DETECTION OF CORONAVIRUS AMONG DOMESTIC ANIMALS

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Abstract
This article presents the results of studies on biological samples collected from 640 swabs taken from dogs and cats across various regions of the Republic of Kazakhstan. These samples were part of a monitoring study on the spread of coronavirus among domestic animals. Total RNAs were isolated using the magnetic sorption method with the ALPREP kit and subsequently analyzed with the ALSENSE-SARS-CoV-2 RT-qPCR kit. Real-time RT-PCR analysis revealed that 104 samples tested positive within 22–37 amplification cycles. These positive samples were then cultured in Vero cell lines to confirm the presence of the virus. The biological activity of the resulting virus-containing suspension was determined using the Reed-Muench method. During cultivation, one viral isolate with a biological activity of 5.83±0.08 lg $TCID_{50}$/ml was obtained. A microphotograph of the virus was taken using an electron microscope to determine its size, shape, and structure, which confirmed its morphology corresponding to the Coronaviridae family. The data obtained further indicate that domestic animals can suffer from and carry coronavirus. It is becoming increasingly evident that the virus can infect and replicate in the organs of various farm and domestic animals.

Key words: biological samples, coronaviruses, PCR analysis, RNA, domestic animals, Vero cell culture, electron microscopy.

Introduction

Coronaviruses comprise a large family of viruses known to infect both humans and animals (Denis *et al*., 2020; Prince *et al*., 2021; Considerations, 2020; Cohen, 2020). However, evidence of mutual transmission between humans and animals remains inconclusive. In this regard, the global scientific community is investigating the potential for cross-species virus transmission. While human-to-human transmission is well-established, the dynamics of transmission between humans and animals are less clear. The threat is not only to human health, but it also poses a significant risk to the animal population. There are numerous reports indicating a high likelihood of COVID-19 transmission from humans to animals, leading to an increase in pet abandonment. Another concern is the possibility of reverse zoonosis, where the COVID-19 virus could be transmitted back to humans from pets such as cats and dogs.

Evidence suggests that the virus may initially circulate in bats before being transmitted to humans via an intermediate host, raising new questions about the potential for human-to-animal transmission. Several instances of this virus transmitting from infected humans to animals have been documented (Ng & Hiscox, 2020). Literature indicates that SARS-CoV-2 infections in animals can range from asymptomatic to symptomatic, with signs varying from mild respiratory and gastrointestinal symptoms to severe conditions such as pneumonia and death (Cui *et al*., 2022).

To understand the spread of coronavirus among animals and explore the possible evolutionary connections between humans and animals, extensive research, including virus isolation, cultivation, and analysis of the biological and physicochemical properties of viruses isolated from animals, is essential.

In this context, Kazakh scientists have explored the susceptibility of various cell lines to the coronavirus. Their research involved 11 different primary and continuous cell lines, finding that the coronavirus caused cytopathic effects in Vero cell cultures (Zhugunissov *et al*., 2022). This highlights the need for further research to optimize the cultivation of virus-containing material in Vero cell cultures. This study aims to examine the biological properties of material containing the virus isolated from domestic dogs.

Materials and Methods

Sampling and Transportation

Sampling from domestic animals exhibiting clinical signs of coronavirus infections was conducted in compliance with the applicable regulatory legal acts in the Republic of Kazakhstan (Prikaz, 2015). Specialists adhered to prescribed procedures and biosafety regulations during sampling, including the use of protective clothing, goggles, gloves, disinfectants, and appropriate handling techniques.

During swab collection, animals were securely restrained to ensure stable fixation. Dry, sterile cotton swabs were used to collect samples from the nasal cavity, oropharynx, rectum, and conjunctiva mucous membranes. Throughout the expedition, swabs were stored in liquid nitrogen.

Clinical Samples

For this study, a total of 640 clinical samples (including mouth washes, rectal, nasal, and ocular swabs) were collected from 160 diseased domestic animals (dogs and cats) across various regions including Turkestan, Karaganda, East Kazakhstan, Astana, and Almaty.

Total RNA extraction was performed using the ALPREP kit, employing the magnetic sorption method according to the manufacturer's protocol (Ali *et al*., 2017).

Virus identification via real-time PCR was conducted using the 'ALSENSE-SARS-CoV-2–RT-qPCR' kit, following the manufacturer's instructions (Wu *et al*., 2020).

Virus isolation in cell culture

Virus isolation in Vero cell culture was achieved through blind passaging for a minimum of three generations (Zhugunissov *et al*., 2022). The infectious activity of the virus was determined by titration following the Reed-Muench method (Wurtz *et al*., 2021) in a Vero cell culture grown in a 96-well culture microplate. Serial 10-fold dilutions of the virus stock ranging from 10^{-1} to 10^{-8} were prepared in DMEM supplemented with 2% PBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Subsequently, 100 µl of each dilution was added to the wells. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 7 days, and cytopathic effects were assessed using an inverted microscope. The titer of virus-containing material was calculated using the Reed-Muench formula and expressed in lg $TCID_{50}$/ml (Reed & Muench, 1938; Korochkin *et al*., 2010; Zhugunissov *et al*., 2022).

Electron Microscopy of Viruses

The virus-containing material was concentrated by ultracentrifugation using a Himac CS-150FNX ultracentrifuge (Japan) at 366,000 g for 20 minutes. After centrifugation, the supernatant was discarded, and the sediment was resuspended in 1X PBS buffer to a volume of 100 µl. Preparations for electron microscopy were made by adsorbing onto copper grids coated with a formvar substrate reinforced with carbon. Negative contrast was achieved using a 2% aqueous solution of phosphotungstic acid, and the samples were examined using a transmission electron microscope JEM-100 CX-II JEOL (Japan) at an accelerating voltage of 80 kV at various magnifications. Photographs were captured from developed and fixed negatives using an Azov photo enlarger.

Statistical Analysis

Statistical processing of the data was performed using the GraphPad Prism 8 software package. The data were subjected to analysis using Student's t-test, deemed reliable at $p < 0.05$ (Ashmarin, Vasiljev, & Ambrosov, 1975).

Results and Discussion

For this study, 640 samples were used to monitor coronavirus infection among domestic animal species from nurseries in Turkestan, Karaganda, East Kazakhstan, Astana, and Almaty. From the real-time PCR results, it was established that out of the 640 biological samples studied, 104 showed positive results for the presence of the virus, see 'Figure 1'. The positive control sample gave a positive result at cycle 22 (Ct-22.00), compared with the fastest detection in sample No. 5 at cycle 21 (Ct-21.88). This sample was isolated from the rectum of dog No. 5. The remaining samples were detected within cycles 25-37 (Ct-25.23 - Ct-37.27).

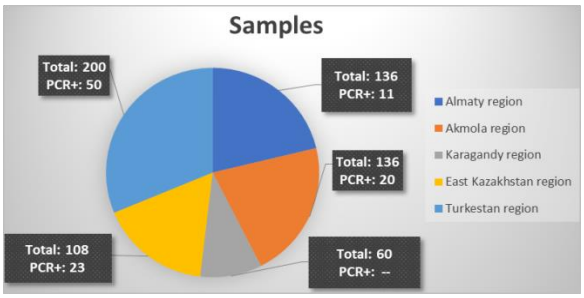

Figure 1. Map of samples with sampling sites. The virus was identified in selected samples using real-time PCR.

Next, the samples that showed a positive result in the PCR analysis (Nos. 1-104) were seeded on Vero cell culture. Observation of morphological changes in cell culture samples was carried out over 4 days. No cytopathogenic effect (CPE) was detected in any of the biological samples at the first passage level. The culture suspension was then frozen at -50 ±0.5°C. Subsequently, blind passaging was performed for three generations. Cell cultures were infected with a thawed virus-containing suspension in the following passage. At the third passage level, on the third day of observation, rounding and destruction of cells in samples No. 5, 7, and 10 were noticeable, indicating the formation of virus CPE. All samples were then sent to the fourth passage. Observation of morphological changes continued over 4 days. On the fourth day, changes in cell shape, such as swelling, rounding, or thinning, were noticeable only in sample No. 5, indicating the formation of CPE. A cytopathogenic agent could not be detected in the other biological samples, leading to their exclusion from further studies. Sample No. 5 was passaged until the eighth passage with results presented in 'Figure 2'.

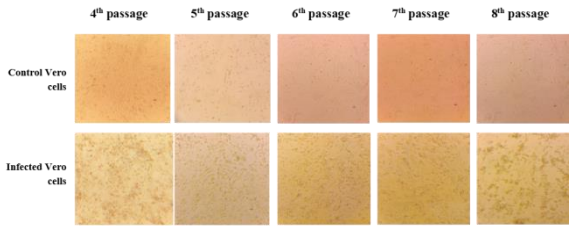

Figure 2. Blind passaging and formation of CPE on a monolayer of Vero cell culture on the fourth day after infection.

From the data obtained in 'Figure 2', it is evident that at the eighth passage level, monolayer destruction occurred through detachment/desquamation of the affected cells 48-72 hours after the appearance of the first signs of cytopathology. From the data presented in 'Figure 1', it is clear that at the fourth passage level, biological sample No. 5 (rectal wash) showed CPE of the virus in the Vero cell culture starting from the third

day after infection. As observed in the figure, in subsequent passage levels, the number of rounded cells increases, while the number of spread-out cells on the adhesion surface (in the monolayer of cell culture) decreases. Pockets of emptiness form due to detached cells, and there is an increase in intercellular space compared with the control culture.

Next, the biological activity of the studied material was determined at all passage levels. The results of a comparative analysis of biological activity are presented in 'Figure 3'.

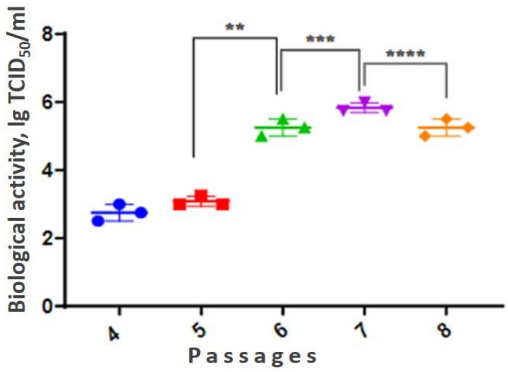

Figure 3. Comparative analysis of the biological activity of the virus in Vero cell culture using the Holm-Sidak method revealed statistically significant differences across passages 4 to 8. Significant differences in biological activity were observed between passages 5 and 6 ($p = 0.000202$), between passages 6 and 7 ($p = 0.024896$), and between passages 7 and 8 ($p = 0.024896$).

The results indicate that the viral material in the studied cell culture samples increases in concentration from one passage to the next, with average values reaching 5.83±0.08 lg $TCID_{50}$/ml. The presence of the virus was additionally confirmed by electron microscopy, see 'Figure 4'.

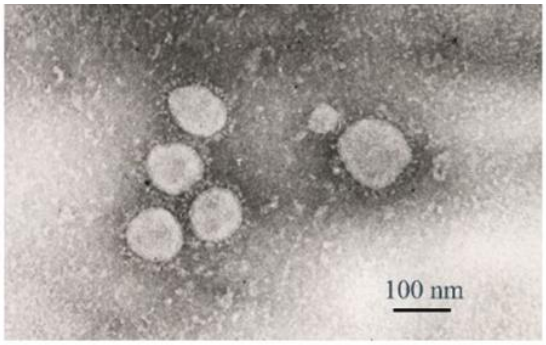

Figure 4. Electron microscopy image of the virus. The image was taken at 10,000 times magnification on a transmission electron microscope JEM-100 CX-II JEOL (Japan).

As observed in 'Figure 4', the virus exhibits a spheroid shape with a diameter of 120-160 nm. Virions possess a lipid envelope with club-shaped peplomers, 5-10 nm in length, formed by trimers of protein S. These peplomers, resembling the teeth of a crown, give the name to the entire family Coronaviridae.

Discussion

SARS-CoV-2, which is believed to have originated from bat coronaviruses, enters host cells through the ACE2 receptor. This process shows varying affinities across different animal species. Infected pets display positive SARS-CoV-2 PCR results and develop antibodies, often mirroring respiratory symptoms observed in their owners, which suggests interspecies transmission (Ferasin *et al*., 2021).

Among domestic animals, alpha, beta, and delta coronaviruses are notable causative agents of infections. These viruses exhibit host specificity and diverse clinical presentations. For instance, alpha coronaviruses affect dogs (enteric form), cats, pigs (vector-borne gastroenteritis), minks, and ferrets, while beta coronaviruses cause diseases in cattle (BCoV), dogs (respiratory form), horses, and pigs (hemagglutinating encephalomyelitis). Delta coronavirus primarily infects pigs (Dzhavadov *et al*., 2020; Nagornyh, Tyumencev, & Akimkin, 2020; Andreeva & Nikolaeva, 2021).

Some animal species have tested positive for SARS-CoV-2, mainly after close contact with humans infected with SARS-CoV-2. These detections include various animals such as birds, various primates, reptiles, ungulates, felines, other carnivores, and domestic dogs (Andreeva & Nikolaeva, 2021).

In this study, coronavirus circulation was detected in stray dogs from an animal detention facility in Almaty. Clinical manifestations in animals, similar to humans, include cough, sneezing, shortness of breath, nasal and eye discharge, vomiting or diarrhea, fever, and lethargy, with asymptomatic infections also documented (Perera *et al*., 2021).

Clinical samples from dogs' rectum, oropharynx, and nose were used for research. Based on the PCR results, a coronavirus isolate with a suspected SARS-Cov-2 species was identified, which was further characterized by real-time PCR and electron microscopy. This revealed the size, shape, and morphology of the virus.

Vero cell culture was utilized for virus isolation and cultivation due to its rapid adaptation for coronavirus, facilitating the exploration of biological, molecular-genetic, and physicochemical properties essential for epidemic prevention and diagnosis (Zhugunissov *et al*., 2022).

Isolation of the virus is crucial to determine and study its biological, molecular, and physicochemical properties since obtaining a new current strain of the virus can help prevent a possible epidemic by creating relevant means of prevention and diagnosis.

As is known today, the biological, molecular-genetic, and physicochemical properties of coronavirus

isolated from pets and animals have not been sufficiently studied. There is no complete information about what evolutionary role pets and animals play in spreading coronavirus in nature (Gautam *et al*., 2020). The results of this study highlight the optimal cultivation conditions required for isolates from animals, namely an incubation temperature of 35-37°C and an incubation time of 48-72 hours, facilitating the full manifestation of virus CPE in Vero cell culture.

Additionally, literature reports have demonstrated the permissiveness of various laboratory cell lines to SARS-CoV-2 growth. The study showed that virus growth was observed on 7 cell lines: 6 monkey cell lines: VERO E6, VERO 81, VERO SLAM, MA104, LLC-MK2, BGM and 1 human cell line Caco-2. Cytopathogenic effects are variable: 48-72 hours pass from the lysis of the cell monolayer to the absence of a cytopathogenic effect. In their opinion, the penetration of coronaviruses into cells depends on the spike protein (S) binding, which can infect not only various human tissues but also animals (Diaz *et al*., 2020).

While some literature suggests dogs exhibit low susceptibility to SARS-CoV-2, recent reports of infected pet dogs from different regions indicate the possibility of transmission through contact with infected humans. Although these dogs showed no clinical signs, our study focused on animals presenting with diarrhea, lacrimation, and excessive salivation, suggesting potential variations in viral pathogenicity (Shi *et al*., 2020; Leroy, Ar Gouilh, & Brugere-Picoux, 2020; Csiszar *et al*., 2020; Loeb 2020; Goumenou, Spandidos, & Tsatsakis, 2022).

Given the similarity of ACE2 receptors in dogs to human ACE2 (hACE2) receptors, raising concerns about their role as potential carriers, further investigations are warranted. However, current evidence does not support the transmission of the virus from infected dogs to animals or humans.

The obtained data will be used to further experiments aimed at determining the pathogenicity of coronavirus viral biomass in vivo and conducting genetic studies.

Conclusions

1. This study analyzed 640 swabs obtained from domestic pets, revealing that 104 samples tested positive for the presence of coronavirus. After reproduction in Vero cell culture, a coronavirus viral biomass with a biological activity of 5.83±0.08 lg$TCID_{50}$/ml was isolated from a dog.
2. The data obtained indicate the potential for coronavirus infection in pets, highlighting the necessity for further research in this area.

Acknowledgements

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References

Ali, N., Rampazzo, R. C., Costa, A. D., & Krieger M. A. (2017). Current Nucleic Acid Extraction Methods and Their Implications to Point-of-Care Diagnostics. *BioMed Research International*, 9306564, 13. DOI: 10.1155/2017/9306564.

Andreeva, A. V. & Nikolaeva, O. N. (2021). Novaya koronavirusnaya infekciya (COVID-19) u zhivotnyh (New coronavirus infection (COVID-19) in animals). *Veterinarnyj vrach*. 2. Retrieved March 21, 2024, from https://cyberleninka.ru/article/n/novaya-koronavirusnaya-infektsiya-covid-19-u-zhivotnyh. (in Russian).

Ashmarin, I. P., Vasiljev, N. N., & Ambrosov, V. A. (1975). *Bystrye metody statisticheskoj obrabotki i planirovanie eksperimentov* (Fast methods of statistical processing and planning of experiments). Leningrad: LGU. (in Russian).

Cohen, J. (2020) From mice to monkeys, animals studied for coronavirus answers. *Science*. 368 (6488). 221-222. DOI:10.1126/science.368.6488.221.

Considerations for sampling, testing, and reporting of SARS-CoV-2 in animals. (2020). Version 1, 7 May. Retrieved March 21, 2024, from https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/COV-19/.

Csiszar, A., Jakab, F., Valencak, T. G., Lanszki, Z., Toth, G. E., & Kemenesi, G. (2020). Companion animals likely do not spread COVID-19 but may get infected themselves. *GeroScience*. 42. 1229-1236. DOI: 10.1007/s11357-020-00248-3.

Cui, S., Liu, Y., Zhao, J., Peng, X., & Lu, G. (2022). An Updated Review on SARS-CoV-2 Infection in Animals. *Viruses*. 14 (7), 1527. DOI: 10.3390/v14071527.

Denis, M., Vandeweerd, V., Verbeeke, R., Laudisoit, A., Reid, T., Hobbs, E., Wynants, L., & van Der Vliet, D. (2020). COVIPENDIUM: information available to support the development of medical countermeasures and interventions against COVID-19. *Transdisciplinary Insights*. DOI: 10.5281/zenodo.4072014.

Diaz, F. J., Jimenez, W. A., Alvarez, L. F., Valencia, G., Donato, K. L., & Muñoz, C. F. (2020). Isolation and characterization of an early strain of SARS-CoV-2 during the 2020 epidemic in Medellin, Colombia. *Biomedica*. 40(2), 148-158. DOI: 10.7705/biomedica.5834.

Dzhavadov, E. D., Petrova, O. G., Ivashkina, L. N., Pechura, E. V., Pleshakova, V. I., Rahmanina, N. A., Poryvaeva, A. P., & Shilova, E. N. (2020). *Koronavirusy u zhivotnyh i cheloveka* (Coronaviruses in animals and humans). BIO.5 (236). 16. (in Russian).

Ferasin, L., Fritz, M., Ferasin, H., Becquart, P., Corbet, S., Gouilh, M. A., Legros, V., & Leroy, E. M. (2021). Infection with SARS-CoV-2 variant B.1.1.7 detected in a group of dogs and cats with suspected myocarditis. *Vet Rec*. 189(9):e944. DOI: 10.1002/vetr.944.

Gautam, A., Kaphle, K., Shrestha, B., & Phuyal, S. (2020). Susceptibility to SARS, MERS, and COVID-19 from animal health perspective. *Open Veterinary Journal*. 10 (2), 164. DOI: 10.4314/ovj.v10i2.6.

Goumenou, M., Spandidos, D. A., & Tsatsakis, A. (2022) Possibility of transmission through dogs being a contributing factor to the extreme Covid-19 outbreak in North Italy. *Molecular Medicine Reports*. 21(6). 2293-95. DOI: 10.3892/mmr.2020.11037.

Korochkin, R. B., Verbickij, A. A., Aleshkevich, V. N., & Sandul, A. V. (2010). *Kultivirovanie virusov v kulturah kletok* (Cultivation of viruses in cell cultures). UMKD. Vitebsk: VGAVM. (in Russian).

Leroy, E. M., Ar Gouilh, M., & Brugere-Picoux, J. (2020). The risk of SARS-CoV-2 transmission to pets and other wild and domestic animals strongly mandates a one-health strategy to control the COVID-19 pandemic. *One Health*. 10.100133. DOI: 10.1016/j.onehlt.2020.100133.

Loeb, J. (2020). Pet dog confirmed to have coronavirus. *The Veterinary Record*. 186, 265. DOI: 10.1136/vr.m892.

Nagornyh, A. M., Tyumencev, A. I., & Akimkin, V. G. (2020) SARS, snova SARS i MERS. Obzor zhivotnyh modelej respiratornyh sindromov cheloveka, vyzyvaemyh koronavirusnymi infekciyami (Review of animal models of human respiratory syndromes caused by coronavirus infections). *Zhurnal mikrobiologii, epidemiologii i immunobiologii*. 97 (5), 431. (in Russian).

Ng, L. F. P. & Hiscox, J. A. (2020). Coronaviruses in animals and humans. *BMJ*, 368, DOI: 10.1136/bmj.m634.

Perera, R. A., Ko, R., Tsang, O. Y., Hui, D. C., Kwan, M. M., Brackman, C .J., To, M. W., Yen, H. L., Leung, K., Cheng, S. M., Chan, K. H., Chan, K. C., Li, K. C., Saif, L., Barrs, V. R., Wu, J. T., Sit, T. C., Poon, L. M., & Peiris, M. (2021). Evaluation of a SARS-CoV-2 Surrogate Virus Neutralization Test for Detection of Antibody in Human, Canine, Cat, and Hamster Sera. *J Clin Microbiol*. 59(2), e02504-20. DOI: 10.1128/JCM.02504-20.

Prikaz Ministra selskogo hozyajstva Respubliki Kazahstan № 7-1/393. '*Ob utverzhdenii Pravil otbora prob peremeshchaemyh (perevozimyh) objektov i biologicheskogo materiala*' (Order of the Minister of Agriculture of the Republic of Kazakhstan No. 7-1/393 'On approval of the Rules for sampling moved (transported) objects and biological material'). April 30, 2015. (in Russian).

Prince, T., Smith, S. L., Radford, A. D., & Tom, S. (2021). SARS-CoV-2 Infections in Animals: Reservoirs for Reverse Zoonosis and Models for Study. *Viruses*. 13 (3), 494. DOI: 10.3390/v13030494.

Reed, L. J. & Muench, H. A. (1938). Simple Method of Estimating Fifty Per Cent Endpoints. *The American Journal of Hygiene*. 27(3), 493-497. DOI: 10.1093/oxfordjournals.aje.a118408.

Shi, J., Wen, Z., Zhong, G., Yang, H., & Wang, C. (2020). Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science*. 368, 1016-1020. DOI: 10.1126/science.abb7015.

Wu, Y., Xu, W., Zhu, Z., & Xia, X. (2020). Laboratory verification of an RT-PCR assay for SARS-CoV-2. *Journal of Clinical Laboratory Analysis*. 34(10), e23507. DOI: 10.1002/jcla.23507.

Wurtz, N., Penant, G., Jardot, P., Duclos, N., & Scola, B. L. (2021). Culture of SARS-CoV-2 in a panel of laboratory cell lines, permissivity, and differences in growth profile. *European Journal of Clinical Microbiology & Infectious Diseases*. 40, 477-484. DOI: 10.1007/s10096-020-04106-0.

Zhugunissov, K., Kerimbayev, A., Kopeev, S., Myrzakhmetova, B., Tuyskanova, M., Nakhanov, A., Khairullin, B., Orynbayev, M., Abduraimov, Ye., Kassenov, M., Zakarya, K., & Kutumbetov, L. (2022). SARS-CoV-2 virus: isolation, growth, thermostability, inactivation and passages. *Experimental Biology*. 1(90), 73-89. DOI: 10.26577/eb.2022.v90.i1.07.