1. Imported cat sample data

	Cat breeds	Sex	Country of origin	Age	Sample code and test results	
					Blood	Rectal swab
1	Highland Fold	M	Russia	7 months	045 negative	**045v positive**
2	British Short Hair	F	Russia	1 year	056 negative	056v negative
3	British Short Hair	F	Russia	2 year, 10 months	089 negative	089v negative
4	Maine Coon	F	Russia	1 year 2 months	117 negative	117v negative
5	Ragdoll	M	Vietnam	6 months	121 negative	121v negative
6	British Short Hair	F	Russia	7 months	-	**123v positive**
7	British Short Hair	F	Vietnam	7 months	125 negative	125v negative
8	British Short Hair	M	Russia	1 year 2 months	545 negative	545v negative
9	British Short Hair	F	Russia	10 months	583 negative	583v negative
10	British Short Hair	M	Vietnam	2 months	681 negative	681v negative
11	British Short Hair	F	Vietnam	1 year 5 months	682 negative	**682v positive**
12	British Short Hair	F	Vietnam	1 year 5 months	684 negative	684v negative
13	British Short Hair	F	Russia	7 months	759 negative	759v Faint band
14	British Short Hair	F	Russia	6 months	900 negative	900v negative
15	British Short Hair	M	Russia	5 months	996 negative	**996v positive**

The primers used were taken from Aksono *et al.* (2023) with modifications on the reverse side. The primers are based on the M gene and are listed in Table 2. The master mix composition was adjusted according to the guidelines from the Superscript TM III RT PCR kit as shown in Table 3. All reagents in the kit were taken out from freezer storage, thawed by hand, vortexed, and spun down. Each reagent was then added to the 2 ml microtube. The master mix was then vortexed and spun down. A total of 23 µl lysate was added to each 100 µl microtube. The extraction result as a template was then added with 2 µl according to its code. The amplification process followed the steps in Table 4. Electrophoresis used 2% agarose gel, Tris Acetate EDTA (TAE) 1x, ethidium bromide (ETBR), gel pilot loading dye 5x, and 100 bp DNA Marker.

Table 2. FIPV identification primer

Primer	Sequence	Position	Amplicon
Primer forward	5' TCTTGCTAACTGGAACTTCAGCTGG 3'	26362 - 26386	677 bp
Primer reverse (**modified**)	5' T**R**ACGCG**Y**TGTCCCTG**KKY**G 3'	27019 - 27038	

Table 3. Master mix composition with Superscript TM III RT PCR

Composition	Volume 1x (µl)
H_2O	11,2
2x Reaction Mix One Step RT-PCR	10
Superscript ™ III Platinum Taq Mix	0,8
Primer Forward	0,5
Primer Reverse	0,5
Template RNA	2
Total volume reaction	**25**

Table 4. RT PCR amplification steps

Steps	Temperature (°C)	Duration	Cycle
Pre-denaturation	48	30 minute	1x
Initial denaturation	94	2 minute	1x
Denaturation	94	15 second	40 cycle
Annealing	56	30 second	
Extension	68	1 minute	
Final extension	68	5 minute	1x
	4	∞	∞

Sequencing was conducted by Genetika Science. The sequencing results were then analyzed using MEGA XI software and nucleotide homology searches were performed using the Basic Local Alignment Search Tool (BLAST) on GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Result and Discussion

A positive RT-PCR result is indicated by a single band at 677 bp. Positive result were observed in one sample from an imported cat from Vietnam (682v) (Figure 1) and three

samples from Russia (045v, 123v, 996v) (Figure 2; Figure 3).

The test result on sample 759v (Figure 2) showed a faint band. Repetition of the test was performed on this sample, but the same result was obtained.

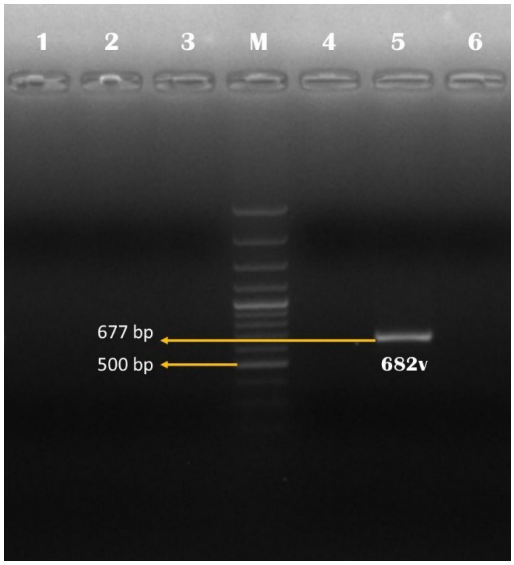

Figure 1. Amplification results. M: Marker, 3: 681, 4: 682, 5: 684, 6: 681v, 7: 682v, 8: 684v.

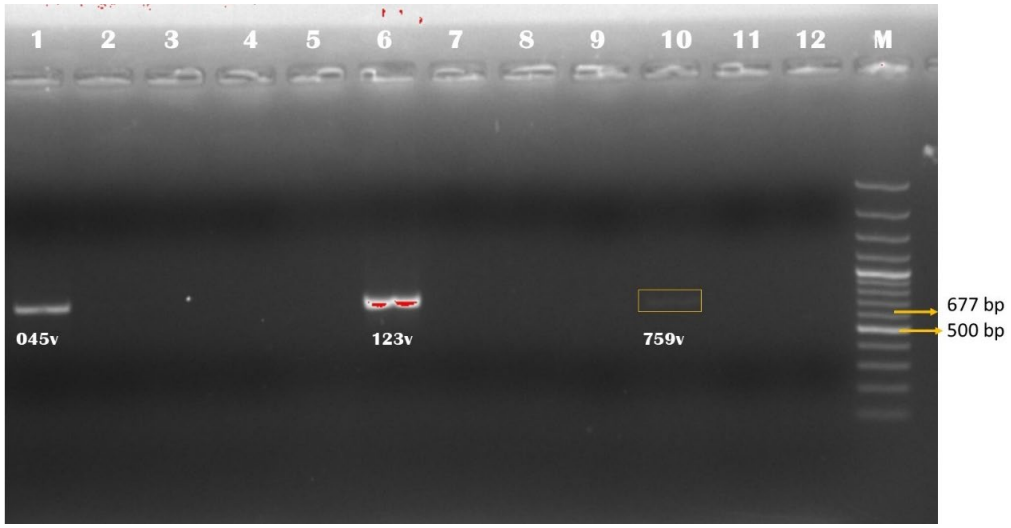

Figure 2. Amplification results. M: Marker, 1: 045v, 2: 056v, 3: 089v, 4: 117v, 5: 121v, 6: 123v, 7: 125v, 8: 545v, 9: 583v, 10: 759v.

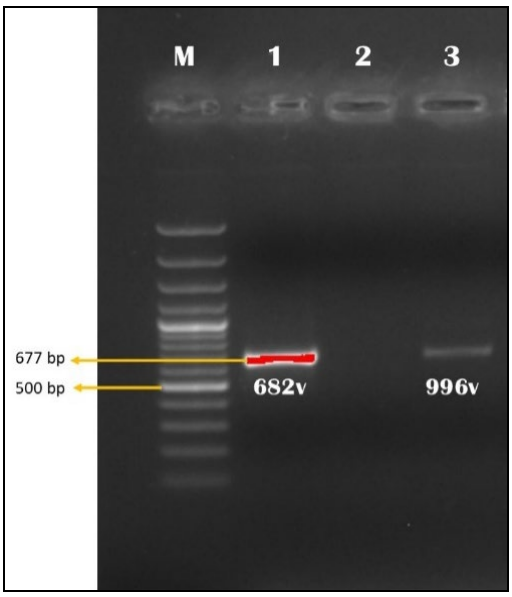

Figure 3. Amplification results. M: Marker, 1: 682v, 2: 900v, 3: 996v.

Sequencing was conducted to determine the molecular characteristic of 123v from Russia and 682v from Vietnam. The sequencing process used the Sanger method. The sequencing results were analyzed using MEGA XI software. The separate forward and reverse sequencing results were combined into a contig. The contig results for FIP sample 123v from Russia were 559 bp and for FIP sample 682v from Vietnam were 646 bp. The contig results in FASTA format were uploaded to the BLAST program on the NCBI website for homology analysis. The FIP sample 123v from Russia had the highest homology (95.36%) with *FECV225* (accession number HQ738731.1), and also had homology (95.17%) with *FIPV369* (accession number HQ738709.1). The FIP sample 682v from Vietnam had the highest homology (96.19%) with *FIPV/DY0528* (accession number MW722890.1).

The sample sequences were analyzed with the blastx program at NCBI to determine the amino acid sequence comparison results. The blastx analysis results for both samples showed homology with the feline coronavirus membrane protein. The sequence of sample 123v from Russia was 98.90% homologous with the membrane protein (feline coronavirus) (accession number ADW94525.1), and the sequence of sample 682v from Vietnam was 99.52% homologous and identical with the membrane protein (feline coronavirus) (accession number UQH66207.1) from China.

This study took samples from imported cats from Vietnam and Russia, where previously there had been unreported cases of FIP from these two countries. The cats sampled were in healthy condition without clinical symptoms or a history of illness. Cats can spread FCoV in three patterns: intermittent, shedding at specific times, and persistent shedding. Younger cats under five years old shed more than older cats. Immunosuppressive conditions increase viral load spread and prolong the shedding period (Felten *et al.*, 2023). The density in catteries and the transportation process, such as importation, can cause stress in cats and lead to immunosuppression. FCoV is

highly contagious through the fecal-oral route (Gozalo *et al.*, 2023).

The use of blood samples for RT-PCR in FIP diagnosis often has low sensitivity, even in cats with experimental FIP infection. False positive results can also occur in cats without FIP (Barker and Tasker, 2020). FCoV detection using RT PCR was initially developed using primers for the highly conserved 3'-untranslated region (3'-UTR). It was initially assumed that only FIPV could be detected in blood, but it turned out that FCoV RNA could also be detected in the blood of asymptomatic cats and cats with diseases other than FIP. Cats with FIP exhibit higher viral loads and copy numbers than healthy FECV-infected cats and higher viral copy numbers are found in the feces of healthy FECV-infected cats when compared to their blood (Felten and Hartman, 2019). This can be seen in study where no positive results were found in blood samples (Figure 4), while positive results only appeared in rectal swab samples.

Detecting the sub-genome mRNA of the FCoV M gene can identify FCoV replication in extra-intestinal specimens that have specific characteristics such as FIPV. The M gene itself is a conserved viral gene that is only expressed during replication. Conserved genes are important genes that will not change throughout evolution. These genes are unique and changes to these genes are likely to cause the organism to die. Several genes and conserved areas that can be used for molecular detection of FIPV include the spike gene (S), nucleocapsid gene (N), ORF1b gene which encodes RNA-dependent RNA polymerase (RdRp), 3'-UTR (untranslated region), the M gene, and the ORF7b gene. FIP virus replicates in peripheral blood monocytes and thus produces mRNA, whereas FECV is unable to replicate in peripheral blood, so no mRNA is produced (Lukiswanto, 2016; Felten *et al.*, 2017; Ibrahim *et al.*, 2022).

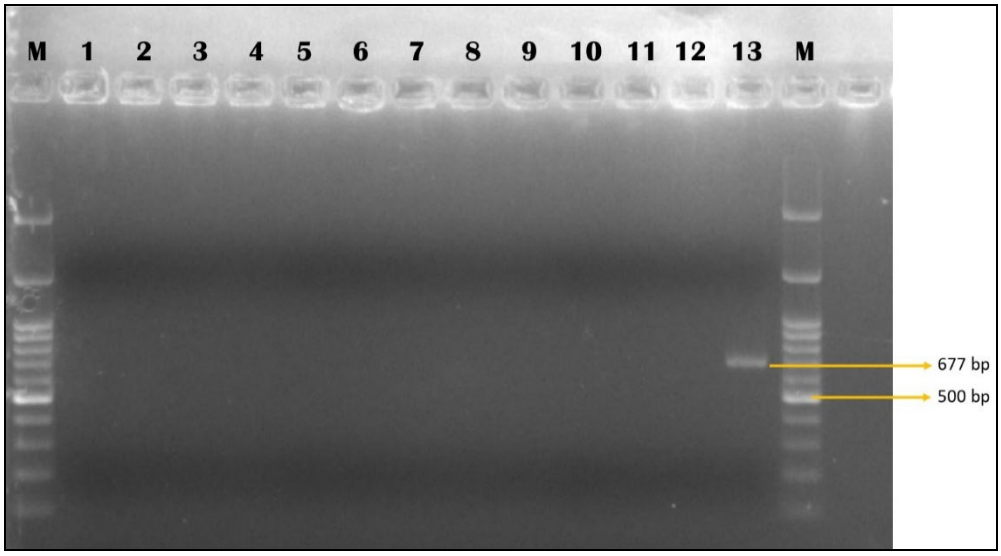

Figure 4. Amplification results. M: Marker, 1: 045, 2: 056, 3: 089, 4: 117, 5: 121, 6: 125, 7: 545, 8: 583, 9: 759, 10: 900, 11: 996, 12: negative control, 13: positive control.

According to Yin *et al.* (2021), FIP has no correlation with sex or breed, but is significantly correlated with age and sterilization status. FIP is more common in young cats or unsterilized male cats. Positive results in this study were shown in both male cats, female cats, young cats and those over one year old. The results prove that asymptomatic imported cats can carry FCoV and can be molecularly detected using rectal swab samples.

Conclusion

In conclusion, this study is the first to detect the presence of FCoV in imported cats using RT PCR. FCoV can be found in imported cats using rectal swab samples, even in those that appear healthy. International relations influence the frequency of cat traffic. The M gene can be used to detect FIPV at the molecular level. The compatibility of FIP vaccines from other countries needs to be tested against the FIPV strains present in Indonesia. Further research is needed to better understand the mechanisms causing genetic diversification and its impact on the pathogenesis of FIP.

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Author's Contribution

Fidyah Fitrawati: Methodology; Software; Validation; Formal analysis; Investigation; Writing - Original Draft; Writing - Review & Editing; Suwarno: Methodology, Visualization, Supervision; Ira Sari Yudaniayanti: Conceptualization, Supervision; Jola Rahmahani: Supervision; Muchammad Yunus: Supervision; Soeharsono: Supervision; Desnywati: Methodology; Software; Haeriah: Methodology; Validation.

Conflict of Interest

The authors declare that there is no conflict of interest in this research. All authors have resolved any financial, personal, or professional matters that might have influenced the work reported in this manuscript.

Data Availability Statement

The data that support the discoveries of this research are accessible from the corresponding author upon sensible ask.

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