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LETTER

Are pangolins scapegoats of the COVID-19 outbreak-CoV transmission and pathology evidence?

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Abstract

The COVID-19 outbreak has infected over 6 million people across the world. The origin of COVID-19 coronavirus (CoV) remains unknown, although pangolins have been suggested as potential hosts. We investigated two pangolins seized in Guangdong Province, China. Molecular screening revealed CoV in one pangolin (“Dahu”), while another (“Meidong”) was infected by *Ehrlichia ruminantium*. Dahu exhibited difficulty breathing, infections of lung, intestines, and nostrils, as revealed by computed tomography imaging and necropsy. Previous phylogenetic analyses showed bat coronavirus RaTG13 is closer to COVID-19 CoV compared to pangolin coronavirus. Over 20 caregivers have had close physical contact with CoV-positive Dahu, but none became infected with CoV. Our data suggest that pangolins are unlikely the natural reservoir or secondary hosts of COVID-19 CoV. Pangolins seems to be victims infected by CoV carried by a not yet unidentified natural reservoir host species, perhaps due to their weakened immune system.

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KEYWORDS

animal conservation, coronavirus, COVID-19, CT imaging, pangolins, pangolin pathology

1 | INTRODUCTION

The COVID-19 outbreak has become a pandemic and global public health issue. Infected people may stay asymptomatic for up to 24 days, but are able to infect other people in the meantime, which increases the likelihood of transmission (Guan et al., 2020). As of June 1, 2020, the deadly COVID-19 coronavirus (SARS-CoV-2) has infected more than 6,246,042 people and killed more than 374,452 people from around the world (Dong, Du, & Gardner, 2020). COVID-19 patients usually show common clinical symptoms of fever, dyspnea, and pneumonia (Chen et al., 2020; Huang et al., 2020; D. Wang et al., 2020).

Several reports have attempted to identify the source or intermediate hosts of the COVID-19 CoV (Lam et al., 2020b; Wahba et al., 2020; Xiao et al., 2020). A recent research group from the South China Agriculture University announced that the CoV found in Malayan pangolins (*Manis javanica*) has relatively high similarity with the COVID-19 CoV Wuhan-Hu-1 sequence (Xiao et al., 2020).

Pangolins are not only endangered, they are one of the most trafficked mammals on Earth (Choo et al., 2016; Gaubert et al., 2018; Heinrich et al., 2016). They are well known for their weak immune system based on observations in captive pangolins as they easily succumb to disease such as pneumonia, gastrointestinal disease, and parasitic infections (Clark, Thai, & Phuong, 2008; Hua et al., 2015; Lihua et al., 2010). Prior to the COVID-19 outbreak, Liu and colleagues detected CoVs in two pangolins based on metagenomic data, proposing CoV infection in pangolins (Liu, Chen, & Chen, 2019). Based on the genetic similarity of the recent isolates, researchers have suggested that pangolins could be potential reservoir or intermediate hosts in the emergence of COVID-19 CoV (Lam et al., 2020b; Liu et al., 2020; Xiao et al., 2020; Zhang, Wu, & Zhang, 2020). Xiao et al (2020) found regions of pangolin CoV with high amino acid similarity to COVID-19 CoV based on phylogenetic and recombination analyses, but they concluded that COVID-19 CoV was more likely to be the result of a recombination event between a pangolin CoV and a bat CoV (Xiao et al., 2020).

Although finding the source or the intermediate host is very important to understand the outbreak and potentially prevent such events in the future, the recent reports are preliminary at best and have raised widespread exaggerated concerns in international media. It is important to recall that the early erroneous implication of palm

civets (*Paradoxurus hermaphroditus*) as primary reservoir for the SARS CoV in 2002, leading to the culling of some 10,000 captive palm civets (Watts, 2004). In this report, we explore pangolin CoV sequence, pathology, and transmission observations to analyze whether pangolins are the natural intermediate hosts of the COVID-19 CoV in order to provide better insight into this critical question.

2 | METHODS

2.1 | Overview

We present an overview of the methods, with further methodological details provided in the Supporting Information. Briefly, all organ tissues of Dahu and Meidong were harvested by veterinary officers at Guangzhou Leader Animal Hospital. Blood samples for the screening of *Burkholderia fungorum* were provided by DWNP, Malaysia. The MinFound ScintCare CT 16 device (MinFound, China) was used to perform computed tomography (CT) scans of the two pangolins. For the diagnostic testing for COVID-19 CoV, specimens were sent to the Suzhou Center for Disease Prevention and Control, China for screening of COVID-19 CoV. To examine the presence of bacteria in pangolins, we used universal bacterial 16S gene primers.

Phylogenetic trees were constructed using MEGA-X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). Sequences were initially aligned using MUSCLE (Edgar, 2004). The alignments were manually curated to ensure accuracy. Maximum-likelihood trees were inferred using the Tamura-Nei DNA substitution model and nodal support was estimated using 1,000 replicates.

2.2 | The rescue of the large number of pangolins in Guangdong

At the end of March 2019, Guangdong and Guangxi Anti-Smuggling Bureaus successfully seized 155 pangolins (103 alive and 52 dead) smuggled into China through Vietnam and arrested 34 suspects (Yan, 2019). The operation involved 310 police forces that successfully destroyed an illegal trade network that smuggled endangered species from overseas to China, and then transported them to elsewhere in China (LegalDaily, 2019). The pangolins were seized together with snakes, eagles, tortoise, geckos, and

other endangered species, wrapped in bags or kept in small cages under poor conditions (Figure S1). Of these 103 living pangolins, 21 of them were transferred to the Guangdong wildlife rescue center, where they were kept in two small rooms and shared food (Figure S1j–l). Sixteen of the 21 pangolins died quickly, leaving five pangolins alive by mid-April, 2019. After comprehensive analysis of the carcasses, most of the dead pangolins showed congested lung, pulmonary fibrosis, splenomegaly, and hepatomegaly, which could be associated with infectious disease (Liu et al., 2019).

On April 17, a team formed by experts from China Biodiversity Conservation and Green Development flew to Guangzhou to rescue the remaining living pangolins. We analyzed two pangolins named “Dahu” and “Meidong” using detailed veterinary and clinical examination.

3 | RESULTS

3.1 | Analysis of Dahu

During the rescue operations, Dahu was found with dyspnea and rhonchi respiratory sounds and subsequently died on April 18, 2019 (Table S1). CT imaging revealed the presence of exudative lesions in the lungs, suggesting inflammation (Figure 1a–c). A soft tissue density formation located in the nostrils and middle ear suggested the presence of rhinitis and otitis media (Figure 1d and e). In addition, CT scan showed the thickening of the colon wall that might indicate the presence of inflammation (Figure 1f and g). CT imaging also showed the presence of ascites in the abdomen. Necropsy revealed the presence of focal hemorrhagic gastroenteritis and lung hyperemia (Figure 2a and b). No gross anatomic alterations were found in the liver, heart, brain, or kidneys (Figure 2c–f).

Ten tissue specimens (liver, blood, heart, spleen, lung, skin, cerebellum, stomach, cerebrum, and intestine) were taken from Dahu on April 20, 2019 and used for further bacterial and viral assays. All samples tested negative for bacteria (Table 1a). In light of the current published papers that claimed pangolins to be the potential intermediate hosts of the deadly COVID-19 CoV (Lam et al., 2020b; Xiao et al., 2020), the specimen collected from Dahu were further examined for the presence of CoV using commercial detection fluorescent oligo probes targeting two viral genes (*N* and *ORF1ab*) that were specifically designed to detect COVID-19 CoV. Almost all tissue specimens from Dahu (except liver) tested positive for the *N* gene, whereas the *ORF1ab* gene tested negative in all tissues, confirming presence of a related CoV with a similar *N* gene but not identical to the human COVID-19 CoV *ORF1ab* gene.

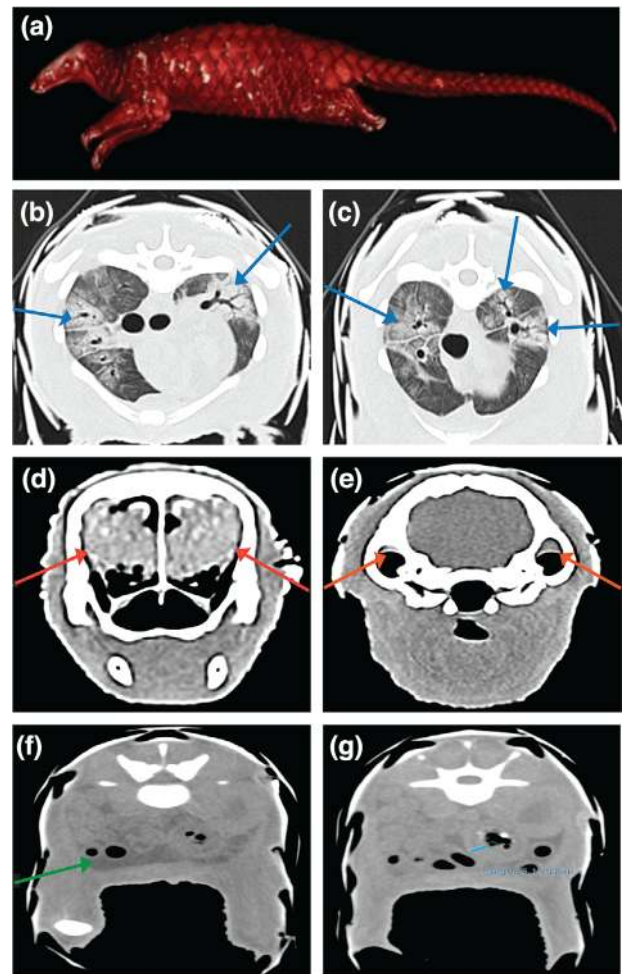


FIGURE 1 CT imaging of the Dahu pangolin. (a) Whole body specimen. (b and c) The diffuse interstitial tissue hyperplasia was found in the two lung bronchi, and the air bronchogram and thickening of interlobular septa involved both lungs (blue arrowheads). No abnormality was observed in the pleural and mediastinal lymph node. (d and e) The density of soft tissue increased in the nasal cavity and tympanum (red arrowheads). (f and g) Possible slight thickening (5.1 mm) of the colonic wall. The abdominal cavity was filled with liquid density shadow (green arrowhead), with the average CT value being about 25 HU

3.2 | Analysis of Meidong

Meidong moved infrequently and showed periodic fever, yellowish nails, loss of appetite, blue eyes with discharge, and yellowish discharge at the dorsal area of the tail (Figure 3a). Meidong died on June 1, 2019 (Table S1). CT imaging showed that the body fat of the animal was reduced likely due to poor nutrition (Figure 3b and c). Postmortem examination revealed the presence of hemorrhage in both lungs (Figure 3d). Blood test showed hypoalbuminemia, which can be a sign of pathologies such as liver failure or bacterial infection such as from *Ehrlichia ruminantium* and *Ehrlichia canis* (Figure 3e) (El-Neweshy, Al Mawly,

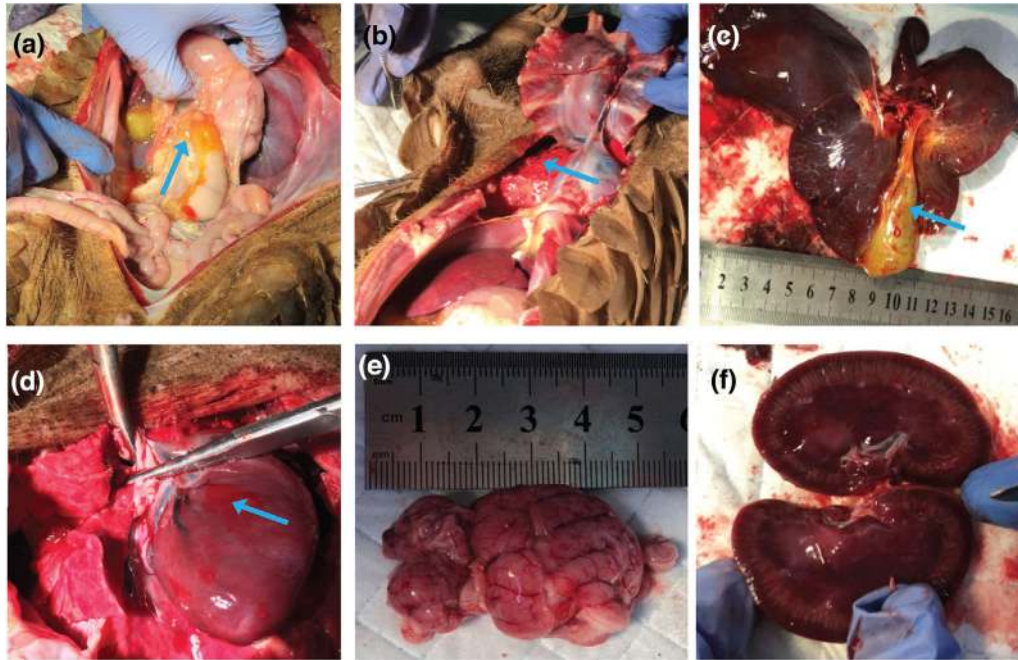


FIGURE 2 Anatomical analysis of the Dahu pangolin. (a) At the junction of cecum and colon, the posterior jejunum and the stomach pyloricus, the local intestinal wall turned yellow (arrowhead) and a few bleeding spots were seen. The mesenteric lymph nodes were slightly enlarged. (b) The abdominal organs were exposed by incision along the midline of the abdomen. We observed moderate amount of pale-yellow seroperitoneum, and a small amount of clear hydropericardium. However, no adult parasites were detected. Congestion was seen in the lung, and no abnormal density of the pulmonary nodules was found. (c) No obvious abnormality was observed in the appearance of the liver, no abnormal nodular hyperplasia was found, the gallbladder was full, and yellow green bile was found inside. (d) The heart was intact, the auricle showed dark red changes, and no pleural effusion. (e) The brain, cerebellum, and brain stem are symmetrical without hydrocephalus. (f) The shape of both kidneys was normal; no kidney stones were found

Aboollo, & El-Manakhly, 2019; Gounden and Ishawaral, 2019; Harrus, Waner, Bark, Jongejan, & Cornelissen, 1999). PCR (Polymerase Chain Reaction amplification of 16S sequences from sterile organ samples including lung, spleen, pancreas, liver, and blood indicated that Meidong likely developed septicemia (Figure 3f and Table 1). Phylogenetic analysis of these sequences revealed the presence of *Ehrlichia ruminantium*, a tick-borne rickettsial pathogen that usually infects mammals (Figure 3g; Table S2). *Ehrlichia ruminantium* infected animals can cause clinical symptoms such as anorexia, hypoalbuminemia, and transient fever in animals with depressed immune systems (Cebra & Cebra, 2012; Robinson and Robinson, 2016). No COVID-19 CoV was detected in all seven tissue specimens that we examined (blood, pancreas, lung, liver, heart, spleen, and intestine). The analyses suggest Meidong may have died from infection caused by *E. ruminantium*.

3.3 | Phylogenetic relationship of pangolins CoV and human COVID-19 CoV

It is possible that the CoV found in Dahu might be the same as the one found in the two Guangdong pangolins

(Liu et al., 2019) since they shared the same locale, clinical symptoms, and history of close confinement in early 2019. As anticipated, Sanger sequencing of the *env* genes in the lung and brain samples of Dahu showed that both sequences were 99.1% similar to the *env* gene of the Guangdong pangolin CoV, suggesting that they are indeed genetically related. Whole-genome analyses have shown that the pangolin CoV is ~90% identical to COVID-19 CoV while bat CoV RaTG13 is the closest to COVID-19 CoV (~96% identity), suggesting they have recently shared a common ancestor (Liu et al., 2020; Zhou et al., 2020). Multiple research groups have recently reported evidence of recombination signals in the genome of the Guangdong pangolin CoV through recombination analyses (Table 2) (Boni et al., 2020; Lam et al., 2020a; Wong, Cregeen, Ajami, & Petrosino, 2020; Xiao et al., 2020). For instance, Lam et al. (2020a) divided the CoV genomes into eight regions based on the predicted recombination breakpoints, and reconstructed the phylogenetic trees of each region (Lam et al., 2020a). The analysis revealed putative recombination events between the pangolin CoV, human COVID-19 CoV, and Bat CoV RaTG13. The COVID-19 CoV was more similar to the Guangdong pangolin CoV (97.4%) at the crucial receptor-binding domain protein sequence than the

TABLE 1 Results of diagnostic testing for COVID-19 CoV and bacterial screening in Malayan pangolins

(a) The Dahu pangolin											Positive control	Negative control
Organ	Liver	Blood	Heart	Spleen	Lung	Skin	Cerebellum	Stomach	Cerebrum	Intestine		
Code	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10		
<i>N</i> gene	*	+	+	+	+	+	+	+	+	+	+	-
<i>N</i> gene CT	38	31	35	35	35	31	33	32	26	22	25	-
ORF1ab	-	-	-	-	-	-	-	-	-	-	+	-
ORF1ab CT	-	-	-	-	-	-	-	-	-	-	25	-
16S gene PCR	-	-	-	-	-	-	-	-	-	-	NA	NA
(b) The Meidong pangolin											Positive control	Negative control
Organ	Blood	Pancreas	Lung	Liver	Heart	Spleen	Intestine					
Code	M01	M02	M03	M04	M05	M06	M07					
<i>N</i> gene	-	-	-	-	*	-	-	-	-	+	-	-
<i>N</i> gene CT	-	-	-	-	37	-	-	-	-	25	-	-
ORF1ab	-	-	-	-	-	-	-	-	-	+	-	-
ORF1ab CT	-	-	-	-	-	-	-	-	-	25	-	-
16S gene PCR	+	+	+	+	+	+	+	+	+	NA	NA	NA

“+” = presence; “*” = suspected; “-” = absence. Positive control was the human COVID-19 CoV; NA, not applicable. To confirm the presence of COVID-19 CoV, a specimen needs to be tested positively for both *N* and *ORF1ab* genes.

bat RaTG13 (89.2%). It was found that bat RaTG13 is the closest relative of COVID-19 CoV based on the remainder of the CoV genome, or at the whole-genome level, suggesting a potential for the COVID-19 CoV to have originated from the recombination of the pangolin CoV and bat CoV (Li, Giorgi, et al., 2020; Xiao et al., 2020). However, all studies so far that have analyzed pangolin CoVs indicate that it is unlikely that human COVID-19 CoV originated directly from pangolins.

3.4 | Pangolin-to-human transmission has not been observed

Custom officials, people at the rescue center, reporters, researchers, and our team members have been exposed or have handled the 155 pangolins seized by the custom officers in Guangdong province since March 2019. For instance, at least 20 people (including those people from the zoo, universities, animal hospital, press media, and volunteers) had close contact with the pangolins within less than 1 m of distance for more than 60 minutes without protection (e.g., masks). Moreover, our team member Ms. Siyuan Zhang looked after these pangolins for more than 70 days. However, to the best of our knowledge, none have been reported to be infected by the pangolin coronavirus since 9 months (March 2019) before the outbreak of COVID-19, suggesting that the pangolin CoV might have limited potential for transmission from pangolin to human. If pangolins were the direct principal hosts of COVID-19 CoV, we would expect many of the people

who had contact with the animals to be infected by the pangolin CoV, tracing the earliest cases of the outbreak to Guangdong, instead of Wuhan. However, we cannot rule out the possibility that the pangolin CoV might have evolved or more likely that a single recombination event, possibly among several wildlife-derived CoVs, led to a new COVID-19 CoV strain that was then capable of infecting humans in Wuhan after being maintained in pangolins for months prior to the COVID-19 outbreak.

Recent studies may shed more light on this and give even more support to our conclusion that there is no evidence of a direct transmission from pangolins to humans (Table 2). For example, Boni et al. (2020) showed that the variable loop of the COVID-19 CoV S protein was unlikely acquired from pangolin CoV as these two sequences are approximately 10% divergent throughout the entire S protein. Furthermore, RaTG13 bat CoV shares closest affinity with COVID-19 CoV based on phylogenetic analyses of two longest breakpoint-free regions. Their divergence-dating analyses also suggested pangolin CoV pre-date the RaTG13/COVID-19 CoV lineage, providing compelling support for COVID-19 CoV having likely originated directly from bat CoVs rather than from pangolin CoVs.

3.5 | Animal-to-animal transmission in pangolins

Illegal traders usually ship poached pangolins in despicable conditions. When large numbers of live pangolins

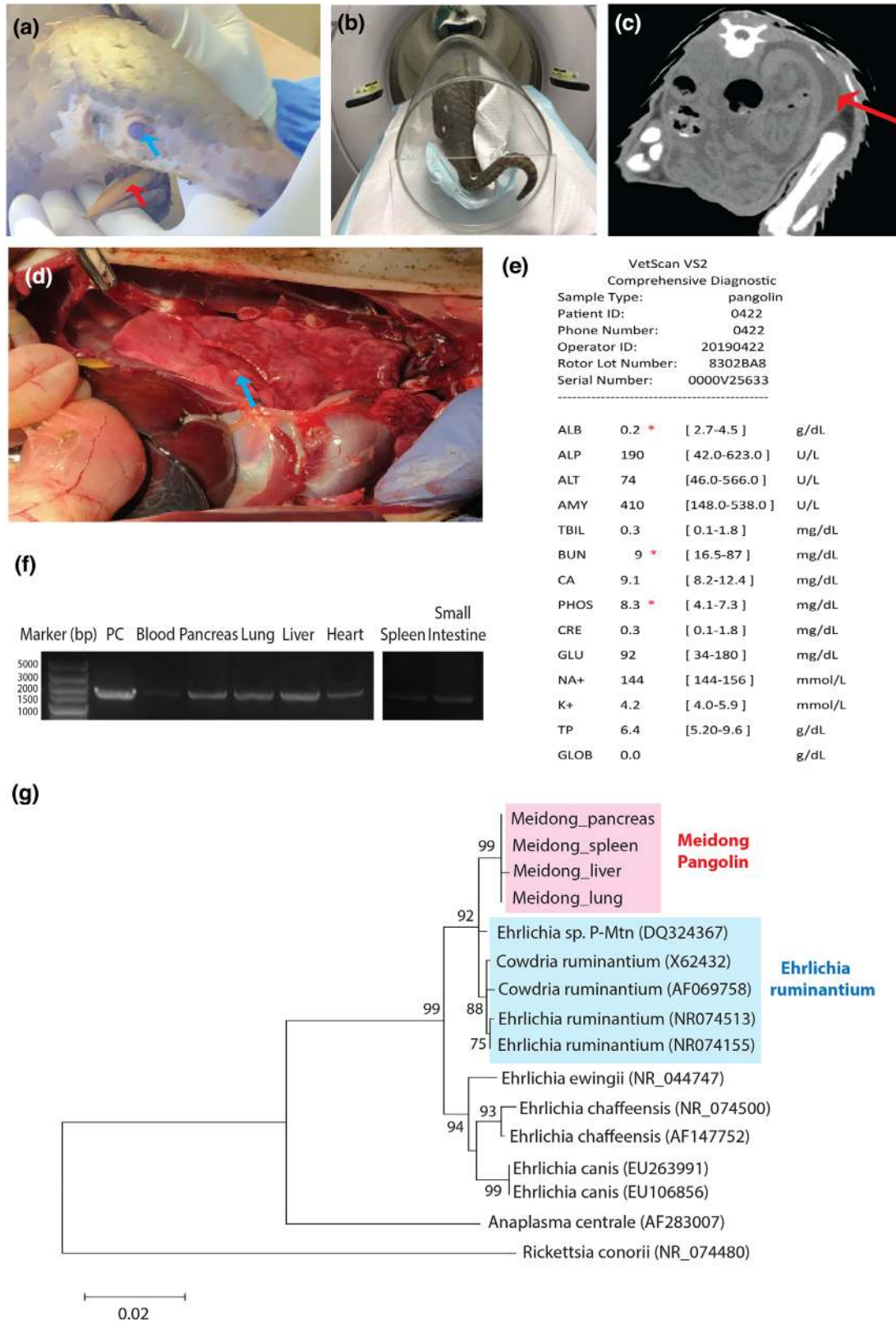


FIGURE 3 Examination of the Meidong pangolin. (a) Blue eye and eye infection were observed. (b and c) CT-scan analysis showed the fat content of the whole animal was generally less. (d) Lung bleeding was observed. (e) Blood testing showed levels of ALB (albumin), BUN (blood urea nitrogen), and PHOS (phosphate) were out of the reference range, as indicated with asterisks. (f) PCR amplification of the 16S gene showed clear bands in all sterile organs that we examined. (g) 16S-based phylogenetic tree of Meidong and related organisms. The phylogenetic tree was generated using the maximum likelihood method. Bootstrap numbers were generated in 1,000 replicates. Nodes with bootstrap values greater than 70 are indicated

TABLE 2 Summary of results from recent studies that included pangolin CoVs in their analyses, and 10 studies have demonstrated that COVID-19 CoV likely did not originate directly from pangolin CoVs

Number of pangolin CoVs analyzed	Methods of analysis	Conclusions	Reference
6 (<i>Manis javanica</i>)	Sequence similarity analyses; recombination-aware phylogenetic analysis of COVID-19 CoV S protein; breakpoint-free regions (BFRs)	<ul style="list-style-type: none"> –Variable loop of COVID-19 CoV S protein was not acquired from pangolin CoV; –10% divergence across S protein between COVID-19 CoV protein and pangolin CoV –RaTG13 bat CoV shares closest affinity with COVID-19 CoV based on phylogenetic analyses of two longest BFRs –Divergence dating analyses suggest pangolin CoV pre-date RaTG13/SARS-CoV-2 lineage 	Boni et al. (2020)
2 (<i>Manis javanica</i>)	Recombination-aware phylogenetic analysis of CoV sequences	–RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV	Cagliani et al. (2020)
8 (<i>Manis javanica</i>)	Sequence similarity analyses; whole genome and recombination-aware phylogenetic analysis of CoV sequences	<ul style="list-style-type: none"> –RaTG13 bat CoV is more closely related to COVID-19 CoV based on phylogenetic analyses of concatenated ORF1ab-S-EM-N genes of CoVs –RaTG13 bat CoV is more closely related to COVID-19 CoV based on phylogenetic analyses of eight different regions of CoV genome sequences 	Lam et al. (2020a)
2 (<i>Manis javanica</i>)	Phylogenetic analyses of CoV genomes	<ul style="list-style-type: none"> –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV –Pangolin CoVs lacked RRAR motif of COVID-19 CoV S protein –COVID-19 CoV did not evolve directly from pangolin CoVs 	Li, Zai, et al. (2020)
8 (<i>Manis javanica</i>)	Sequence similarity analyses; recombination-aware phylogenetic and selection analyses of CoV sequences	<ul style="list-style-type: none"> –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV –Some regions of SARS-CoV-2 genome are most closely related to pangolin CoVs –SARS-CoV-2 RBM sequence shows signal of cross-species recombination between the bat and the pangolin CoVs 	Li, Giorgi, et al. (2020) (re-analysis of data from Liu et al., 2019)
3 (<i>Manis javanica</i>)	Sequence similarity analyses; whole genome phylogenetic analyses of CoVs and individual phylogenetic analysis of S gene	<ul style="list-style-type: none"> –Pangolin CoV is 90.32% identical to COVID-19 CoV (whole genome) –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV (whole genome) –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV (S protein) –RBM of pangolin CoV and COVID-19 CoV 98.6% identical –COVID-19 CoV did not evolve directly from pangolin CoVs 	Liu et al. (2020)
8 (<i>Manis javanica</i>)	Sequence similarity analyses	–RaTG13 bat CoV is more similar to COVID-19 CoV than pangolin CoV	Wahba et al. (2020)(re-analysis of data from Liu et al., 2020)

(Continues)

TABLE 2 (Continued)

Number of pangolin CoVs analyzed	Methods of analysis	Conclusions	Reference
1 (<i>Manis javanica</i>)	Sequence similarity analyses; whole genome phylogenetic analyses of CoVs and individual phylogenetic analyses of the RdRp and S genes	<ul style="list-style-type: none"> –Pangolin CoV is 90.1% identical to COVID-19 CoV (whole genome) –Pangolin CoV is closely related to a cluster composed of COVID-19 CoV and RaTG13 bat CoV –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV –Pangolin CoV only differs by one amino acid from COVID-19 CoV in RBD of S protein –COVID-19 CoV may have originated from recombination of Pangolin-CoV-like virus with bat CoV RaTG13-like virus 	Xiao et al. (2020)
2 (<i>Manis javanica</i>)	Sequence similarity analyses; whole genome phylogenetic analyses of CoVs and RdRp gene	<ul style="list-style-type: none"> –Pangolin CoV is 91.02% identical to COVID-19 CoV –RaTG13 bat CoV is 96.2% identical to COVID-19 CoV –RaTG13 bat CoV is more closely related to COVID-19 CoV than pangolin CoV –Pangolin CoV and COVID-19 CoV share five identical amino acids in their receptor-binding domains that are important for interaction with human ACE2 receptor –Pangolins are reservoirs of CoVs related to COVID-19 CoV 	Zhang et al. (2020) (re-analysis of data from Liu et al., 2019)
7 (<i>Manis javanica</i>)	Sequence similarity analyses; whole genome phylogenetic analyses of CoVs and individual phylogenetic analyses of S gene, RBD, and RdRp gene	<ul style="list-style-type: none"> –RBD of pangolin CoV and COVID-19 CoV 97.4% identical –RmYN02 CoV from <i>Rhinolophus malayanus</i> is the closest relative to COVID-19 CoV based on whole genome analysis –RmYN02, RaTG13 and COVID-19 CoVs were clustered together apart from pangolin CoVs in RdRp gene trees. –Bats harbor CoVs that are most closely related to COVID-19 CoV 	Zhou et al. (2020)

are transported with other animal species, and held in captivity in overcrowded and unhygienic conditions, the likelihood for a pangolin to acquire any pathogen from other animals increases. This situation was observed in the case of pangolins seized in 2019 by the Guangdong anti-smuggling authority, where most pangolins died quickly, largely due to infections.

In 2012, when the Department of Wildlife and National Parks seized a batch of more than 40 Malayan pangolins, one of them was infected by *B. fungorum* (Choo et al., 2016). To examine whether other pangolins were also infected by *B. fungorum*, we used 16S gene PCR assays to screen the blood of 39 other pangolins. Five

pangolins (FB1, FB2, FB3, FB4, and FB5) from the batch and two control pangolins (SB1 and SB2) from an independent batch were screened (Table S1). Four pangolins (each from first batch: FB1–FB5) displayed clear positive bands, indicating septicemia (Figure 4a). Sanger sequencing and phylogenetic analysis confirmed that they carried *B. fungorum* (Figure 4b). These *B. fungorum* sequences are near identical (99–100%), suggesting that the *B. fungorum* were transmitted among pangolins (Table S3). Based on these limited observation, animal-to-animal transmission may be common in seized pangolins, perhaps due to their reported poor immunity (Choo et al., 2016; Hua et al., 2015).

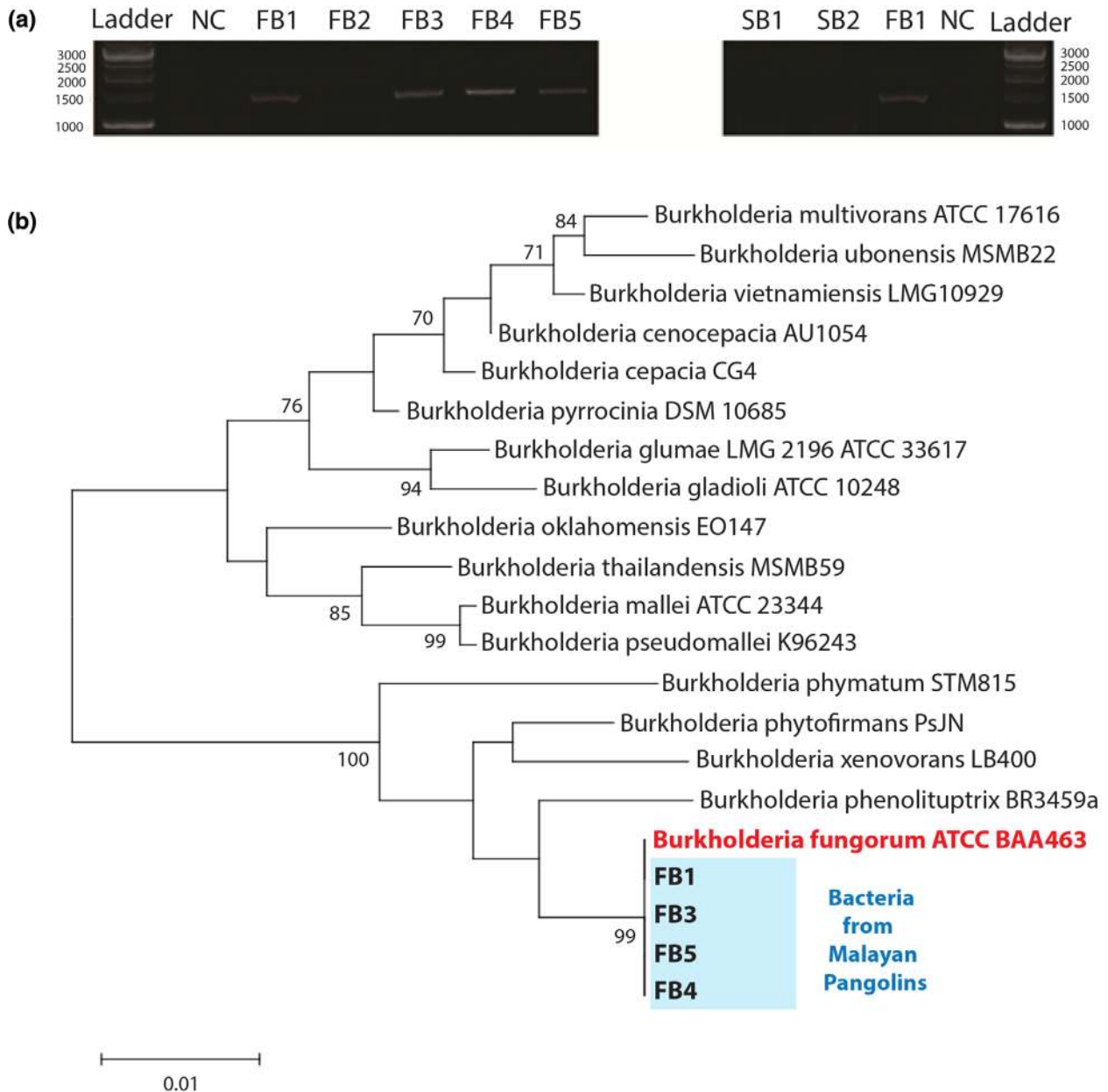


FIGURE 4 Identification of *Burkholderia fungorum* in Malayan pangolins. (a) PCR assays using the blood of seven pangolin individuals from two independent batches (Batch 1: FB1-5; Batch 2: SBI-2). Using the universal 16S gene primers to identify the presence of bacteria in all pangolin samples. NC (without DNA) is the negative control. (b) PCR products were sequenced using Sanger sequencing. The phylogenetic tree was generated using the 16S gene sequences and the maximum likelihood method. Bootstrap numbers were generated in 1,000 replicates. Nodes with bootstrap support values of 70 or greater are indicated

4 | DISCUSSION

Several groups have suggested that pangolins are the reservoir hosts or intermediate hosts in the emergence of COVID-19 CoV based on their high genome similarity (Liu et al., 2020; Xiao et al., 2020; Zhang et al., 2020). More extensive CoV screens and analyses around COVID 19 CoV transmission need to be conducted and replicated to be considered more than speculative. Pangolins are actually

unlikely to be the natural reservoir or intermediate hosts due to several reasons discussed below.

First, bat RaTG13 CoV rather than pangolin CoVs are the closest relative to COVID-19 CoV (Table 2). Bats were shown to be the reservoir species for SARS CoV in 2002 (L. F. Wang et al., 2006). Nonetheless, in the early stages of the 2002 SARS outbreak, the discovery of a related CoV in captive palm civets led to the erroneous implication of this species as the reservoir host that transmitted SARS CoV to

humans. This led to the culling of over 10,000 palm civets (Watts, 2004).

Second, transmission of the pangolin CoV to humans did not occur for at least 20 people among animal caregivers or researchers who had continuous unprotected physical contact with the CoV-infected animals since March 2019.

Third, very few species have been screened for COVID-19 CoV since the beginning of the outbreak. It was recently demonstrated that a tiger in the Bronx Zoo (United States), ferrets, and cats can be infected with COVID-19 CoV (Elasrar, 2020; Shi et al., 2020). Other animals such as dogs, pigs, and cows all carry various CoVs (Saif, 2004). There exist some ~6,495 mammalian species (Burgin, Colella, Kahn, & Upham, 2018); the vast majority of these remain untested for COVID-19 CoV or CoVs in general. Pangolins were tested because of illegal wildlife trade where they lead all other species in trafficking and confiscation. The conservation threat to pangolins is certain real, but this is no justification for unproven false claims regarding zoonotic transmission.

Fourth, two research groups examined about 42 pangolins, but only a few pangolins (e.g., about seven pangolins) were found with CoV (Lam et al., 2020a; Liu et al., 2019). Moreover, these studies could be biased because they mainly studied the diseased or dead pangolins especially those with lung infection. The proportion of pangolins carrying CoV may be overestimated since many other pangolins were not tested. However, these combined results indicate a relatively low CoV prevalence in pangolin populations. Perhaps, the wildlife trade not only places people at risk, but also precious and endangered wildlife species such as pangolins.

Fifth, free ranging pangolins lead an isolated ecological existence in nature, limiting spread of deadly pathogens. A similar ecological behavior strategy likely spared African cheetahs from new CoV outbreaks that can be very deadly in crowded captive settings (100% morbidity and 60% mortality) (Wilkerson et al., 2004).

Sixth, pangolins have reduced immunity to pathogenic microbes (Hua et al., 2015) perhaps due in part to a constitutive genetic loss of the immunity-related IFN γ gene function (Choo et al., 2016). Pangolins display poor skin and mucosal immunity and are frequently afflicted with the infections of skin and mucosa-protected organs such as lungs and intestines. Dahu and the Guangdong pangolins were seized together with other wildlife and handled by many people since March 2019. Therefore, we cannot rule out the possibility that they might have been infected with CoV from other seized species or even humans, making it more complicated to trace the source of the CoV.

Seventh, recent studies reported CoVs mainly from diseased or dead pangolins confiscated by anti-smuggling operations (Lam et al., 2020b; Liu et al., 2019). However, many reservoir host species carrying viruses remain asymptomatic as is the case with species of bats (Hu, Ge, Wang, & Shi, 2015). CoV prevalence reports on living or healthy pangolins have not been reported to our knowledge. Therefore, it could be concluded that pangolins cannot be considered the natural hosts, or the principal reservoir species for COVID-19 CoV, but were likely the coincidental victim of CoV transmission.

Since pangolins are endangered species, it would be impractical to find extensive pangolin samples for analysis and validation within a short period of time. However, it would be interesting in the future to perform a timely noninvasive large-scale screening for CoV in healthy wild populations of pangolins to further confirm the prevalence and diversity of CoVs.

The human contact discussed in this study poses the opportunity for pangolin to human transmission as well as human to pangolin transmission, an inference we need to consider. Besides that, we cannot be certain of the timing of CoV infection and prevalence in wild pangolins prior to the December 2019 human outbreak. The CoV clearly was present at or just after the time of pangolin confiscation (March 2019). The prior infection or timing remains an open question. The CoV-infected-Malayan pangolins were smuggled into China through Vietnam; therefore, it remains unclear whether they were infected inside or outside of China.

Our study provides new information on one potential constraint surrounding the conservation of endangered pangolins. We propose that future conservation of these species should also give more attention to possible infections by pathogens and that surveillance and further studies must be carried out both in captive pangolins and wild populations. Furthermore, we advise people to stop poaching and eating pangolins because they are usually associated with different kind of pathogens given their poor immunity. It is possible that these pathogens could be transmitted and harm human health.

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AUTHOR CONTRIBUTIONS

S.W.C. and J.Z. conceived this project. S.W.C., S.Z., X.T., J.Z., and F.T.S. performed animal sampling. S.W.C., K.Y.T., and X.T. designed primers, PCR, and Sanger sequencing experiments. S.W.C. and K.Y.T. performed data analyses and interpretation. S.Q. J.Z. and X.T. handled and performed diagnostic tests for COVID-19 CoV. S.W.C. wrote the manuscript. S.J.O., S.P., J.Z., X.T., S.Z., K.P.K., F.T.S., and A.A. joined the data interpretation and revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ACCESSIBILITY STATEMENT

All data are available in the main text and supporting information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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