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Lack of detection of SARS-CoV-2 in wildlife from Kerala, India in 2020–21

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Abstract

Spillover of SARS-CoV-2 into a variety of wild and domestic animals has been an ongoing feature of the human pandemic. The establishment of a new reservoir in white-tailed deer in North America and increasing divergence of the viruses circulating in them from those circulating in the human population has highlighted the ongoing risk this poses for global health. Some parts of the world have seen more intensive monitoring of wildlife species for SARS-CoV-2 and related coronaviruses but there are still very large gaps in geographical and species-specific information. This paper reports negative results for SARS-CoV-2 PCR based testing using a pan coronavirus end point RDRP PCR and a Sarbecovirus specific E gene qPCR on lung and or gut tissue from wildlife from the Indian State of Kerala. These animals included: 121 *Rhinolophus rouxii* (Rufous Horsehoe Bat), six *Rhinolophus bedommei* (Lesser Woolly Horseshoe Bat), 15 *Rossettus leschenaultii* (Fulvous Fruit Bat), 47 *Macaca radiata* (Bonnet macaques), 35 *Paradoxurus hermaphroditus* (Common Palm Civet), five *Viverricula indica* (Small Indian Civet), four *Herpestes edwardsii* (Common Mongoose), ten *Panthera tigris* (Bengal Tiger), eight *Panthera pardus fusca* (Indian Leopard), four *Prionailurus bengalensis* (Leopard cats), two *Felis chaus* (Jungle cats), two *Cuon alpinus* (Wild dogs) and one *Melursus ursinus* (sloth bear).

DATA SUMMARY

Demographic information for the samples described is presented in full in the supplementary information, available in the online version of this article. No other new data or code was generated as part of this project.

INTRODUCTION

There have been numerous reports of SARS-CoV-2 spill over from the human pandemic into multiple species. Prominent events with large numbers of animals in multiple sites and spill over back into the human population include domestic cats (*Felis Cattus*) [1, 2], farmed American mink (*Neogale vison*) [3, 4] and Syrian hamsters (*Mesocricerus auratus*) [5, 6]. SARS-CoV-2 has also established ongoing transmission in wild white-tailed deer (*Odocoileus virginianus*) in the USA, with infection back into the human population confirmed. Worryingly the variants found in the deer population have begun to significantly diverge from those in the human population creating an unpredictable reservoir of novel variants [7–9]. It would also appear from laboratory studies that the range of species able to be infected by SARS-CoV-2 is very dependent on the strain of virus and it is likely that as it continues to evolve in people that the species range of susceptibility will not be stable [10, 11].

There have been a very large number of reports of other species either able to be infected experimentally or with infection detected in sporadic case reports. These are reviewed in [12] but include a large number of cricetid rodents, felids, mustelids, other small carnivores and primates. Many of these reports have been from animals held in zoological collections where they are in close contact with humans, and it is not clear whether these species in their natural environment are at risk or not. Indeed there is a marked contrast in disease transmission between farmed mink at high population density, with almost 100% of animals infected in a very short period of time in some outbreaks [13] and the sporadic reports, despite intense monitoring, in wild animals,

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One supplementary material is available with the online version of this article.

which are largely solitary [14–17]. These behavioural considerations may be as important as biological barriers to which species the virus establishes in.

In addition, we also still have very large gaps in knowledge of the distribution of sarbecoviruses in bats from the Rhinolophoidea; horseshoe bats and roundleaf bats, their natural hosts. There has been intensive sampling of bats in SE Asia, driven by the original SARS-CoV outbreak in 2006 [18]. This effort has established that Sarbecoviruses (SARS like betacoronaviruses) are largely only found in Rhinolophoidid bats. There are however about 180 species of these bats spread across Eurasia and Africa with coronaviruses detected in about 30 of them [19]. Central and South Asia alongside Sub-Saharan Africa are notable absences in *Sarbecovirus* detection studies [20] with that gap only just beginning to be filled [21–25].

There has been remarkably little study of SARS-CoV-2 in animals in India despite the countries devastating human pandemic [26]. One study in Gujarat (a north western state) of 413 domestic animals of a variety of species reported 23.79% of animals qPCR positive on nasal or rectal swabs, the positive animals being dogs, cattle and buffalo with sequence confirmation of one canine isolate [27]. Sequencing effort was targeted in areas with a large number of human cases potentially explaining the very high qPCR positivity in this study. A serological study of 320 captive Bengal tigers, Asiatic lions and leopards from eight Indian states demonstrated that 48 (15%) of these animals had seroconverted to SARS-CoV-2 by October 2021. A small number of Indian elephants (24) and 40 spotted and swamp deer were all seronegative [28]. There have also been reports of PCR positive Asiatic lions in zoos [29] with 2/18 animals in Uttar Pradesh (northern India) and 1/20 in Rajasthan (north west India) qPCR positive on nasal or rectal swabs, with sequence confirmation of the isolates, other felids housed at these institutions did not test positive. Four out of 24 Asiatic lions in Chennai (Tamil Nadu state, south east India) were also found to be qPCR positive and sequence confirmed in a zoo [30], two of these animals died. The only report in a wild animal in India is a solitary juvenile Asiatic leopard found dead in Uttar Pradesh with qPCR positivity and sequence confirmation in [31], this was the only animal out of more than 500 qPCR screened samples positive. In all these cases the felid infections were consistent with the circulating human variants at the time.

India's size and number of climate zones mean that biodiversity is very high with pressures from the world's largest human population and known problems with illegal wildlife trade and human/wildlife conflict contributing to multiple zoonotic disease outbreaks [32–34]. The western ghats rainforest along the west coast of India is a biodiversity hotspot with 133 mammal species recorded. It is also an area of intense human wildlife interaction and conflict, with large species such as tigers and elephants causing considerable destruction in human settlements. Consequent to this, zoonotic disease outbreaks are frequent, with the Kyasanur forest and its eponymous virus part of this ecosystem. Surveillance systems and monitoring in this region are however seriously under-resourced with little systematic surveillance of either animals or their viruses [34].

This study sought to partially bridge these gaps with targeted trapping and testing of Rhinolophus bats and opportunistic testing of carnivore and primate species either found dead (roadkill) or culled as part of nuisance animal control activities in the state of Kerala in south west India.

METHODS

Sample collection

A total of 260 animals from 13 species (Table 1) were targeted for coronavirus monitoring. For the two horseshoe bat species, palm civets and common mongoose, the targeted numbers were calculated in Epitools [35], two stage sampling for demonstration of disease freedom (cluster size unknown) based on assumption of 5% prevalence of Coronavirus and 50% of populations affected. Prior assumptions were based on previous studies of rodent coronaviruses in wild populations [36]. This gave an estimate of seven clusters with 17 individuals in each cluster to be samples (119 animals per species). The species targeted were the two most common horseshoe bats in this environment (others are rare) and the most common small carnivore predators of bats in these sites.

Bats were trapped using mist or harp nets, Subject to inhalational anaesthesia with isoflurane with throat and cloacal swabs collected. Later bats were euthanized by extending the anaesthesia and tissue samples were collected by necropsy. Samples were stored in RNAlater for nucleic acid extraction. Small carnivores (common palm civet, small Indian civet, common mongoose, leopard cat, jungle cat), bonnet macaques and larger carnivores (Bengal tiger, leopard, sloth bear and wild dog), samples were collected as part of routine necropsy procedures from dead animals in the study area. All the carcasses were fresh (within 12 h of death) and samples were preserved in RNA later and stored at −80 degrees Celsius. All procedures were conducted under the supervision of an experienced wildlife veterinarian.

Ethical approval was granted by the University of Nottingham School of Veterinary Medicine and Science Committee for Animal Research and Ethics (CARE). Permission for field work in forest areas for scientific research and sample collection was as per the permit number KFDHQ- 1979/ 2021-CWW / WL 10 issued by the Chief Wildlife Warden, Kerala state, India.

Table 1. Species and sample type screened for SARS-CoV-2

Species	Lung sample	Gut sample	Total no. of animals	No. of sites	Sample collection dates
Bats					
Rufous horseshoe bat	121	98	121	13	May 2021–June 2022
Lesser woolly horseshoe bat	6	–	6	4	May 2021–June 2022
Fulvous fruit bat	15	–	15	3	May 2021–June 2022
Primates					
Bonnet macaque	47	–	47	22	Feb 2019–Feb 2022
Carnivores					
Common palm civet	35	–	35	19	Feb 2018–May 2021
Small Indian civet	5	–	5	4	March 2018–March 2020
Common mongoose	5	–	4	4	Sep 2021–March 2022
Tiger	10	–	10	5	April 2020–April 2022
Leopard	8	–	8	6	January 2019–March 2022
Leopard cat	4	–	4	4	January 2021–January 2022
Jungle cat	2	–	2	2	September–May 2021
Wild dog	2	–	2	2	May 2019–Nov 2021
Sloth	1	–	1	1	Archive sample
Total			402		

RNA extraction, reverse transcriptase (RT) and RNA-dependent RNA polymerase (RDRP) gene coronaviruses generic conventional PCR

All sample processing and PCR was performed in India at the Kerala state forest department and SciGenom labs, Kerala.

RNA extraction from lung tissue, faecal samples, rectal and oronasal swabs, and cell culture supernatant as positive control, was carried out using the Invitrogen Viral RNA extraction kit as per manufacturer's instructions. The positive control sample used throughout this study was cDNA from the OC43 Coronavirus ATCC strain VR1558. RT was performed with the Applied Biosystems cDNA reverse transcription kit as per manufacturer's instructions. All cDNA products were stored at −20°C for conventional PCR. An endpoint SARS-CoV-2 specific PCR assay [36, 37] was used to amplify the RDRP gene with the Takara R050 A PrimeSTAR GXL taq according to manufacturer's instructions.

RNA and cDNA quality control was assessed via partial amplification of 108 bp of the beta actin gene using a published conventional PCR protocol [38]. Primers were F: CAGCACAATGAAGATCAAGATCATC and R: CGGACTCATCGTACTCCTGCTT.

RESULTS

No animal sample tested positive for SARS-CoV-2. Locations of samples are shown in Fig. 1.

DISCUSSION

This study found no evidence of widespread circulation of SARS-CoV-2 or related coronaviruses in Indian wildlife. Some of the species tested here, such as bonnet macaques, palm civets and mongoose are very commonly found in and around human habitation and represent significant pest or nuisance species in terms of aggressive interactions with humans and potential zoonoses or cross species transmission to and from domestic animals [39–41]. These species are high risk for SARS-CoV-2 spill over and it is at least reassuring that these animals tested negative. Though with the large caveats that sampling was PCR based, a small number of animals and could easily have missed infections. Follow up work with serological testing for SARS-CoV-2 antibody (indicating previous infection) would be an extremely useful follow up to this project, with of course the caveat that widely available serological assays have not been validated for these species, making results difficult to interpret [28].

Studies of felids in zoo (captive) populations in India have demonstrated a high rate of seroconversion [28] and PCR positive animals have been detected in zoos [29, 30] and in one wild leopard [31]. Our results here, while a small number of opportunistic samples, add to evidence that SARS-CoV-2 is not a widespread issue in wild Indian felids [31].

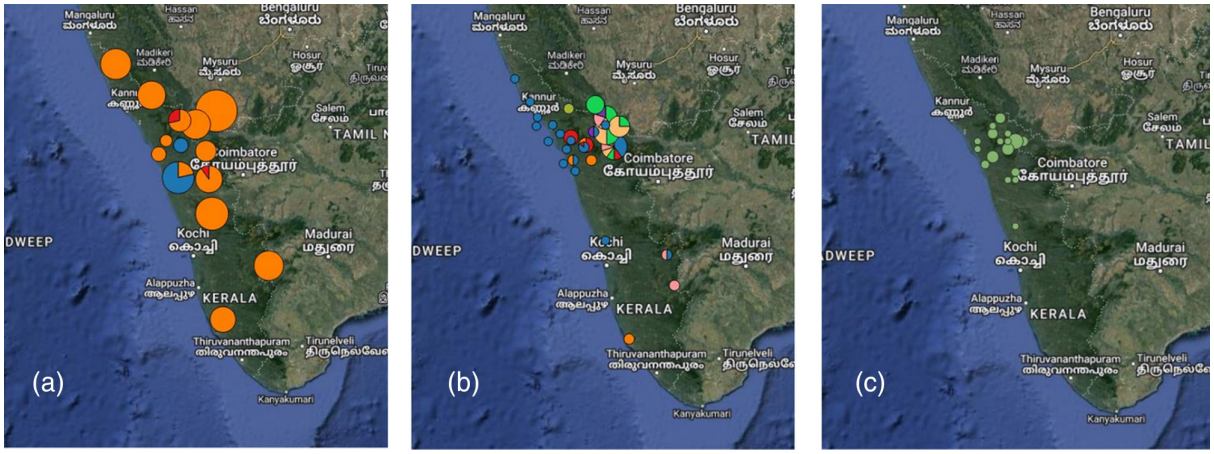

Fig. 1. (a) Locations of bat samples, orange = rufous horseshoe bat, red = lesser woolly horseshoe bat, blue = fulvous fruit bat, pie chart sizes are proportional to the number of animals at each location. (b) Locations of carnivore samples, blue = common palm civet, red = small Indian civet, orange = common mongoose, bright green = tiger, yellow = leopard, pink = leopard cat, purple = jungle cat, light green = wild dog, grey = sloth bear, pie chart sizes are proportional to the number of animals at each location. (c) Locations of bonnet macaque samples, circles are proportional to the number of animals at each location (maps drawn in QGIS v 3.3.1).

A completely negative finding in the two horseshoe bat species was unexpected, particularly as these species are the natural hosts of SARS-like viruses and the PCR assays used in this study should have detected known horseshoe bat sarbecoviruses. Our similar study of UK horseshoe bats did however demonstrate that presence or absence of sarbecoviruses can be very species specific with lesser horseshoe bats having a 44% positivity rate on faecal or rectal swab samples but no detection at all in greater horseshoe bats [42]. Studies in SE Asia present with very different results with high positivity rates and sarbecoviruses detected in multiple species [43]. Of note the species in which SARS-CoV-2 like sarbecoviruses and recombinant viruses are commonly found, *R. sinicus, R. ferrumequinum, R. pusillus, and R. affinis* are either rare (*R. pusillus*) or not found in Kerala. These species are all cave roosting bats that form large colonies which may be a key factor in facilitating sarbecovirus diversity and cross species transmission.

Our sampling numbers and targets should have been able to detect sarbecoviruses in rufous horseshoe bats where target numbers were achieved. Target numbers were not achieved in other species, primarily due to extreme adverse weather conditions (flooding) in Kerala during the sampling period. Most known roost sites for the lesser woolly horseshoe bat (which frequently roosts in sites such as drain coverts) were found abandoned. Trapping success rates for small carnivores were also less than optimal. Nonetheless we present our negative results in the interest of providing the only data to date on Indian horseshoe bat populations. This adds to data indicating that sarbecovirus spill-over out of the horseshoe bat population may be a distinctly regional (SE Asian) phenomena [19, 43].

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Author contributions
A.Z., J.M. and V.O. performed field work and PCR testing. S.K. and S.S. performed data analysis and curation. A.B. and R.T. conceived the study, held the funding, managed the project and performed data analysis. R.T. wrote the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval was granted by the University of Nottingham School of Veterinary Medicine and Science Committee for Animal Research and Ethics (CARE), Permission for field work in forest areas for scientific research and sample collection was as per the permit number KFDHQ- 1979/ 2021-CWW / WL 10 issued by the Chief Wildlife Warden, Kerala state, India.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000686.v2.1

Craig David Smith; University of Dundee School of Life Sciences, 17 Gullane Road, UNITED KINGDOM, Dundee

Date report received: 17 January 2024
Recommendation: Accept

Comments: This is a study that would be of interest to the field and community.

Author response to reviewers to Version 1

Dear Dr Smith,

Thankyou for the constructive reviewers comments, we have revised or replied as outlined below and hope that the revised manuscript finds favour for formal publication this round,

Yours Sincerely,

Reviewer 1 Comments to Author:

This study aimed to determine the potential spread of SARS-CoV-2 to wildlife in India. the authors pursued this line of investigation based on prior reports in the literature of either virus or antibodies to SARS CoV2 found in other wildlife species, especially deer and mink. The importance of such a study would provide additional data on potential wildlife species that could serve as reservoirs of this virus and pose potential threats to human populations.

The authors employed trapping of Rhinolophus bats and opportunistic testing of carnivore and primate species either found dead or culled as part of nuisance animal control activities. Unfortunately, or fortunately the results were negative for virus by PCR. the authors did not look for antibodies, data that would have strengthen the paper.

Overall, methodology is appropriate and while this paper presents negative data, this data does add information about wildlife species infected with the SARS CoV2 virus.

We would agree with the reviewer's assessment that while the data are negative it is still important to know which species are not affected by the virus as well as those that are.

Serology would have been a helpful addition to understanding the role of this coronavirus in wildlife.

We debated the inclusion of serology in the original study but there are a number of caveats to its use in wildlife. While there are sandwich ELISA kits for SARS-CoV-2 serology now available which are species agnostic and suitable for field screening or routine diagnostic use, as was the situation in this study, the underlying cross reactivity, sensitivity and therefore false positive and negative rates are unknown for most species. Particularly for poorly characterised species such as many in this study we have no idea what existing coronaviruses they may carry or how these may cross react with SARS-CoV-2 antibodies. Use of these assays in situations of high prevalence such as that in the white-tailed deer in North America is useful for estimating past exposure. In situations of low prevalence (such as this study) the false positivity rate of these assays is problematic, usually these studies require confirmation of seropositivity with live virus or pseudotype assays, neither of which are readily accessible in Kerala.

While we can run such assays in Nottingham shipment of animal samples from India to the UK (or even interstate in India) is also problematic due to the variety of legal permits (CITES, import and export phytosanitary permits, Nagoya protocol, biohazard classification) required.

The issue of how to interpret low level cross reactivity in ELISAs, which is usually demonstrated to be false positivity in comparison with gold standard virus neutralisation assays has been an ongoing issue in Wildlife studies of SARs-CoV-2 exposure for example:https://www.tandfonline.com/doi/full/10.1080/22221751.2023.2217940

https://onlinelibrary.wiley.com/doi/10.1111/tbed.14534

https://pubmed.ncbi.nlm.nih.gov/35841263/

There are also practical issues with serology in the animals and samples available as horseshoe bats are very small (with limited sera able to be collected) and opportunistic sampling of other species meaning suitable sera samples were not always available.

Ultimately due to the uncertain value of serology testing we felt the PCR (and sequencing were there any positives) was of more value in this situation.

Reviewer 4 Comments to Author: The manuscript by Zachariah et al screened a variety of wildlife species from Kerala, India for SARS-CoV-2 infection by pan-coronavirus PCR and SARS-CoV-2 envelope gene qPCR. No animals were PCR positive for coronaviruses during the sampling period from 2020-2021. The authors collected lung tissue, faeces and rectal and oronasal swabs from animals as part of the necropsy procedure on these species.

The major limitation of this work is the lack of serology for SARS-CoV-2. It could be expected that animals would remain PCR positive for SARS-CoV-2 for much shorter periods of time than antibodies would be detectable? Why did the authors not perform ELISAs for SARS-CoV-2 antibody in addition to PCR?

We have outlined the caveats to serology testing in wildlife in low prevalence situations and why this can be very difficult to interpret in responses to reviewer one.

As the animals were deceased when samples were taken, the authors could have screened tissues that have been shown to have the highest levels of SARS-CoV-2 in other wildlife species including white tailed deer (such as medial retropharyngeal lymph nodes and palatine tonsil). Could the authors justify their sampling strategy?

We would agree after discussion with researchers performing the deer studies that retropharyngeal lymph node would be a good tissue to target in future. It seems self-explanatory in retrospect that nucleic acid remnants would linger for an extended period of time in lymph nodes (the reason why such a high detection rate was found in white tailed deer in that tissue) but at the time this study was commissioned that was not yet reported. The only reason that tissue was targeted in the USA studies was that these samples were already being collected and stored for chronic wasting disease of deer monitoring. It was not at all clear when this was performed that RNA would be detectable for such an extended period of time after infectious virus was cleared. It was more logical to collected samples from known sites of virus replication or excretion and indeed in a companion study to this one in the UK we did detect coronaviruses in similar species from similar samples (indicating that these methods were indeed adequate to pick this up)https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.001917#tab2

This also gives useful information on probable routes of transmission, respiratory vs faecal/oral as veterinary coronaviruses vary in whether they are primarily gastrointestinal or respiratory viruses. Indeed sarbecoviruses in bats appear to be primarily gastrointestinal infections – there is no guarantee that upper respiratory tract lymph nodes would be enriched for coronavirus detection in the animals in this study. In addition, in very small animals like horseshoe bats it can be very difficult to identify lymph nodes accurately, white tailed deer are in the order of 50kg, horseshoe bats in the order of 20g weight. For the sake of consistency and practicality for the field teams we opted for samples that we could collect reliably in all species and interpret results with some certainty.

The manuscript would benefit from proofreading prior to acceptance as there are a number of typographical errors in the current version.

We have proofread the manuscript again and hopefully found these now.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000686.v1.5

Craig David Smith; University of Dundee School of Life Sciences, 17 Gullane Road, UNITED KINGDOM, Dundee

Date report received: 18 December 2023
Recommendation: Minor Amendment

Comments: The work presented is clear and the arguments well formed.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000686.v1.3

Anonymous.

Date report received: 08 December 2023
Recommendation: Minor Amendment

Comments: The manuscript by Zachariah et al screened a variety of wildlife species from Kerala, India for SARS-CoV-2 infection by pan-coronavirus PCR and SARS-CoV-2 envelope gene qPCR. No animals were PCR positive for coronaviruses during the sampling period from 2020-2021. The authors collected lung tissue, faeces and rectal and oronasal swabs from animals as part of the necropsy procedure on these species. The major limitation of this work is the lack of serology for SARS-CoV-2. It could be expected that animals would remain PCR positive for SARS-CoV-2 for much shorter periods of time than antibodies would be detectable? Why did the authors not perform ELISAs for SARS-CoV-2 antibody in addition to PCR? As the animals were deceased when samples were taken, the authors could have screened tissues that have been shown to have the highest levels of SARS-CoV-2 in other wildlife species including white tailed deer (such as medial retropharyngeal lymph nodes and palatine tonsil). Could the authors justify their sampling strategy? The manuscript would benefit from proofreading prior to acceptance as there are a number of typographical errors in the current version.

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000686.v1.4

David Benfield; The Ohio State University OARDC: The Ohio State University Ohio Agricultural Research and Development Center, Animal Sciences - Food Animal Health Program, 1680 Madison Avenue, Wooster, UNITED STATES
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Date report received: 31 August 2023
Recommendation: Minor Amendment

Comments: This study aimed to determine the potential spread of SARS-CoV-2 to wildlife in India. the authors pursued this line of investigation based on prior reports in the literature of either virus or antibodies to SARS CoV2 found in other wildlife species, especially deer and mink. The importance of such a study would provide additional data on potential wildlife species that could serve as reservoirs of this virus and pose potential threats to human populations. The authors employed trapping of Rhinolophus bats and opportunistic testing of carnivore and primate species either found dead or culled as part of nuisance animal control activities. Unfortunately, or fortunately the results were negative for virus by PCR. the authors did not look for antibodies, data that would have strengthen the paper. Overall, methodology is appropriate and while this paper presents negative data, this data does add information about wildlife species infected with the SARS CoV2 virus. Serology would have been a helpful addition to understanding the role of this coronavirus in wildlife.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000686.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000686.v1.2