Taiwan Veterinary Journal, Vol. 42, No. 1 (2016) 19–26
DOI: 10.1142/S1682648515500171

World Scientific
www.worldscientific.com

DETECTION OF BAT CORONAVIRUS AND SPECIFIC ANTIBODIES IN CHESTNUT BAT (*SCOTOPHILUS KUHLII*) POPULATION IN CENTRAL TAIWAN

Bo-Gang Su*, Hong Chang Chen†, Hsi-Chi Cheng‡ and Yi-Ning Chen*,§
*Department of Bioscience Technology, Chung Yuan Christian University
200 Chung Pei Road, Chung Li 32023, Taiwan, ROC
†Institute of Ecology and Evolutionary Biology, National Taiwan University
No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, ROC
‡Endemic Species Research Institute, Council of Agriculture
No. 1 Minsheng E. Road, Jiji Township, Nantou County 55244, Taiwan, ROC

Received 24 August 2015
Accepted 14 September 2015
Published 21 December 2015

ABSTRACT

Bats can serve as natural reservoirs for severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome coronavirus (MERS-CoV). Investigating the prevalence of bat CoV is critical for assessing the risks of the outbreaks of emerging CoV. Chestnut bats (*Scotophilus kuhlii*) were captured in this study for detecting the partial RNA-dependent RNA polymerase (RdRp) gene in their feces through reverse transcription polymerase chain reaction (RT-PCR) and antibodies to the nucleocapsid (N) protein of bat CoV through western blotting (WB) analysis. Three recombinant N protein fragments (N1, N2, N3) of the isolated *Scotophilus* bat CoV/CYCU-S1/TW/2013 were expressed by *Escherichia coli*. WB analyses were performed with bat serum samples and the sera of a patient who recovered from a SARS-CoV infection. Fragment N2 contained a highly conserved motif among CoVs whereas N1 and N3 protein fragments were specific to the *S. kuhlii* bat CoV. A total of 32 fecal and 19 serum samples were collected in Changhua County and Yunlin County during 2013 and 2014. About 17 fecal samples tested positive for the RdRp gene with an overall prevalence of 53%. Sequences comparison showed that the *Scotophilus* bat CoV isolates in Taiwan belonged to the genus *Alphacoronavirus* and were closest to Scotophilus bat CoV/Hainan/China/2005 and Diliman1552G1/Philippines/2008, followed by porcine epidemic diarrhea coronavirus. Only one bat serum sample reacted positively to all 3 N protein fragments. Cross-reactivity was observed between N2 protein fragment and the sera of a patient recovered from a SARS-CoV infection. The results indicated that *Scotophilus* bat CoV was circulating endemically in chestnut bat population in Taiwan.

Keywords: Bat coronavirus; Nucleocapsid protein; RNA-dependent RNA polymerase gene; Antibodies; Taiwan.

§Corresponding author: Yi-Ning Chen, Department of Bioscience Technology, Chung Yuan Christian University, 200 Chung Pei Road, Chung Li 32023, Taiwan, ROC. Tel: +886-3-265-3538; Fax: +886-3-265-3599; E-mail: yining@cycu.edu.tw

INTRODUCTION

Outbreaks of coronaviral diseases caused by severe acute respiratory syndrome-coronavirus (SARS-CoV) and Middle East respiratory syndrome (MERS)-CoV have prompted investigations of the natural reservoirs and transmission mechanism of CoV among different animal species. Bats have been proven to be natural reservoirs of SARS- and MERS-CoV in addition to other emerging zoonosis viruses including Ebola, Marburg, rabies, influenza, hanta, Nipah, and Hendra viruses.[1] The discovery that a bat SARS-related CoV can respond to the same type of cell receptor, angiotensin-converting enzyme 2 (ACE2), from humans, civet cats, and bats to cross-infect cell among various species confirmed the interspecies transmission of bat CoV.[2] More studies have also shown that both of MERS-CoV and Bat CoV HKU4 can use the cell receptor from humans and bats to cross-infect cells prepared or derived from humans, bats, camels, and other animal species.[3,4]

Coronavirus is the largest RNA virus with a single positive-stranded RNA genome of 26–32 kb. The distinct CoVs have been discovered in all types of animals because its unique transcription mechanism facilitates RNA recombination.[5] The CoV genome is organized in the order of 5′- polymerase - spike (S) - envelope (E) - membrane (M) - nucleocapsid (N) -polyA-3′. The polymerase gene encodes 15–16 nonstructural proteins (nsp) including RNA-dependent RNA polymerase (RdRp), whose amino acid sequences are the most common information used for phylogenetic analysis.[6] The N protein is critical for viral replication because of its interactions with the viral genome and nuclear proteins.[7] The N protein is also the most common antigen used for detecting antibodies to CoV because it is the most abundant viral protein during CoV infections and relatively conserved among different CoVs.[8]

The *Alpha-*, *Beta-*, *Gamma-*, and *Delta-CoV* genera were suggested for classifying all CoVs.[9] Bats host a higher number of different CoV species than any other animal species among the *Alpha-* and *Beta-CoV* genera, leading to the hypothesis that bat CoV is the common ancestor for all CoVs in mammals.[10] Of the 20 recognized CoVs today, 6 were bat CoVs and have been detected in 11 of 18 bat families worldwide,[11] including countries around Taiwan such as China, Hong Kong, Japan, the Philippines, and Thailand.[6,8,12–15] Therefore, investigating the prevalence of bat CoVs in the bat population in Taiwan is critical for public health. To acquire a comprehensive understanding of the ecology of bat CoV in Taiwan, coronaviral RNA and antibodies specific to bat CoV were both detected because coronaviral infections in animals typically last only weeks or months.[16–18]

After the order Rodentia, bats constitutes the second largest mammalian order (Chiroptera) and are distributed in every part of the world except Antarctica. Because of their ability to fly and migrate, their long life span, and their tendency to live in large social groups, bats are predisposed to maintain and transport viruses to other mammals, thus causing possible outbreaks of epidemics.[19] Bat CoVs were detected in young and lactating female bats at a significantly higher rate.[18,20] Therefore, maternity rooting sites, which are inhabited predominantly by lactating females and newborns, are speculated to be the major locations for CoV amplification in the breeding season, namely the spring and summer.[18,20] Chestnut bats (*Scotophilus kuhlii*) generally migrate to Taiwan for breeding from April to October and roost in the palm trees of school campuses and human structures. Their habitats near human communities and pig farms poses an increased chances of contacts between bats and pigs and human, thus enabling possible CoV transmission. In the current study, we focused on Chestnut bats in detecting bat CoV and the antibodies specific to bat CoV.

MATERIALS AND METHODS

Sample Collection

The protocol for capturing and sampling of bats in the present study was approved by the Chung Yuan Christian University Animal Care and Use committee (approval number 103008) and Agriculture Bureau of Regional Government. Chestnut bats (*S. kuhlii*) roosting in the palm trees of Chang-An Elementary School at Changhua County (23°50′51″N/120°23′12″E) during 2013 and Taiwan Sugar Corporation Factory at Yunlin County (23°34′05″N/120°17′51″E) during 2014 were captured by the experienced bat biologists from Taiwan Endemic Species Research Institute (TESRI) using an extended butterfly net. The captured bats were put into cotton bags individually and allowed to produce fecal pellets for the collection in 500 μL of RNA *later*® RNA stabilization solution (Qiagen, Germantown, MD, USA). Blood samples were taken by heparin (30 U/μL)-rinsed tips from the wing veins nicked by 26 G needles. No more than 5% of total blood volume of individual bat (1/20 of body weight) was collected for their survival.[21] About 50 μL of blood or 20 μL of serum after the centrifugation at 5000 rpm for 20 min was collected from a chestnut bat weighted 20 g in average.

RNA Extraction

Viral RNA was extracted from the fecal pellets in RNA*later*® solution by using QIAamp Viral RNA kit (Qiagen). In brief, 140 μL of the supernatant of the RNA*later*® solution with fecal samples after the centrifugation at 5000 rpm for 20 min was added to 560 μL of AVL buffer containing 5.6 μL of carrier RNA in AVE buffer. The mixture was incubated at room temperature for 10 min and then added 560 μL of 96–100% ethanol. Next, 630 μL of solution was applied to the QIAamp column in a 2-mL collection tube and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and the QIAamp column was washed by AW1 and AW2 buffers. Finally, viral RNA was eluted from the QIAamp column in 60 μL of AVE buffer after the centrifugation at 8000 rpm for 1 min.

Reverse Transcription

The extracted RNA was reverse transcribed to cDNA by using iScript Select DNA Synthesis kit (Bio-Rad, Hercules, CA, USA) containing the mixture of random primers and oligo dT primers according to the manufacturer's instructions. Briefly, the 20 μL-reaction comprising 13 μL of viral RNA, 4 μL of 5 x iScript reaction buffer, 2 μL of random and oligo dT primers, and 1 μL of iScript reverse transcriptase was incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and then stopped at 4°C.

PCR Detection of RdRp Gene Fragment

The presence of CoV in the fecal sample was confirmed by the semi-nested PCR assay targeting RdRp gene fragment (440 bp) using primer pair, RdRp-F and RdRp-R, in the first PCR, and RdRp-F and RdRp-nest R in the second PCR. Sequences of primers are listed in Table 1. Both PCR shared the same temperature program: denatured at 95°C for 10 min, 34 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 45 s, and the last extension step at 72°C for 7 min. The expected amplicons of 440 bp were confirmed by 1.5% agarose gel by electrophoresis and purified by Zymoclean™ Gel DNA Recovery Kit (Zymo, Irvine, CA, USA) for sequencing by Genomics company (New Taipei City, Taiwan).

Expression of Nucleocapsid Protein Fragments

The full length of N gene (1191 bp) was amplified from the cDNA by primers, Sco-N9-F and ACoV.3UTR-R, and cloned into Strata Clone Blunt Vector (Agilent, La Jolla, CA, USA) as pSC-B-Sco1-N9-3UTR. The N1 (222 bp), N2 (699 bp), and N3 (288 bp) gene fragments were amplified by primer pairs, Sco-N1F-BamHI and Sco-N1R-EcoRI, Sco-N2F-BamHI and Sco-N2R-EcoRI, Sco-N3F-BamHI and Sco-N3R-EcoRI, and then cloned into pTriEx™-3 vector (Millipore, Darmstadt, Germany) assigned as pTri-Sco-N1, pTri-Sco-N2, and pTri-Sco-N3, respectively (Fig. 1, Table 1). All PCRs used for amplifying N gene fragments were performed with Q5® Hot Start High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA) to minimize sequence errors from the process of PCR. The temperature program of PCR included the first step of denaturation at 98°C for 2 min, 35 cycles of 98°C for 30 s, 52°C for 30 s, 72°C for 62 s, and the last extension step at 72°C for 2 min. The three plasmids containing N1, N2, or N3 gene were transformed into *Escherichia coli* Rosetta strain, separately. N protein fragments were expressed by transformed *E. coli* Rosetta™ strain (Millipore) after induction with 0.5 mM of isopropyl-beta-D-thio galactopyranoside (IPTG) for 4 h at 37°C.

Purification of Nucleocapsid Protein Fragments

N protein fragments were extracted by BugBuster® Protein Reagent Kit (Millipore) and purified by Ni-NTA® His-Bind purification kit (Millipore). Protein concentration was quantified by OD value at 595 nm with Bio-Rad protein assay reagent (Bio-Rad) based on the standard curve established by bovine serum albumin (BSA) from 0 to 1 μg/μL. The quality of proteins was confirmed by 20% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie® Brilliant Blue (CBB) R259 (Sigma, St. Louis, MO, USA).

Western Blotting Analysis

Purified N protein fragments were separated by 20% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). First, the membrane was blocked by tris-buffered saline (TBS) buffer (1.4 M NaCl, 27 mM KCl, 250 mM Tris) with 5% BSA at room temperature for 40 min. Second, the membrane was cut into strips to incubated with different primary antibodies at 37°C for 1 h separately, including the 1/250 diluted bat serum samples, 1/250 diluted human serum from a patient recovered from the infection of SARS-CoV or the 1/16,000 diluted mouse anti-His monoclonal antibody (mAb) (Sigma) for positive control. After the washings

Table 1. Primers Amplifying Genes of Bat Coronavirus.

Primers	Sequences (5′ to 3′)
RdRp-F	GGTTGGGACTATCCTAAGTGTGA
RdRp-R	CCATCATCAGATAGAATCATCAT
RdRp-nest-R	ATCAGATAGAATCATCATAGAGA
Sco-N9-F	TGGCGACTACTATGCTGCTG
ACoV.3UTR-R	ATTACAGGCTTACCGTTGTGT
Sco-N1F-BamHI	TAGGATCCGATGGCTTCTGTAAAA
Sco-N1R-EcoRI	TAGAATTCGGCAAATCAACTCGGTC
Sco-N2F-BamHI	TAGGATCCGCCATCCAACTGGCAC
Sco-N2R-EcoRI	CTGAATTCGGCTTGGTGGCAACATT
Sco-N3F-BamHI	TAGGATCCGGAAATGGCTGATGGT
Sco-N3R-EcoRI	ATGAATTCGGATTCTGGGTGTCAAAAACC

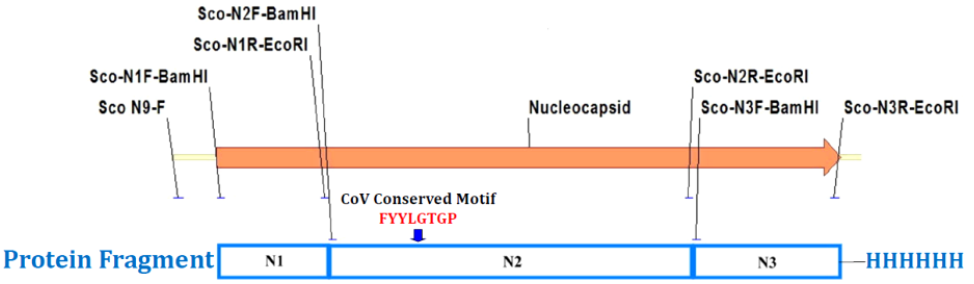

Fig. 1 Illustration of nucleocapsid protein fragment N1, N2, and N3 with the relative positions of different primers and conserved motif.

with TBST buffer (TBS with 0.1% Tween 20) for 30 min, different HRP-conjugated secondary antibodies, including goat anti-bat IgG (1/10,000) for bat serum, goat anti-mouse IgG (1/12,000) for anti-His mAb, and goat anti-human IgG (1/80,000) for the human serum, were applied to membrane strips at 37°C for 1 h. After the washings with TBST buffer for 30 min, the blotting reactions were developed by Immobilon Western Chemiluminiescent HRP substrate (Millipore).

RESULTS

Detection of Bat Coronavirus

For chestnut bats (*S. kuhlii*), a total of 14 bat fecal samples at Changhua County during 2013 and 18 bat fecal samples and 19 bat serum samples at Yunlin County during 2014 were collected. A conserved 440 bp region within the RdRp gene of bat CoV was detected in 8 fecal samples (57%) during 2013 (Table 2) and 9 fecal samples (50%) during 2014 (Table 3). Sequences of partial RdRp gene (375 bp) of 11 *Scotophilus* bat CoV isolates, CYCU-S1, S3, S5, S6, S8, S9, S11, S13/TW/2013, CYCU-S34, S37, and S38/TW/2014, were submitted to GenBank with accession number from KT381902 to KT381912. Blast analysis indicated that all 11 isolates belonged to the genus *Alpha-CoV* and were closest to *Scotophilus* bat CoV 512/Hainan/China/2005 (NC009657) from *S. kuhlii* in the Hainan island of China in 2005 and *S.* bat CoV Diliman1552G1/Philippines/2008 (AB539080) from *S. kuhlii* in the Diliman of Philippines in 2008 with 93–98% sequence identity. The closest CoV from animal species other than bats to the Scotophilus bat CoV isolated in Taiwan was porcine epidemic diarrhea coronavirus (PEDV)-CV777 (AF353511) with 70–73% sequence identity.

Expression of N Protein Fragments

All purified N protein fragments were presented as a single band in both CBB staining and western blotting (WB) analysis using mouse anti-His mAb (Fig. 2). The recombinant proteins constructed by pTriEx3 system had extra 10 amino acid residues than the original protein because of the addition of the carboxyl terminal HSV-tag and 6 x His-tag in the recombinant proteins.

Table 2. Detection of Bat Coronavirus in the Fecal Samples from Chestnut bats (*S. kuhlii*) at Chang-An Elementary School in Changhua County Changhua County During 2013.

ID	Sex[a]	Age[b]	RdRp/PCR[c]
S1	M	Adult	**Pos.**
S2	M	<1 yr	Neg.
S3	F	Adult	**Pos.**
S4	F	Adult	Neg.
S5	M	Adult	**Pos.**
S6	M	Adult	**Pos.**
S7	M	Adult	Neg.
S8	F	Adult	**Pos.**
S9	M	<1 yr	**Pos.**
S10	F	Adult	Neg.
S11	F	<1 yr	**Pos.**
S12	F	Adult	Neg.
S13	F	Adult	**Pos.**
S14	F	Adult	Neg.

[a]Sex of bat was determined as male (M) or female (F) based on external genitals.

[b]Age of one-year-old was determined by ephiphyseal-diaphyseal closure in the metacarpal-phalangeal joint.

[c]RdRp stands for RNA-dependent RNA polymerase gene fragment of coronavirus targeted by PCR. Positive result, Pos., shows a 440 bp-band after gel electrophoresis. Negative result, Neg., shows no band.

Therefore, the sizes of the recombinant N, N1, N2, and N3 protein fragments were 65 kD, 16 kD (aa 1–71), 38 kD (aa 72–301), and 25 kD (aa 302–394), respectively.

Detection of Antibodies to Bat Coronavirus

Antibodies to the N protein fragments of *S. kuhlii* bat CoV CYCU-S1/TW/2013 were detected by WB analysis. The results of PCR targeting RdRp gene and the antibody responses to three N protein fragments from the tested serum samples were recorded (Table 3). The serum of patient recovered from the infection of SARS and one bat serum (S43) reacted positively to N2 protein fragment. Only bat serum S43 also reacted positively to N1 and N3 protein fragments (Fig. 3, Table 3). The correlation between the presence of viral RdRp gene in feces and antibodies to N protein fragments cannot be analyzed because no fecal samples were collected from bat S43. All other serum samples reacted negatively to all three N protein fragments even though viral RdRp gene was detected in 9 out of 18 bats showing negative antibody responses.

Table 3. Detection of Bat Coronavirus in the Fecal Samples and Antibody Responses in the Serum Samples from Chestnut Bats (*S. kuhlii*) at Taiwan Sugar Corporation Factory at Yulin County During 2014.

			PCR[c]	Western Blotting[d]		
ID	Sex[a]	Age[b]	RdRp	N2	N1	N3
S15	M	<1 yr	Neg.	Neg.	Neg.	Neg.
S19	M	Adult	**Pos.**	Neg.	Neg.	Neg.
S20	F	Adult	**Pos.**	Neg.	Neg.	Neg.
S23	M	Adult	Neg.	Neg.	Neg.	Neg.
S24	F	Adult	Neg.	Neg.	Neg.	Neg.
S25	M	Adult	Neg.	Neg.	Neg.	Neg.
S26	M	Adult	Neg.	Neg.	Neg.	Neg.
S29	F	<1 yr	**Pos.**	Neg.	Neg.	Neg.
S31	F	<1 yr	Neg.	Neg.	Neg.	Neg.
S32	F	Adult	Neg.	Neg.	Neg.	Neg.
S33	F	Adult	**Pos.**	Neg.	Neg.	Neg.
S34	F	<1 yr	**Pos.**	Neg.	Neg.	Neg.
S36	M	Adult	Neg.	Neg.	Neg.	Neg.
S37	F	<1 yr	**Pos.**	Neg.	Neg.	Neg.
S38	F	Adult	**Pos.**	Neg.	Neg.	Neg.
S42	M	Adult	**Pos.**	Neg.	Neg.	Neg.
S43	M	Adult	n.a.	**Pos.**	**Pos.**	**Pos.**
S45	M	Adult	**Pos.**	Neg.	Neg.	Neg.
S48	M	Adult	Neg.	Neg.	Neg.	Neg.
αHis[e]	n.a.	n.a.	n.a.	**Pos.**	**Pos.**	**Pos.**
SARS[f]	n.a.	n.a.	n.a.	**Pos.**	Neg.	Neg.

[a]Sex of bat was determined as male (M) or female (F) based on external genitals.

[b]Age of 1-year-old was determined by ephiphyseal-diaphyseal closure in the metacarpal-phalangeal joint.

[c]RdRp stands for RNA-dependent RNA polymerase gene fragment of coronavirus targeted by PCR. Positive result, Pos., shows a 440 bp-band after gel electrophoresis. Negative result, Neg., shows no band. "n.a." means no sample available.

[d]WB analyses were performed on N1 (aa 1–71), N2 (aa 72–301), and N3 (aa 302–394) protein fragments of Scotophilus bat coronavirus CYCU-S1/TW/2013.

[e]Expression of protein fragments fused with His-tag was confirmed by mouse anti-His monoclonal antibody.

[f]Cross-reactivity of N protein-based serological detection was tested by the serum from a patient recovered from the infection of SARS.

DISCUSSION

It is the first report presenting the status of CoV in the population of chestnut bats (*S. kuhlii*) in central Taiwan. The *Alpha-CoV* has been investigated and similar to the bat CoV identified in the same bat species (*S. kuhlii*) sampled in the Hainan Province of China in 2005 and at the Diliman of Philippines in 2008.[14,22] The

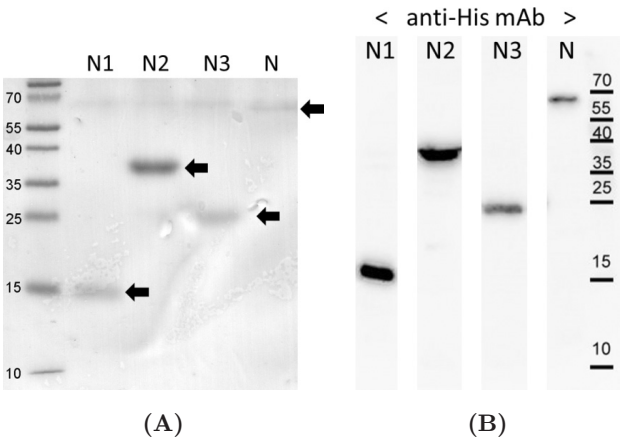

Fig. 2 Expression and WB of the recombinant N protein fragments of *Scotophilus* bat coronavirus CYCU-S1/TW/2013. **(A)** The N protein fragments separated on 20% SDS-PAGE stained with CBB. **(B)** WB results of the N protein fragments by using 1:16,000 of mouse anti-Histidine monoclonal antibody (Sigma) followed by 1:12,000 of HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (Thermo). Fragment N1 (16 kD), N2 (38 kD), N3 (25 kD), and the full length of N protein (65 kD), are shown in lane N1, N2, N3, and N.

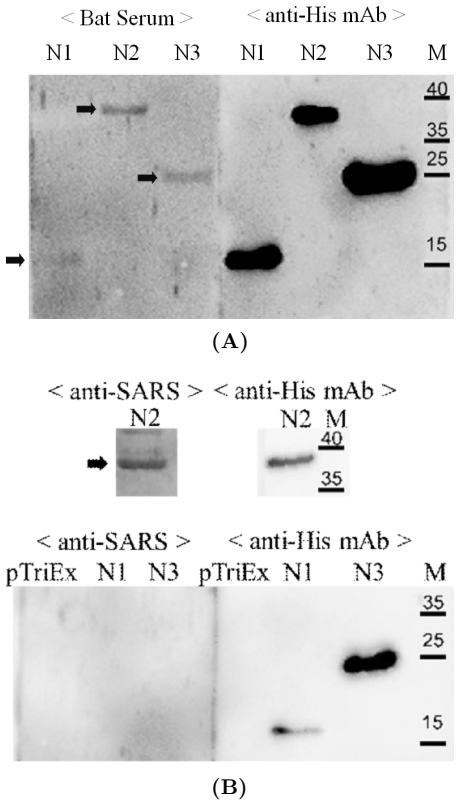

Fig. 3 WB results between three nucleocapsid protein fragment (N1, N2, and N3) of *Scotophilus* bat CoV/CYCU-S1/TW/2013 and the tested serum samples. **(A)** Expression of N protein fragments was confirmed by anti-His monoclonal antibody. Bat serum sample S43 can react to all three N protein fragments. **(B)** Cross-reactivity was only presented between N2 protein fragment and the serum from a patient recovered from the infection of SARS-CoV.

nucleotide sequence identity among isolates CYCU-S1/TW/2013, 512/Hainan/China/2005, 515/Hainan/China/2005, 527/Hainan/ China/2005, and Diliman1552G1/Philippines/2008 from different geographic origins was over 93%. All 4 samples collected in Philippines (100%) were tested positive for RdRp gene, 4 out of 43 samples positive (12%) in the Hainan island of China, and 17 out of 32 samples (53%) in Taiwan. With similar sample size, the prevalence of bat CoV was higher in Taiwan than that in Hainan, China. The bats tested positive for bat CoV did not show any clinical signs and appeared healthy. Chestnut bats as known as lesser Asiatic yellow bat in China (*S. kuhlii*) can be found in Philippines, Pakistan, Hainan Island, Taiwan, and Borneo.[23] High similarities of sequences among the bat CoV isolates from Hainan, Taiwan, and Philippines suggested that *Scotophilus* bat CoV is enzootic in the population of *S. kuhlii* in eastern Asia. It would be interesting to further study whether *Scotophilus* bat CoV circulates endemically in three different geographic locations or the same *Scotophilus* bat population can migrate from Philippines to Taiwan, and further to the Hainan Island of China while carrying *Scotophilus* bat CoV with them.

Very few serological investigations of bat CoV were conducted in bat populations because it is very difficult to collect sufficient amount of bat serum samples for testing. The surveillance in Philippines showed that 66.5% of bat serum samples (119/179) had antibodies to the recombinant N protein of bat *Beta-CoV* Philippines/Diliman 1525G2/2008 isolated from the lesser dog-faced fruit bats (*Cynopterus brachyotis*), while only 29.6% of bats (53/179) were tested positive for RdRp gene by reverse transcription polymerase chain reaction (RT-PCR).[8] The serological study in Guangdong of Southern China revealed that 43% (75/175) and 64% (224/350) of tested serum samples were positive by WB analysis and ELISA based on the recombinant N protein of *Beta-CoV Rousettus leschenaultia*/HKU9/China/2007 (Ro-Bat CoV HKU9, HM211098), respectively. Many bats having antibodies to Ro-Bat CoV HKU9 were tested negative by RT-PCR targeting RdRp gene although the antibody titers were significantly lower in bats tested negative than those in bats tested positive by RT-PCR.[24] In both cases in Philippines and China, the detection of antibodies to bat CoV can provide higher detection rate than the detection of bat coronaviral

RNA. However, the detection rate of antibodies specific to bat CoV was only 5% (1/19), lower than 50% (9/18) of detection rate by of RT-PCR targeting RdRp gene in the present study. Three juveniles at the age less than one year old were tested positive for RdRp gene but negative for specific antibody. It could result from that three bats were too young to produce antibodies or there was not enough time to produce antibodies after the infection of bat coronavirus. However, the sensitivity of the serology assay in the present study needs to be further improved.

Cross-reactivity was observed between the antibody to SARS-CoV (*Beta-CoV* subgroup b) and the N2 protein fragment containing the pan-CoV conserved motif (FYYLGTGP) from *S. kuhlii* bat CoV CYCU-S1/TW/2013 (*Alpha-CoV*) in the present study. Agnihothram's group has proposed that cross-reactivity to N proteins only happened within subgroups of the genus *Alpha-* or *Beta-CoV* but not among different subgroups.[25] However, antigenic cross-reactivity have occurred not only among different subgroups in the genus *Beta-CoV*, like SARS-CoV (*Beta-CoV* subgroup b) and human coronavirus (HCoV)-OC43 (*Beta-CoV* subgroup a),[26] but also between different genera, such as SARS-CoV (*Beta-CoV* subgroup b) and different *Alpha-CoV*, including *Scotophilus* bat CoV in this study, HCoV-229E, porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and canine coronavirus (CCoV).[27,28] Non-specific antibody responses to N protein can hamper disease control strategies due to misleading diagnostic results when human respiratory disease can be caused by different human CoV, including HCoV-229E and HCoV-NL63 in *Alpha-CoV*, HCoV-OC43 and HCoV-HKU1 in *Beta-CoV* subgroup a, SARS-CoV in *Beta-CoV* subgroup b, MERS-CoV in *Beta-CoV* subgroup c. To rectify the problem of cross-reactivity, recombinant S protein can be used for the detection of specific antibodies.[28] Stockman's group designed different fragments of N protein to differentially detect cross-reactive and specific antibodies.[29] A similar observation was also seen in our study that the N2 protein fragment can react to the antiserum against SARS-CoV, HCoV-229E, and HCoV-OC43 while the N1 and N3 protein fragments can only react to the antiserum against the corresponding CoV.[29] Therefore, the N1 and N3 protein fragments developed in the current study can be used to differentially detect antibodies specific to *S. kuhlii* bat CoV CYCU-S1/TW/2013.

In conclusion, the prevalence of bat CoV in the population of chestnut bats (*S. kuhlii*) in central Taiwan was 53% and the *Scotophilus* bat CoV-specific antibodies can be detected by recombinant N protein fragments. Further researches are needed to understand the transmission mechanism of *Scotophilus* bat CoV within local bat population or migratory bats traveling around Philippines, Taiwan, and the Hainan island of China. It is also very important to evaluate the possibility of CoV transmission between *Scotophilus* bats and pigs due to the high similarity of *Scotophilus* bat CoV and PEDV.

ACKNOWLEDGMENT

This work was supported by the Ministry of Science and Technology (NSC 102-2313-B-033 -001). We thank Dr. Yu- Siang Lee at Veterinary Medical Teaching Hospital of National Chung Hsing University for assisting the bleeding of the captured bats and Dr. Hsiang-Kuang Tseng at MacKay Memorial Hospital for providing the serum from a patient recovered from the infection of SARS-CoV.

REFERENCES

1. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T, Bats: Important reservoir hosts of emerging viruses, *Clin Microbiol Rev* **19**:531–545, 2006.
2. Ge XY, Li JL, Yang XL, Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor, *Nature* **503**:535–538, 2013.
3. Cai Y, Yu SQ, Postnikova EN, Mazur S, Bernbaum JG, Burk R, Zhang T, Radoshitzky SR, Muller MA, Jordan I, Bollinger L, Hensley LE, Jahrling PB, Kuhn JH, CD26/DPP4 cell-surface expression in bat cells correlates with bat cell susceptibility to middle east respiratory syndrome coronavirus (MERS-CoV) infection and evolution of persistent infection, *PLoS One* **9**:e112060, 2014.
4. Eckerle I, Corman VM, Muller MA, Lenk M, Ulrich RG, Drosten C, Replicative capacity of MERS coronavirus in livestock cell lines, *Emerg Infect Dis* **20**:276–279, 2014.
5. Lai MM, SARS virus: The beginning of the unraveling of a new coronavirus, *J Biomed Sci* **10**:664–675, 2003.
6. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, Seebens A, Niedrig M, Pfefferle S, Yordanov S, Zhelyazkov L, Hermanns U, Vallo P, Lukashev A, Müller MA, Deng H, Herrler G, Drosten C, Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences, *J Virol* **84**:11336–11349, 2010.
7. Chen H, Wurm T, Britton P, Brooks G, Hiscox JA, Interaction of the coronavirus nucleoprotein with nucleolar antigens and the host cell, *J Virol* **76**:5233–5250, 2002.
8. Tsuda S, Watanabe S, Masangkay JS, Mizutani T, Alviola P, Ueda N, Iha K, Taniguchi S, Fujii H, Kato K, Horimoto T, Kyuwa S, Yoshikawa Y, Akashi H, Genomic

and serological detection of bat coronavirus from bats in the Philippines, *Arch Virol* **157**:2349–2355, 2012.
9. Chan JF, To KK, Tse H, Jin DY, Yuen KY, Interspecies transmission and emergence of novel viruses: Lessons from bats and birds, *Trends Microbiol* **21**:544–555, 2013.
10. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, Bai R, Teng JL, Tsang CC, Wang M, Zheng BJ, Chan KH, Yuen KY, Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus, *J Virol* **86**:3995–4008, 2012.
11. Drexler JF, Corman VM, Drosten C, Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS, *Antivir Res* **101**:45–56, 2014.
12. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY, Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats, *Proc Natl Acad Sci USA* **102**:14040–14045, 2005.
13. Shirato K, Maeda K, Tsuda S, Suzuki K, Watanabe S, Shimoda H, Ueda N, Iha K, Taniguchi S, Kyuwa S, Endoh D, Matsuyama S, Kurane I, Saijo M, Morikawa S, Yoshikawa Y, Akashi H, Mizutani T, Detection of bat coronaviruses from Miniopterus fuliginosus in Japan, *Virus Genes* **44**:40–44, 2012.
14. Tang XC, Zhang JX, Zhang SY, Wang P, Fan XH, Li LF, Li G, Dong BQ, Liu W, Cheung CL, Xu KM, Song WJ, Vijaykrishna D, Poon LL, Peiris JS, Smith GJ, Chen H, Guan Y, Prevalence and genetic diversity of coronaviruses in bats from China, *J Virol* **80**:7481–7490, 2005.
15. Wacharapluesadee S, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T, Group C betacoronavirus in bat guano fertilizer, Thailand, *Emerg Infect Dis* **19**:1349–1351, 2013.
16. Chen YN, Wu CC, Bryan T, Hooper T, Schrader D, Lin TL, Specific real-time reverse transcription-polymerase chain reaction for detection and quantitation of turkey coronavirus RNA in tissues and feces from turkeys infected with turkey coronavirus, *J Virol Methods* **163**:452–458, 2010.
17. Lau SK, Li KS, Huang Y, Shek CT, Tse H, Wang M, Choi GK, Xu H, Lam CS, Guo R, Chan KH, Zheng BJ, Woo PC, Yuen KY, Ecoepidemiology and complete genome comparison of different strains of severe acute respiratory syndrome-related Rhinolophus bat coronavirus in China reveal bats as a reservoir for acute, self-limiting infection that allows recombination events, *J Virol* **84**:2808–2819, 2010.
18. Osborne C, Cryan PM, O'Shea TJ, Oko LM, Ndaluka C, Calisher CH, Berglund AD, Klavetter ML, Bowen RA, Holmes KV, Dominguez SR, Alphacoronaviruses in New World bats: Prevalence, persistence, phylogeny, and potential for interaction with humans. *PLoS One* **6**:e19156, 2011.
19. Bennett M, Bats and human emerging diseases, *Epidemiol Infect* **134**:905–907, 2006.
20. Gloza-Rausch F, Ipsen A, Seebens A, Gottsche M, Panning M, Felix Drexler J, Petersen N, Annan A, Grywna K, Muller M, Pfefferle S, Drosten C, Detection and prevalence patterns of group I coronaviruses in bats, northern Germany, *Emerg Infect Dis* **14**:626–631, 2008.
21. Voigt CC, Cruz-neto A, Energetic analysis of bats. in Kunz TH, Parsons S (eds.), *Ecological and Behavior Methods for the Study of Bats* 2nd edn., The Johns Hopkins University Press, Baltimore, pp. 623–645, 2009.
22. Watanabe S, Masangkay JS, Nagata N, Morikawa S, Mizutani T, Fukushi S, Alviola P, Omatsu T, Ueda N, Iha K, Taniguchi S, Fujii H, Tsuda S, Endoh M, Kato K, Tohya Y, Kyuwa S, Yoshikawa Y, Akashi H, Bat coronaviruses and experimental infection of bats, the Philippines, *Emerg Infect Dis* **16**:1217–1223, 2010.
23. Simmons N, Order Chiroptera. in Wilson DE, Reeder DM, (eds.), *Mammal Species of the World: A Taxonomic and Geographic Reference*, The Johns Hopkins University Press, Baltimore, pp. 312–529, 2005.
24. Lau SKP, Poon RWS, Wong BHL, Wang M, Huang Y, Xu H, Guo R, Li KSM, Gao K, Chan KH, Zheng BJ, Woo PCY, Yuen KY, Coexistence of different genotypes in the same bat and serological characterization of *Rousettus* bat coronavirus HKU9 belonging to a novel *Betacoronavirus* subtype, *J Virol* **84**:11385–11394, 2010.
25. Agnihothram S, Gopal R, Yount BL Jr, Donaldson EF, Menachery VD, Graham RL, Scobey TD, Gralinski LE, Denison MR, Zambon M, Baric RS, Evaluation of serologic and antigenic relationships between middle eastern respiratory syndrome coronavirus and other coronaviruses to develop vaccine platforms for the rapid response to emerging coronaviruses, *J Infect Dis* **209**:995–1006, 2014.
26. Che XY, Qiu LW, Liao ZY, Wang YD, Wen K, Pan YX, Hao W, Mei YB, Cheng VC, Yuen KY, Antigenic cross-reactivity between severe acute respiratory syndrome-associated coronavirus and human coronaviruses 229E and OC43, *J Infect Dis* **191**:2033–2037, 2005.
27. Sun ZF, Meng XJ, Antigenic cross-reactivity between the nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus and polyclonal antisera of antigenic group I animal coronaviruses: Implication for SARS diagnosis, *J Clin Microbiol* **42**:2351–2352, 2004.
28. Woo PC, Lau SK, Wong BH, Chan KH, Hui WT, Kwan GS, Peiris JS, Couch RB, Yuen KY, False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide, *J Clin Microbiol* **42**:5885–5888, 2004.
29. Stockman LJ, Haynes LM, Miao C, Harcourt JL, Rupprecht CE, Ksiazek TG, Hyde TB, Fry AM, Anderson LJ, Coronavirus antibodies in bat biologists, *Emerg Infect Dis* **14**:999–1000, 2008.