Serological Study of SARS-CoV-2 Antibodies in Japanese Cats: Analysis of Risk Factors Among Cat Lifestyles

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Abstract

Background

Little is known about the epidemic status of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in cats living in Japan, and about the influence of cat lifestyles on the SARS-CoV-2 infection epidemic in cats.

Results

We developed protein A/G-based ELISA, which was standardized using positive rabbit antibodies. The measured values of this ELISA were consistent with those of conventional anti-feline IgG-based ELISA. We then collected blood samples from 1,969 cats that visited veterinary clinics in Japan from June to July 2020 and examined for the presence of anti-SARS-CoV-2 antibodies. Using protein A/G-based ELISA, nine cats were found to have SARS-CoV-2 S1-specific IgG, among which four had RBD-specific IgG. Among those nine samples, one showed neutralizing activity. Based on these, we estimated that the prevalence of SARS-CoV-2 neutralizing antibodies in cats living in Japan was 0.05% (1/1,969 samples). This prevalence did not differ much from the prevalence of neutralizing antibodies for SARS-CoV-2 in humans conducted in Japan at that time. Furthermore, we searched for factors associated with the prevalence of neutralizing antibodies in cats using our data and information from five countries (China, Croatia, France, Germany, and Italy). The prevalence of SARS-CoV-2 in cats was correlated with the rate of keeping indoor-only.

Conclusions

Protein A/G-based ELISA has the potential to be a standardized method to measure anti-SARS-CoV-2 antibodies in cats. The infection status of SARS-CoV-2 in cats in Japan is linked to that in humans, and the epidemic of SARS-CoV-2 infection in cats may be controlled by their living environment.

Background

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide and remains rampant [1]. SARS-CoV-2 infections have become widespread [2], even in vaccinated individuals [3, 4]. To stop the pandemic of COVID-19, we must control SARS-CoV-2 in the human living environment. The main transmission route of SARS-CoV-2 is thought to be the adhesion of droplets from infected people to the respiratory mucosa [5]. By contrast, although a cluster caused by mink-to-human transmission has been reported [6], transmission through animals has not been emphasized to date. Because SARS-CoV-2 can be transmitted to both humans and animals, it is necessary to understand the transmission of SARS-CoV-2 in animals.

Several cases of confirmed SARS-CoV-2 infection in cats and dogs have been reported [7]. As of November 10, 2021, all of these infections are presumed to have been transmitted from humans, and there have been no reported cases of transmission from cats and dogs to humans [8]. However, as well as mink, cats have been reported to be more susceptible to SARS-CoV-2 than other animal species [9], and laboratory transmission experiments have shown that cats can transmit droplet or airborne infections to other cats [10]. In addition, it was recently reported that cats can induce the emergence of SARS-CoV-2 variants that induce breakthrough infection [11]. Because cats are one of the most common pets worldwide, studies on the prevalence of SARS-CoV-2 infection in cats are of high importance.

The prevalence of SARS-CoV-2 neutralizing antibodies was used to estimate the cumulative number of COVID-19 cases until vaccination began, because SARS-CoV-2 neutralizing antibodies are reported to persist for at least 3 months once infected [12]. The prevalence of SARS-CoV-2 neutralizing antibodies in cats was used to estimate the cumulative number of SARS-CoV-2 infections. In cats, because no vaccine against SARS-CoV-2 is available, the prevalence of SARS-CoV-2 neutralizing antibodies can still be used to estimate the cumulative number of SARS-CoV-2 infections in cats. However, at present, the seroprevalence of SARS-CoV-2 antibodies in cats in various countries is limited, and to our knowledge, no information for Japan has been reported to date.

Therefore, in the present study, we estimated the cumulative number of SARS-CoV-2 infections in cats living in Japan as of July 2020. We developed an ELISA system that can measure anti-SARS-CoV-2 IgG, and conducted ELISAs using serum or plasma from cats that visited veterinary clinics in Japan from June 1 to July 31, 2020. We determined the seroprevalence of SARS-CoV-2 antibodies by performing neutralizing antibody tests on ELISA-positive samples. We then surveyed the owners of the 1,969 cat samples to determine the environment in which they kept their cats. We added information from five countries (China, Croatia, France, Germany, and Italy) where the prevalence of neutralizing antibodies had already been reported, and searched for factors associated with the prevalence of neutralizing antibodies in cats.

Results

Samples were collected from June 1, 2020, to July 31, 2020, in Japan

Before we collected the samples, the necessary sample size was determined. At the time, only one report, in Wuhan, China, was available for the seroprevalence of SARS-CoV-2 IgG antibody in cats [13]. The 99.9% CI of the seroprevalence of SARS-CoV-2 IgG antibodies in cats was 3.63–25.47% in

Wuhan, China. Referring to the cumulative COVID-19 cases per 1,000 people in Japan and Wuhan, China, at that time from the World Health Organization database (accessed on May 25, 2020), the levels of SARS-CoV-2 infection in cats were assumed to be similar in these countries. Thus, the assumed seroprevalence in Japan was tentatively set to ca. 3.0% for the sample size calculation. Based on this seroprevalence assumption in Japan, the sample size was calculated to be 1,562 cases (α error = 0.05, β error = 0.80) using goodness-of-fit tests. Considering the loss of samples because of a lack of information (i.e., clinical history and cat lifestyle), 1,969 cat blood samples were collected from June 1, 2020, to July 31, 2020, in this study (Table 1).

Samples were collected with owners' consent by veterinarians at 101 animal hospitals in Japan from June 1, 2020, to July 31, 2020. The sampling period was set at 2 months after the outbreak of the virus, when the second wave of infections occurred in Japan (Additional file 1: Supplementary Figure 1).

Table 1
Cat blood samples collected in Japan from June 1 to July 31, 2020.

	Prefecture								**Total**
	Tokyo	**Chiba**	**Kanagawa**	**Osaka**	**Kyoto**	**Aichi**	**Shizuoka**	**Iwate**	
Number of hospitals	20	30	19	5	13	7	6	1	101
Number of samples	420	479	317	42	235	253	208	15	1,969
Serum	277	369	275	35	213	173	151	15	1,508
Plasma	143	110	42	7	22	80	57	0	461
Age (mean ± SD)	8.06 ± 4.69	7.47 ± 5.30	8.61 ±5.06	7.03 ± 4.55	6.75 ± 4.48	7.70 ± 4.50	7.94 ±4.67	10.09 ± 5.95	7.70 ± 4.93
Sex									
Male	33	46	17	8	19	37	18	3	181
Castrated male	182	191	148	18	94	71	93	6	803
Female	33	46	19	3	32	54	18	1	206
Spayed female	172	196	133	13	90	91	79	5	779
Feeding style									
Single	277	278	186	24	126	148	113	9	1,161
Multiple	134	182	131	18	99	102	88	6	760
Unknown	9	19	0	0	10	3	7	0	48
Living environment									
Indoor-only	396	409	291	40	208	190	167	13	1,714
Free access outside	19	38	22	2	13	19	26	2	141
Outdoor living	5	32	4	0	14	44	15	0	114
Symptom									
Fever (≥ 39.0^{o}C)	103	23	17	1	5	11	4	2	166
Respiratory symptom	29	25	21	0	9	13	7	5	109
GI symptom*	50	63	43	4	25	35	18	6	244

* GI, gastrointestinal

Development of ELISA using protein A/G conjugated with horseradish peroxidase (protein A/G-based ELISA)

The correlation of IgG reactivity to the S1 protein, as assessed by protein-A/G based ELISA, with that assessed via conventional ELISA using anti-feline IgG conjugated with horseradish peroxidase (anti-feline-IgG based ELISA) was examined. We examined 34 samples collected during the COVID-19 pandemic period, including 9 positive and 25 negative samples in the protein-A/G-based ELISA, detecting the anti-S1-protein IgG antibody, and 162

negative control samples (Figure 1). The comparison of the IgG reactivity measured by protein-A/G-based ELISA with that measured by anti-feline-IgG-based ELISA revealed the presence of a strong correlation (r^2 = 0.83 and p < 0.0001).

Seroprevalence of anti-SARS-CoV-2 antibodies in cats in Japan

The screening of the 1,969 samples by ELISA to S1 protein identified nine samples with IgG reactivity (seroprevalence: 0.46%) (Figure 2a). In addition, ELISA of these nine positive samples to detect the anti-RBD-protein IgG antibody revealed four samples with IgG reactivity (seroprevalence: 0.20%) (Figure 2b). Comparing IgG reactivity to the S1 protein with that to the RBD protein showed that these four samples had high IgG reactivity to both proteins (Figure 2c).

Because ELISA can produce false-positive results owing to IgG cross-reactivity among coronaviruses [14], the neutralization activity of these nine positive samples to SARS-CoV-2 in the *in vitro* setting was determined. One sample neutralized SARS-CoV-2, which was diluted to 1:80, whereas the remaining eight samples (diluted up to 1:20) did not. It was recorded for the sample that neutralized SARS-CoV-2 that the cat had no respiratory or gastrointestinal symptoms or fever in the previous 3 months. It was kept indoors only and had no history of escape from the house (Table 2). Accordingly, the seroprevalence of SARS-CoV-2 antibodies in cats in Japan was assumed to be 0.05% (1/1,969 samples).

Comparing the percentage of SARS-CoV-2 neutralizing antibodies in humans [0.10%, 8/7,980 samples, June 1-7, 2020, Japan] [15] with the percentage of SARS-CoV-2 neutralizing antibodies in cats in this study, there was no significant difference (Fisher's exact test, p = 0.44). Comparing the prevalence of SARS-CoV-2 neutralizing antibodies in cats regarding the publication of the reports (i.e., China, Croatia, France, Germany, and Italy) [13, 16–20] (Additional file 1: Supplementary Table 1), The seroprevalences in cats living in Japan was the lowest.

Table 2
Information of the nine cats with positive results in the protein-A/G-based ELISA, detecting anti-S1-protein IgG.

Sample No. *	Sampling date (2020)	Sample type	Age	Breed	Lifestyle			Clinical symptom			Protein A/G based ELISA targeted:		Neutralizing Ab titer
					Sex†	Living environ-ment	Roommate animal	Fever‡	Respir-atory§	GI¶	S1 (A^{492})	RBD (A^{492})	
●1	11th July	Serum	9y11mo	Mixed	M	Indoor-only	-	-	-	-	1.25	2.65	1:80
2	21st June	Serum	2y5mo	Somali	MC	Indoor-only	-	-	-	-	1.15	0.06	<1:20
3	19th June	Serum	8y9mo	Mixed	MC	Indoor-only	-	-	-	-	1.12	0.25	<1:20
4	29th July	Serum	10y3mo	Mixed	FS	Indoor-only	-	-	-	-	1.02	2.78	<1:20
5	1st July	Serum	9mo	Bengal	F	Indoor-only	-	-	-	-	0.98	2.70	<1:20
6	19th July	Plasma	7mo	Mixed	FS	Indoor-only	-	+	-	-	0.91	0.09	<1:20
7	18th July	Serum	1y11mo	Mixed	F	Indoor-only	-	-	-	-	0.76	0.19	<1:20
8	17th July	Plasma	10y4mo	Mixed	F	Free-access-outside	-	-	+	-	0.73	0.20	<1:20
9	4th July	Serum	8y5mo	Munch-kin	MC	Indoor-only	-	-	-	-	0.57	1.17	<1:20

* Sample No. 1 contained a neutralizing antibody to SARS-CoV-2, which is shown as a circle. Samples No. 1–4 showed positive results in the ELISA detecting anti-RBD-protein IgG, which are underlined.

† MC, male castrated; FS, female spayed.

‡ Body temperature above 39°C was considered the presence of fever.

§ The respiratory symptoms included cough, sneezing, conjunctivitis, and nasal and/or ocular discharge.

¶ GI, gastrointestinal. The gastrointestinal symptoms included abdominal pain, belching, stomachache, bloating, loss of appetite, vomiting, abdominal pain, constipation, diarrhea, and/or melena.

Cat-lifestyles in Japan

The cat-lifestyle in Japan was investigated because the frequency of interaction between cats and people can be determined by the owner. The factors associated with cat-lifestyle included living environment (i.e., housing-indoors-only, free-access-outside, and living-outdoors styles), neutering, and multiple animal holdings (Table 1). In Japan, the housing-indoors-only style was ca. 88%, representing the major living environment for cats. The ratio of neutering was ca. 80%. The rate of housing multiple animals was ca. 40%.

Table 3
Correlation of the seroprevalence of SARS-CoV-2 antibodies in cats with factors associated with cat-lifestyles.

	Country						Correlation analysis‡			
							of seroprevalence in cats relative to:		of seroprevalence in cats/confirmed COVID-19 cases per 1,000 relatives to:	
	Japan	China	Croatia	France	Germany	Italy	r-value	p value	r- value	p value
Prevalence of neutralizing antibodies specific to SARS-CoV-2 (CL 95%) in cats*	0.05% (0.003 – 0.29)	2.10% (1.74 – 2.86) †	0.76% (0.40 – 4.20)	6.25% (0.32 – 28.33)	0.22% (0.04 – 0.79)	5.76% (3.25 – 10.02)				
Reference(s)	This study	[13, 16]	[17]	[19]	[20]	[18]				
Cumulative COVID-19 cases per thousand people†	0.286	0.057	0.963	3.059	2.921	3.848	0.540	0.149		
Factor of cat-lifestyle										
Living environment										
Housing-indoor-only	88.04%	37.00%	No data	34.20%	69.70%	18.26%	**-0.900**	**0.042**	-0.502	0.225
Free-access-outside	6.06%	2.53%	No data	8.40%	30.30%	57.02%	0.032	0.342	-0.500	-0.225
Living outdoor	5.90%	60.47%	No data	57.40%	No data	24.72%	0.369	0.375	**0.996**	**0.042**
Neutered	80.25%	No data	25.60%	No data	79.80%	70.50%	-0.800	0.208	-0.610	0.208
Housing multiple animals	39.60%	20.00%	No data	No data	64.40%	60.60%	0.105	0.167	-0.794	0.167
Reference(s)	This study	[21]	[22]	[23]	[24, 28]	[26, 27]				

* Regarding the reports for seroprevalence of anti-SARS-CoV-2 antibodies in cats in China, Croatia, France, Germany, and Italy, the seroprevalence in cats is varied (see Supplemental Table 1).

† Cumulative COVID-19 cases per thousand people are cited from the SARS-CoV-2 surveillance data in the World Health Organization database (https://covid19.who.int/).

‡Spearman's rank correlation coefficient tests were used. Positive *r* values denote a positive correlation; negative *r* values denote a negative correlation. The bold marker shows the numerical values harboring significant differences.

Analysis of factors associated with cat-lifestyles with seroprevalence in cats in six countries

The ratios of factors of cat-lifestyle also varied among five countries (China, Croatia, France, Germany, and Italy) [21–27]. Estimating the important factor of cat-lifestyle, we statistically analyzed the correlation of the factors of cat-lifestyle with seroprevalence in cats among these -countries (Table 3). Comparing the factors of cat-lifestyle, the ratio of housing-indoors-only style and that of neutering in Japan were the highest among them. On the other hand, the ratio of housing multiple animals in Japan was lower than in Germany and Italy and higher than in China. The factors included not only housing indoor only, free access outside, and living outdoors in the living environment but also neutering and multiple animal holdings. The ratio of housing-indoors-only alone was negatively correlated with viral seroprevalence ($r = -0.900$, $p = 0.042$); other factors were not. Considering the epidemic

situation of COVID-19 in humans, the seroprevalence in cats was positively correlated with the ratio of living outdoors (r = 0.996, p = 0.042); other factors were not.

Discussion

In the present study, to analyze the epidemic situation of SARS-CoV-2 in cats, we collected blood samples from 1,969 cats that visited veterinary clinics in Japan in June and July 2020 to determine the presence of SARS-CoV-2-specific antibodies. Of these samples, one showed neutralizing activity (seroprevalence: 0.05%, 1/1,969 samples), which was not significantly different from the concurrent human seropositivity at that time [15]. This suggests that the prevalence of SARS-CoV-2 infection in cats may be related to the COVID-19 epidemic status in humans. We next surveyed the cat lifestyle from owners of the 1,969 cats by questionnaire. We added information from five countries (China, Croatia, France, Germany, and Italy) where the prevalence of neutralizing antibodies had already been reported, and searched for factors associated with the prevalence of neutralizing antibodies in cats. The percentage of cats living indoors-only and outdoors correlated with the prevalence of SARS-CoV-2 neutralizing antibodies in cats.

We performed ELISAs for S1 and RBD of SARS-CoV-2 followed by a neutralization test. Although sero-epidemiological studies targeting the nucleocapsid of SARS-CoV-2 have been conducted [29, 30], the nucleocapsid-protein-specific IgG of cats infected with SARS-CoV-2 cross-reacted with feline coronavirus (FCoV) and other coronaviruses [30, 31]. By contrast, S1 has a unique structure in each coronavirus [32], and feline S1-specific IgG infected derived from FCoV, Feline infectious peritonitis virus, or SARS-CoV-2 barely cross-reacted with other coronaviruses [14]. RBD-specific IgG of SARS-CoV-2 also barely cross-reacted with RBD of feline coronaviruses [20]. In addition, recent analysis of 15 different antibody titers in the blood of COVID-19 patients revealed in humans that measurement of IgG against spike proteins, especially S1 and RBD, correlates with the presence of neutralizing antibodies and is an excellent indicator of past infection [33]. Because identification of the presence of neutralizing antibodies requires special laboratories equipped with biosafety level 3 facilities and involves the risk of analyzing multiple samples, screening for the presence of S1- and RBD-specific IgG and then confirming the presence of neutralizing antibodies in IgG-bearing samples are currently considered to be an appropriate criterion standard.

ELISA to determine the presence of feline anti-SARS-CoV-2 IgG in previous reports used anti-feline IgG antibodies [13, 16, 17, 19, 20]. However, as of September 30, 2021, no feline anti-SARS-CoV-2 IgG antibodies are commercially available. Because of the general unavailability of controls, the results of each ELISA cannot be compared across publications. An ELISA system using Protein A/G has recently been used to investigate infectious diseases across animal species [34, 35]. Because SARS-CoV-2 is spreading worldwide, it is important to compare the results across species. The detection system developed in the present study can generalize antibody titers because rabbit anti-SARS-CoV-2 antibodies are commercially available. Future research using this analysis system is warranted.

Similarities in seroprevalence between humans and cats have been observed in Japan. This suggests that the opportunity for SARS-CoV-2 infection in cats is influenced by the extent of COVID-19 prevalence in humans. Cats kept by COVID-19 patients or people with a history of COVID-19 have a high probability of being infected with SARS-CoV-2 [19, 36]. The prevalence obtained in the present study is supported by these reports.

Changes in our lifestyle, represented by social distancing and staying at home, have reduced the spread of COVID-19 [37, 38]. The behavior of cats is largely determined by their owners. Therefore, we suspected that cat lifestyle is involved in the prevalence of SARS-CoV-2 infection in cats. Because of the small number of positive samples in this study, we examined antibody prevalence and cat lifestyles in countries that had reported the prevalence of SARS-CoV-2 neutralizing antibodies in cats. We consider that whether cats were kept indoors or outdoors may have played a role in the epidemic. Cats kept outdoors may not only form their own feline social networks, but may also be casually petted by people passing. As we have found similarly, previous reports indicate that cats are often asymptomatic even when they are affected by SARS-CoV-2 [8]. Therefore, infected outdoor cats without clinical symptoms can transmit SARS-CoV-2 to cats and to humans by contact and droplet transmission [39]. For cats housed indoors, the public maintains a physical distance, whereas cats housed outdoors are not considered to maintain an appropriate distance from the public. Therefore, housing cats indoors is considered one of the most effective preventive measures to control the spread of SARS-CoV-2 between cats and humans.

There are several limitations to the present study. First, due to ethical constraints, we were unable to interview owners about their history of COVID-19. Therefore, it remains unclear to what extent transmission from cats carrying neutralizing antibodies to SARS-CoV-2 to humans occurs. In addition, because of the limited number of positive samples we obtained, we compared our results with previous reports. Therefore, we expected that there would be variations in the survey methods, and we were not able to analyze directly the relationship between cat housing methods and the spread of SARS-CoV-2 in cats. It is hoped that future research will fill in the gaps in our analysis.

As of July 2020, 0.05% of cats in Japan had a history of infection with SARS-CoV-2, which was similar to the epidemic situation to humans. Because cats are animals that live near humans, the infection status of SARS-CoV-2 is considered to be linked to that in humans. Therefore, follow-up of the epidemic status of SARS-CoV-2 in cats is warranted. Factors that have been implicated in the prevalence of SARS-CoV-2 in cats are whether the cats are housed indoors or outdoors. The epidemic of SARS-CoV-2 infection in cats may be controlled by the efforts of cat owners.

Methods

Collection of samples with clinical history and data pertaining to cat-rearing styles

According to a literature review on May 25, 2020, the seroprevalence in cats was assumed based on the lower limit of a 99.9% confidence interval (CI). The sample size was calculated based on the seroprevalence using G*Power 3.1.9.6 (downloaded from https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower on April 25, 2020).

Samples were collected with owners' consent by veterinarians at 101 animal hospitals in Japan from June 1, 2020, to July 31, 2020. The sampling period was set at 2 months after the outbreak of the virus, when the second wave of infections occurred in Japan. Veterinarians completed a questionnaire on the clinical history of the animals within 3 months of blood collection. Cat-rearing-style information, such as living environment, neutering, and multiple animal holdings, was surveyed from cat owners visiting animal hospitals. Living-environment information was categorized into three types: hosing-indoors-only, free-access-outside, and living-outdoors (i.e., stray cats and feral cats) styles. Moreover, as negative controls, we also used cat blood samples, which were collected from January 1, 2015, to March 31, 2015, for the Azabu University bioresource banking project. This study was carried out in compliance with the ARRIVE guidelines and, was approved by the Animal Ethics Committee of Azabu University (No. 210407–7).

Detection methods for anti-SARS-CoV-2 antibodies in feline serum/plasma samples

Enzyme-linked immunosorbent assay (ELISA) using protein A/G conjugated with horseradish peroxidase was used to detect the anti-S1-protein IgG antibody or anti-RBD-protein IgG antibody [34]. Based on previous reports [13, 20], 100 ng per well of recombinant S1 protein (#S1N-C52H3, Acro Biosystems, Newark, DE, USA) and 50 ng per well of recombinant receptor-binding domain (RBD) protein (#230–30162–100; Ray Biotech, Peachtree Corners, GA, USA) were used to coat half-well plates (Costar, Washington, DC, USA) in 50 mM carbonate buffer. All plates were incubated overnight at 4°C, washed once, and blocked with 0.5% bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) in PBS containing 0.05% Tween-20 (PBS-T) at 25°C for 1 h. After washing three times, the plates were incubated with serum samples diluted at 1:100 in PBS-T with 5% skim milk (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and 10% fetal bovine serum (Bio West, Riverside, MO, USA) for 1 h at 37°C.

The plates were then washed three times and incubated with protein A/G conjugated with horseradish peroxidase (1:25,000; Thermo Fisher Scientific, Waltham, MI, USA) for 1 h. After washing three times, the plates were incubated with *O*-phenylenediamine dihydrochloride (Sigma Aldrich) at 25°C for 30 min. The reaction was terminated by adding 4 N H_2SO_4 (Fujifilm Wako Pure Chemical Corporation). The absorbance at 492 mm (A_{492}) was measured using a spectrophotometer (Multiskan JX; Thermo Fisher Scientific). All samples were assayed in parallel on the same plate. For blood samples showing higher values than the detection range, an additional three serial dilutions were assayed. All experiments were performed in triplicate. PBS-T was used in all washes. The cutoff value was set as the mean ± 3 · standard deviation (SD) of the negative control samples. Samples with a value higher than the cutoff value were considered positive results.

In the protein A/G-based ELISA system, an anti-S1 rabbit polyclonal antibody (1:100; #GTX135356, Genetex, Irvine, CA, USA) and an anti-RBD rabbit monoclonal antibody (1:80,000; #40592-R001, Sino Biological, Beijing, China) were used as positive controls. IgG reactivity in the positive controls was adjusted to 1.0 A_{492}. The samples of the positive control that were serially diluted to 1:2, 1:4, and 1:8 from the dilution condition of 1.0 A_{492} were adjusted as 5, 2.5, and 1.25 A_{492}, respectively. This ELISA is comparable to the conventional ELISA using an anti-feline IgG conjugated with horseradish peroxidase (Figure 1). Moreover, the neutralization activity of diluted samples to SARS-CoV-2 was examined by a neutralization assay using SARS-CoV-2 strain 2019-nCoVJPN/TY/WK-521/2020 and VeroE6/TMPRSS2 cells, as described elsewhere [40].

Data collection of seroprevalence and cat-rearing style in countries by reviewing the relevant literature and cumulative cases of COVID-19 per 1,000 people

The data pertaining to the seroprevalence of SARS-CoV-2 antibodies in cats, which were obtained based on a neutralizing antibody assay, were collected by a systematic literature search of PubMed databases using various combinations of the keywords "epidemiology", "prevalence", "surveillance", "survey", "SARS-CoV-2", "feline", "ELISA", "IgG", "neutralizing", and "cat" published between May 1, 2020, and April 31, 2021. The standard protocol and checklists of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) were followed [41]. The data pertaining to the cat-rearing styles were collected based on a literature search of PubMed databases using various combinations of the keywords "lifestyle", "rearing", "environment", "surveillance", and "cat", and the name of each country published between January 1, 2015, and April 31, 2021. The inclusion criteria were defined as using over 50 samples in the surveillance and targeted toward the rate of multiple animal holdings, neutering rate, and living environment. Of note, the investigation period could not be restricted to 2020 because of a lack of information. The epidemic situation of COVID-19 in humans in each country was based on the reported cumulative COVID-19 cases per 1,000 people from data from the World Health Organization database (https://covid19.who.int/).

Statistical analysis

The CI of seroprevalence in each report was calculated based on the total number of participants and the positive rates in each report. When multiple reports were available in a country, the mean values of the seroprevalence and those of lower and upper limit values of CIs were calculated.

The correlation of IgG reactivity to the S1 protein, as assessed by protein-A/G-based ELISA, with that assessed via conventional ELISA using anti-feline IgG conjugated with horseradish peroxidase (anti-feline-IgG-based ELISA) was analyzed using Pearson's correlation coefficient test.

Correlations of the seroprevalence of SARS-CoV-2 antibodies in cats with factors associated with cat-rearing styles were analyzed using the Kolmogorov–Smirnov normality test, followed by Spearman's correlation coefficient test. The prevalence of anti-SARS-CoV-2 IgG in cats and humans was compared using Fisher's exact test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA).

Abbreviations

COVID-19	Coronavirus disease 2019
CI	Confidence interval
ELISA	Enzyme-linked immunosorbent assay
Ig	Immunoglobulin
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.05% tween-20
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analysis
RBD	Recombinant receptor-binding domain
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation

Declarations

Ethics approval and consent to participate

All sampling procedures complied with national and Japanese regulations, and the animal ethics committee approved this study of Azabu University, Japan (approval number 210407–7). The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not application.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

To the best of our knowledge, the named authors have no conflicts of interest, financial or otherwise.

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Author's contributions

I.I., R.A., and T.S. designed the serological and cat rearing style survey. I.I., R.A., S.H., M.H., M.Y., Y.M., T.M., and M.S. collected the samples and data. I.I. processed the ELISA and the comparative analysis of cat lifestyles. S.M. performed neutralizing antibody assay of study. I.I. drafted the manuscript. S.H. and M.S. reviewed and edited the manuscript. I.I., J.U., T.M., and M.S. contributed to the study design of serological analysis. All authors read and approved the final manuscript.

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Figures

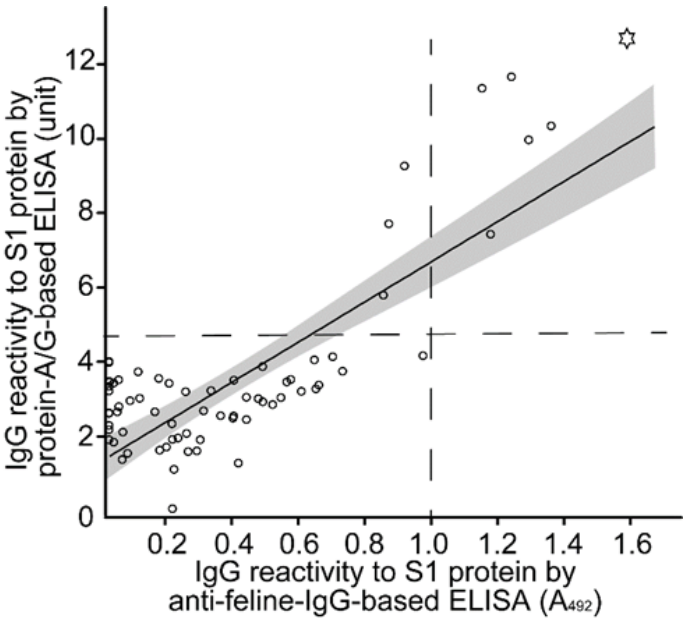

Figure 1

Reliability of ELISA using protein A/G conjugated with horseradish peroxidase (protein-A/G-based ELISA). The correlation of IgG reactivity to the S1 protein, as assessed by protein-A/G based ELISA, with that assessed via conventional ELISA using anti-feline-IgG based ELISA was examined. The absorbance at 492 mm (A_{492}) was measured for anti-feline-IgG based ELISA. The threshold value was set as the mean value + 3 ´ SD of negative control samples, which is shown as a dashed line. Samples with a higher value than the threshold value were considered as positive results. The regression line and 95% confidence intervals are indicated by the black line and gray area, respectively. The circles indicate individual tested samples. The star indicates the sample with neutralizing activity to SARS-CoV-2.

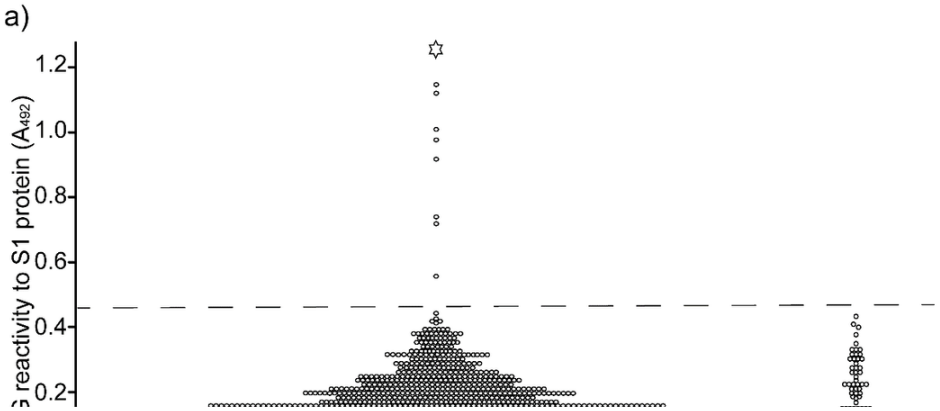

Figure 2

IgG reactivity to SARS-CoV-2 proteins in cats in Japan. IgG reactivity was measured by ELISA using protein A/G conjugated with horseradish peroxidase. The absorbance at 492 mm (A_{492}) was measured for anti-feline-IgG-based ELISA, adjusted by the positive control sera derived from the rabbit. Samples with a value higher than the cutoff value were considered positive results. The circles indicate individual tested samples. The star indicates a sample showing neutralizing activity to SARS-CoV-2 in the neutralizing antibody assay. a) IgG reactivity to the S1 protein among 1,969 samples. The mean A_{492} ± standard deviation (SD) of 145 negative control samples was 0.133 ± 0.111 A_{492}, and the cutoff value (mean + 3SD) is 0.469 A_{492}, shown as the dotted line. b) IgG reactivity to the RBD protein in nine samples that were positive for anti-S1-protein IgG. The mean A_{492} ± SD of 28 negative control samples selected randomly was 0.231 ± 0.267 A_{492}, and the cutoff value (mean + 3SD) is 1.033 A_{492}, shown as the dotted line. We screened 75 samples during the COVID-19 pandemic, including 9 positive samples and 66 negative samples in the protein-A/G-based ELISA, detecting anti-S1-protein IgG. c) Comparison of IgG reactivities with those to the S1 protein, as measured by protein-A/G-based ELISA. Among the nine positive samples in the protein-A/G-based ELISA detecting anti-S1-protein IgG, four cats showed IgG reactivity to the RBD protein.

Supplementary Files

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