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DETECTION OF SPECIFIC ANTIBODIES TO THE NUCLEOCAPSID PROTEIN FRAGMENTS OF SEVERE ACUTE RESPIRATORY SYNDROME-CORONAVIRUS AND *SCOTOPHILUS* BAT CORONAVIRUS-512 IN THREE INSECTIVOROUS BAT SPECIES

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ABSTRACT

Bats are the natural reservoirs of severe acute respiratory syndrome-coronavirus (SARS-CoV). Six *Alphacoronavirus* and five *Betacoronavirus* have been detected in many bat species, including SARS-related CoV and Middle East respiratory syndrome (MERS)-related CoV. In Taiwan, SARS-related CoV, belonging to *Betacoronavirus*, has been detected in *Rhinolophus monoceros*. *Scotophilus* bat CoV-512, belonging to *Alphacoronavirus*, has been detected in *Scotophilus kuhlii*, *Miniopterus fuliginosus*, and *Rhinolophus monoceros* by using reverse transcription polymerase chain reaction (RT-PCR). To understand the infection history of CoV in these three insectivorous bat populations, CoV-specific antibodies were surveyed by using western blot (WB) analysis and indirect enzyme-linked immunosorbent assay (ELISA). The carboxyl terminal fragment of nucleocapsid protein (N3) of SARS-CoV and *Scotophilus*

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bat CoV-512 were used as the antigen in the assays. Of the 52 serum samples obtained from *Scotophilus kuhlii*, 29 samples (56%) were tested positive for *Scotophilus* bat CoV-512-specific antibodies through ELISA. Of the 63 serum samples obtained from *Rhinolophus monoceros*, 9 samples were tested positive for only SARS-CoV-specific antibodies, 7 samples were tested positive for only *Scotophilus* bat CoV-512-specific antibodies, and 16 samples (25.4%) were tested positive for both antibodies through WB analysis. Only 1 of 18 *Miniopterus* bat serum samples tested positive for *Scotophilus* bat CoV-512-specific antibodies through ELISA. Lactating female bats had higher positive rates of CoV-specific antibodies than non-lactating female and male bats did. Our findings were crucial for understanding CoV infection history in three insectivorous bat species and important for the control of bat-borne zoonosis diseases.

Keywords: Insectivorous bats; Enzyme-linked immunosorbent assay; Nucleocapsid proteins; Coronavirus; Taiwan.

INTRODUCTION

Numerous novel coronaviruses (CoVs) have been discovered after the investigation of the natural reservoirs of severe acute respiratory syndrome (SARS)-CoV which has caused a total of 8096 cases worldwide.[1] In the subfamily *Coronavirinae*, 30 CoV species are divided into 4 genera from *Alpha-* to *Deltacoronavirus* and 10 CoV species in the genera *Alpha-* and *Betacoronavirus* are found in different bat species.[2] In the feces of 9 bat species found in Taiwan, *Scotophilus* bat CoV-512, *Miniopterus* CoV-1A, and SARS-related CoV were detected through reverse transcription polymerase chain reaction (RT-PCR), which targets RNA-dependent RNA polymerase gene (RdRp) fragment.[3] Molecular detection and sequencing are the most common tools used for discovering novel CoV species.[4] However, the surveillance of CoV by genomic assays has many limitations that are caused by the degradation of viral RNA, numerous PCR inhibitors in fecal samples, and the insufficient quantity and low quality of PCR products for sequencing.[4–6] Serological assays based on specific antigen fragments can differentiate antibodies against different CoV species and provide information regarding the cumulative exposure history to a specific antigen.[7,8]

Coronaviruses have four major structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The immunogenicity of S and N proteins is high; therefore, they are the most common antigens used in serological assays. Assays based on the N protein are more sensitive than those based on the S protein because the N protein is the most abundant protein during CoV infection and is relatively conserved among different CoVs compared to S protein.[9] However, S protein-based assays are more specific than are N protein-based assays because S proteins different highly in different CoVs and induce neutralizing antibodies in hosts.[9] The N protein is favored for developing new serological assays because of the ease of expression of the N protein in a prokaryotic expression system such as that of *Escherichia coli*.[10] Furthermore, the N protein is smaller in size than the S protein and does not require glycosylation for protein production.[10]

Serological data for CoV-specific antibodies in bats are scant because of difficulties in collecting bat serum samples, particularly from insectivorous bats with small body sizes.[5,8,11,12] It has been speculated that African bats have been harbored SARS-related CoV since long before the outbreaks of SARS-CoV in 2002.[8] Antibodies reactive with the SARS-CoV antigen were detected in 47 of 705 bat serum samples collected from 4 frugivorous and 3 insectivorous bat species in Africa between 1986 and 1999 by using a commercial SARS-CoV enzyme-linked immunosorbent assay (ELISA) kit, which was developed for detecting antibodies specific to SARS-CoV in human serum.[8] Antibodies to SARS-CoV were also detected in frugivorous *Rousettus* bats and insectivorous *Rhinolophus* bats in China by using a ELISA, Western blot (WB) assay, or immunofluorescent antibody (IFA) assay on whole SARS-CoV particles, recombinant N or S protein of SARS-CoV, or SARS-CoV-infected cells.[5,13] Antibodies to another *Betacoronavirus*, *Rousettus* bat CoV HKU 9, were detected in *Rousettus* bats in China and in 5 frugivorous and 2 insectivorous bat species in the Philippines.[5,12] Generally, serological assays have higher detection rate than RT-PCR, and ELISA is more sensitive than WB assay.[5,8,12] We attempted to detect antibodies to the recombinant N protein fragments of *Scotophilus* bat CoV-512 in the serum samples obtained from *Scotophilus kuhlii* through WB assay in previous study and the detection rate of specific antibodies was very low.[14] This study has optimized the conditions for WB assays and developed an N protein fragment-based ELISA for detecting the specific antibodies to *Scotophilus*

bat CoV-512 and SARS-CoV in the serum samples obtained from *Scotophilus (S.) kuhlii*, *Miniopterus (M.) fuliginosus*, and *Rhinolophus (R.) monoceros*.

MATERIALS AND METHODS

Sample Collection and Processing

All bats captured in this study were released after sampling. The procedures for capturing and sampling of bats were approved by the Institutional Animal Care and Use Committee (IACUC) at Chung Yuan Christian University with the approval number 103008 and 103028. Field sampling permissions were obtained from the Agriculture Bureau of Regional Governments. Chestnut bats (*S. kuhlii*) were captured from the palm at Beigang sugar processing factory trees by an extended butterfly net (23°34′05″N/120°17′51″E) in September of 2014 and August of 2015. Eastern bent-winged bats (*M. fuliginosus*) and Formosan lesser horseshoe bats (*R. monoceros*) were captured by a harp trap from the same irrigation culvert at Dongshan (23°19′04″N/120°25′27″E) in October of 2014 and September of 2015. Reproductive status of female bats was determined by the swelling of nipples. The bats were considered lactating when they have swollen breasts and the bats were considered non-lactating when their nipples were not exposed. The bats with insufficient ephiphyseal-diaphyseal closure in the metacarparpal-phalangeal joints were considered younger than one-year-old and others were considered adults. Less than 5% of total blood volume (1/20 of body weight) of individual bat was taken from wing veins nicked by 26–30G needles.[15] The average body weights of *S. kuhlii*, *M. fuliginosus*, and *R. monoceros* were 20, 11, and 5 g, respectively. Both WB and ELISA used the serum produced by the centrifugation at 5000 rpm from the clotted blood samples (CT15RE, Hitachi, Tokyo, Japan). Fecal pellets produced by individual bats were collected in RNAlater® RNA stabilization solution (Qiagen, Germantown, MA, USA) for further RT-PCR analysis.[3]

Reverse Transcription Polymerase Chain Reaction Analysis

The presence of CoV in the fecal samples was detected by RT-PCR targeting RdRp gene fragment (440 bp) according to previous studies.[3] Briefly, viral RNA was extracted from each fecal sample by using QIAamp Viral RNA Mini Kit (Qiagen) and reverse transcribed into cDNA by using iScript Select DNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The semi-nested PCR used primer RdRp-F and RdRp-R in the first PCR and primer RdRp-F and RdRp-nest R in the second PCR to amplify a 440-bp RdRp fragment. The expected PCR products were confirmed by 1.5% agarose gel and sequenced for the identifications of *Scotophilus* bat CoV-512 and SARS-related CoV according to the blast analysis and sequence alignment results.

Recombinant Nucleocapsid Protein Fragments

According to previous study, three N gene fragments of *Scotophilus* bat CoV-512/CYCU-S1/TW/2013 (GenBank number: KT346372) were cloned as pTri-Sco-N1, pTri-Sco-N2, and pTri-Sco-N3.[14] The N3 gene fragment of SARS-related CoV (SARS-N3) was cloned from SARS-CoV Tor2 strain (GenBank number: AY274119) as pTri-SARS-N3. Gene fragment was amplified from pCMV-SARS-CoV-TOR2-N by using primers SARS-N3F-BamHI (5′-TAGGATCCGGTGACACCATCTGGC-3′) and SARS-N3R-EcoRI (5′-TAGAATTCGGAGCCTG GGTGCTG T-3′). N3 protein fragments were extracted by BugBuster® protein reagent kit (Merck Millipore, Darmstadt, Germany) and purified by Ni-NTA® His-Bind purification kit (Millipore). Protein concentration was quantified by OD595 nm with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) 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% SDS-PAGE stained with Coomassie® Brilliant Blue (CBB) R259 (Sigma-Aldrich, St. Louis, MO, USA).

Western Blotting Analysis

To detect specific antibodies to *Scotophilus* bat CoV-512 and SARS-CoV, we modified the procedures of WB analysis in previous study.[14] Briefly, 1 μg of purified N3 protein fragments with His-tag (Sco-N3 and SARS-N3) were separated by 20% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad).

The membrane was cut into strips to incubate separately with a 1:250 of *Scotophilus* and *Rhinolophus* bat serum samples at 4°C for 16 h, modified from the reaction condition of 37°C for 1 h. Antibody–antigen interaction was detected with a 1:20,000 of horseradish peroxidase (HRP)-conjugated goat anti-bat IgG heavy and light chain antibody (Bethyl, Montgomery, TX, USA) at 4°C for 2 h, modified from the reaction condition of 37°C for 1 h, and Immobilon Western Chemiluminescent HRP substrate (Millipore). Anti-His monoclonal antibody

(Sigma-Aldrich) was used as a positive control and PBS without primary antibody was used as a negative control.

Enzyme-Linked Immunosorbent Assay for *Scotophilus* Bat CoV 512-Specific Antibodies

To detect the presence of IgG antibodies to *Scotophilus* bat CoV-512 in serum samples from *S. kuhlii* and *M. fuliginosus*, the N3 protein fragment-based ELISA was developed and optimized. Serum sample S24 and S67 from the *S. kuhlii* bat individuals showing negative RT-PCR and WB results were used as negative control serum. Serum sample S73 from the *S. kuhlii* bat individual showing positive RT-PCR and WB result was used as positive control serum. One serum sample H10 from *Hipposideros armiger terasensis* bat individual showing negative RT-PCR and WB result was also used as negative control serum. The conditions producing bigger differences of OD450nm values between negative control samples and positive control samples were chosen. Two concentrations of antigen (1 μg/well and 2.5 μg/well), two concentrations of BSA blocking solution (1% and 3%), three dilutions of primary antibody (1:50, 1:100, 1:250), and two dilutions of secondary antibody (1:5000 and 1:10,000) were tested. The optimization results are presented in the supplement Fig. S1 and the optimized concentration of N3 protein fragment for coating was 1 μg/well, the concentration of BSA blocking solution was 1%, the dilution of primary antibody was 1:100, and the dilution of secondary antibody was 1:5000. The procedures of ELISA began with the coating of 1 μg/well of purified His-tagged N3 protein fragment of *Scotophilus* bat CoV-512 (Sco-N3) on MaxiSorp Nunc-immuno 96-microwell plates (Roskilde, Denmark). Next, the plates were blocked with PBS with 1% BSA at room temperature for 1 h, washed with PBS with 0.05% Tween 20 for three times, and incubated with bat serum samples (primary antibody) diluted in 1:100 at room temperature for 1 h. After three times of washing, HRP-conjugated goat anti-bat IgG heavy and light chain antibody (Bethyl) diluted in 1:5000 was used as secondary antibody at room temperature for 1 h. Antibody and antigen interaction was detected by adding teteramethylbenzidine (TMB, BD, San Jose, CA, USA) for 15 min. The reaction was stopped by adding 2N H_2SO_4 and OD450nm was measured by Synergy HT Multi-Mode Microplate Reader (Bio-Tek). Based on the results of WB and ELISA from 31 *S. kuhlii* sera, the cut-off value for negative and positive responses by ELISA was calculated by receiver operating characteristic (ROC) curve.[16] Antibody-positive response of *Miniopterus fuliginosus* sera were determined as outlier by Smirnov–Grubbs rejection test.[16] The area under the curve (AUC) was calculated to determine the distinguishing ability of ELISA for the detection of Sco-N3 specific antibody in *S. kuhlii* sera (R project, 1 is perfect, > 0.9 is outstanding, > 0.8 is excellent, > 0.7 is acceptable). Agreement between different assays was analyzed by logistic regression and Cohen's kappa statistical analysis (R project, 0.81–1 is complete agreement, 0.61–0.8 is high level, 0.41–0.6 is middle level, 0.21–0.4 is low level, 0–0.2 is minimum level).

Statistical Analysis

Seroprevalence data was compared by using Fisher's exact test for categorical variables, including gender, maturation status, and reproductive status.[16] p values of < 0.05 were regarded as statistically significance.

RESULTS

Scotophilus Bat CoV-512-Specific Antibodies in *S. kuhlii*

Only 18 out of 26 serum samples collected in 2014 and 13 out of 26 serum samples collected in 2015 from *S. kuhlii* had enough volume for both WB and ELISA. According to the results of Sco-N3-based WB and Sco-N3-based ELISA on the same 31 serum samples, the cut-off value of Sco-N3-based ELISA determined by ROC was OD450nm 0.878. Amongst a total of 52 serum samples from *S. kuhlii*, 29 samples (56%) were positive through Sco-N3-based ELISA (Fig. 1(A)). The median OD450 nm value was 1.18 for positive samples and 0.63 for negative samples. The distinguishing ability of ELISA for the detection of Sco-N3 specific antibody in *S. kuhlii* sera was acceptable (0.7717) by AUC (Fig. 1(B)). There were 8 samples tested positive and 12 negative through both WB and ELISA. Amongst 23 serum samples tested negative through Sco-N3-based WB analysis, 11 serum samples showed positive through Sco-N3-based ELISA. The range of OD450nm values are listed in Table 1. The results of WB and ELISA had slight correlation ($k = 0.36$) and ELISA had higher sensitivity than WB. There are 21 bats having both serum samples for Sco-N3-based ELISA and fecal samples for RT-PCR targeting RdRp partial gene. 10 out of 21 bats had fecal samples tested positive through RT-PCR and 5 out of these 10 bats had serum samples tested positive through ELISA. Out of 11 bats having fecal samples tested negative through RT-PCR,

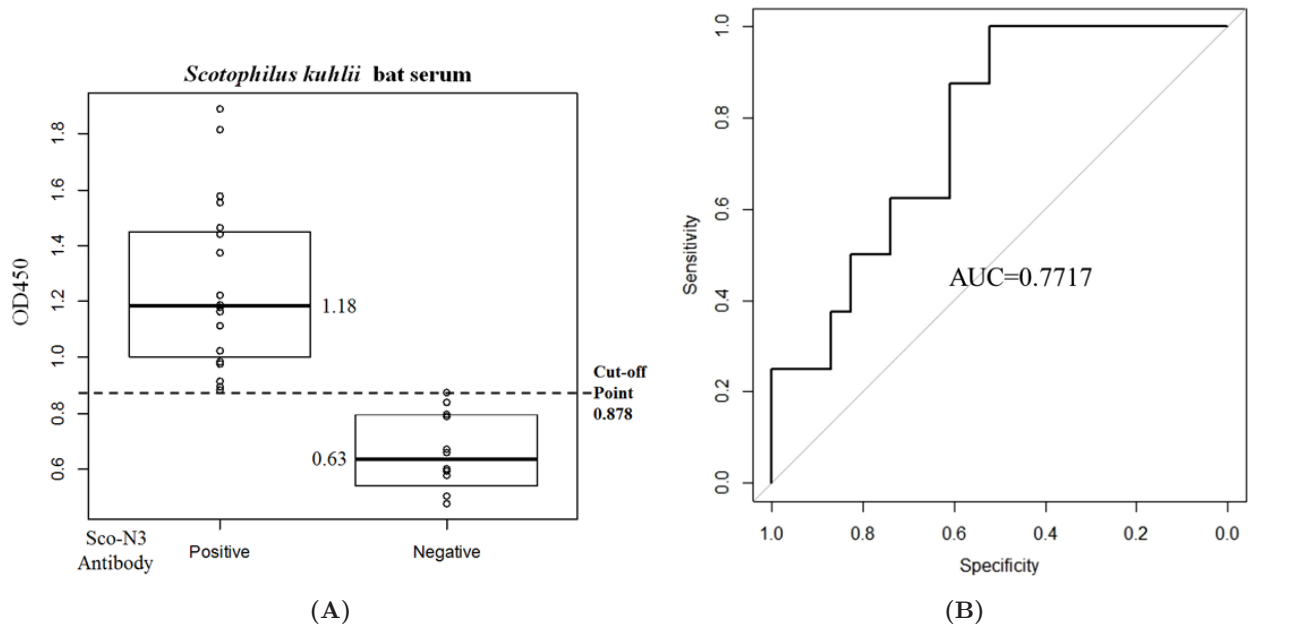

Fig. 1 Antibody response to the N3 fragments of *Scotophilus* bat coronavirus-512 (Sco-N3) through ELISA in *S. kuhlii*. **(A)** Distribution of antibody responses dotted by each circle representing the average OD450nm readings of duplicate wells for one individual sample. The bold lines in the middle of boxes are medians. The upper and lower borders of boxes are upper quartiles and lower quartiles, respectively. The cut-off point was determined by the results of Western blotting (WB) and ELISA on the same 31 serum samples. There are 29 positive samples with the median of 1.18 and 23 negative samples with the median of 0.63. **(B)** The AUC shows acceptable ability of Sco-N3-based ELISA to distinguish positive or negative antibody response.

Table 1. Antibody Responses to the N3 Fragment of *Scotophilus* Bat CoV-512 (Sco-N3) through ELISA and Western Blotting (WB) in 31 Serum Samples Collected from *S. kuhlii*.

		Sco-N3 ELISA (Cut-Off Value OD450nm 0.878)		
		Positive Number (OD Range)	Negative Number (OD Range)	
Sco-N3 WB	Positive	8 (0.881–1.888)	0	8
	Negative	11 (0.893–1.575)	12 (0.479–0.876)	23
		19	12	31

6 of them had serum samples tested positive through ELISA. Among seven *Scotophilus* bats younger than 1-year-old were tested, two had both viral RNA and antibodies, one had viral RNA but no antibodies, two had antibodies but no viral RNA, and two had no viral RNA nor antibodies. No correlation was found between the results of ELISA and RT-PCR ($k = -0.045$). Amongst 8 samples tested positive through both WB and ELISA, 6 samples came from females (6/17, 35%) and 2 samples came from males (2/14, 14%). The seroprevalence determined by ELISA was also higher in female *S. kuhlii* (19/30, 63%) than that in male *S. kuhlii* (10/22, 45%). The lactating adult females (4/6, 67%) had higher detection rate of antibodies through ELISA than non-lactating adult females (2/6, 33%). Adult bats (22/39, 56%) and young bats less than one-year-old (7/13, 54%) had similar seroprevalence determined by ELISA. Molecular and serological results for *S. kuhlii* are listed in Table S2.

Scotophilus Bat CoV-512-Specific and SARS-CoV-Specific Antibodies in *R. monoceros* sera

Antibodies specific to *Scotophilus* bat CoV-512 and SARS-CoV were detected by WB analysis in the sera of *R. monoceros*. Representative WB pictures are shown in Fig. S2. Amongst 62 serum samples, 16 samples showed positive for both Sco-N3 and SARS-N3, 9 samples were positive for Sco-N3, 7 samples were positive for SARS-N3, and 30 samples were negative for both Sco-N3 and SARS-N3 (Table 2). From 30 bats having both serum samples for WB and fecal samples for RT-PCR, 2 bats had feces tested positive for CoV and both of them had antibodies to only SARS-N3. Amongst 28 bats had feces tested negative for CoV, 5 bats had antibodies to both Sco-N3 and SARS-N3, 2 bats had antibodies to

Table 2. Antibody Responses to the N3 Fragment of *Scotophilus* Bat CoV-512 (Sco-N3) or Severe Acute Respiratory Syndrome-Coronavirus (SARS-N3) through ELISA and Western Blotting (WB) in 62 Serum Samples Collected from *R. monoceros*.

		Sco-N3 WB		
		Positive	**Negative**	
SARS-N3 WB	Positive	16	7	23
	Negative	9	30	39
		25	37	62

only Sco-N3, and 4 bats had antibodies to only SARS-N3. There was a little correlation between the results of RT-PCR and WB analysis ($k = 0.152$). The detection rates of antibodies to Sco-N3 (21/48, 44%) or to SARS-N3 (19/48, 40%) in females were higher than those (4/28, 29%; 4/28, 29%) in males. A total of 10 of 22 lactating females had antibodies to both Sco-N3 and SARS-N3, 3 lactating females had antibodies to Sco-N3, and 3 had antibodies to SARS-N3. A total of 2 of 13 non-lactating females had antibodies to Sco-N3 and one had antibodies to SARS-CoV. Lactating females had significantly higher detection rates of antibodies to Sco-N3 ($p = 0.004$) and SARS-N3 ($p = 0.016$) than those of non-lactating females (Table 3). Molecular and serological results for *R. monoceros* are listed in Table S3.

Scotophilus Bat CoV 512-Specific Antibodies in *M. fuliginosus*

Antibodies to Sco-N3 were detected through ELISA in the serum samples collected from *M. fuliginosus*. One sample was determined as positive for Sco-N3 by using Smirnov–Grubbs rejection test on the OD450nm values of 18 serum samples. The fecal samples from all bats were tested negative through RT-PCR (Fig. 2).

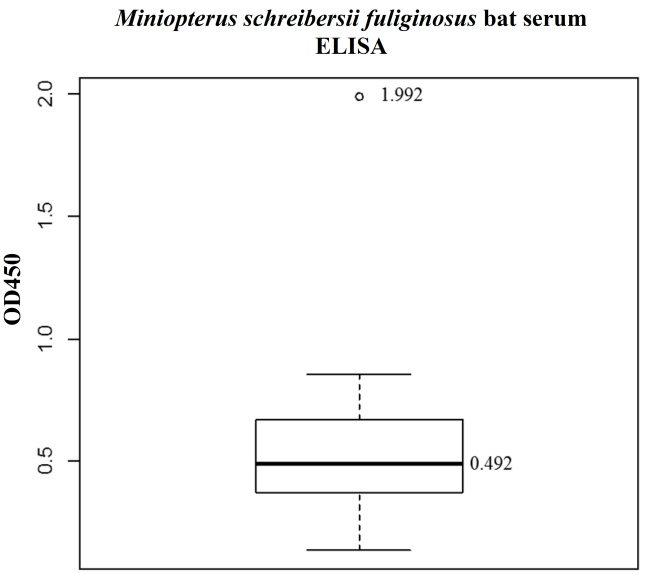

Fig. 2 Antibody response to the N3 fragments of *Scotophilus* bat coronavirus-512 (Sco-N3) through ELISA in *M. fuliginosus*. Distribution of antibody responses dotted by each circle representing the average OD450nm readings of duplicate wells for one individual sample. One sample was determined as positive against Sco-N3 by Smirnov–Grubbs rejection test on the OD values of 18 serum samples. The bold line in the middle of box is median (OD450nm 0.049). The upper and lower borders of box are upper quartiles and lower quartiles, respectively.

DISCUSSION

The current study has provided valuable information about the antibody responses specific to SARS-related CoV and *Scotophilus* bat CoV-512 in the bat population of Taiwan. Previous study detected both SARS-related and *Scotophilus* bat CoV-512 in *R. monoceros* from different individuals.[3] It is difficult to determine whether there were co-infections of SARS-related CoV and *Scotophilus* bat CoV-512 in the same bat individuals because the presence of co-infections would be masked by the biased amplification of the dominant virus species

Table 3. Antibody Responses to the N3 Fragment of Severe Acute Respiratory Syndrome-Coronavirus (SARS-N3) or *Scotophilus* Bat CoV-512 (Sco-N3) in *R. monoceros* of Different Gender and Reproductive Status.

	Anti-SARS-N3 pos./all (prevalence)[b]	***p* Value[a]**	**Anti-Sco-N3 pos./all (prevalence)[‡]**	***p* Value[†]**
Male	4/14 (29%)		4/14 (29%)	
Female	19/48 (40%)	0.367	21/48 (44%)	0.541
Lactating	13/22 (59%)		13/22 (59%)	
Non-lactating	1/13 (8%)	0.004*	2/13 (15%)	0.016*

Notes: [a]Seroprevalence data was compared by using Fisher's exact test for gender and reproductive status (R core team, 2013). p values of < 0.05 were regarded as statistically significance. *$p < 0.05$ is statistically significant.

[b]The seroprevalence was calculated by the percentile ratio of antibody-positive samples to total samples tested.

by PCR. By using multiple sets of virus species-specific primers, the co-infections of bat CoV 1B and HKU8 were observed and different genotypes of bat CoV HKU9 were found in the same *Rousettus* bat individuals.[5,17] This study was the first to analyze the coexistence of antibodies specific to different CoV species in the same bat individuals. Both antibodies to SARS-related CoV and *Scotophilus* bat CoV-512 found in 16 of 62 *R. monoceros* indicated that the same bat individuals can be infected with two different CoV species from two different CoV genera even though the data did not provide information about co-infection or sequential infection of two CoVs. Many serosurveillance data on human populations showed the coexistence of antibodies to HCoV 229E, NL63, HKU1, and OC43 in the same individuals.[18–20] The exposures to different CoV species in human individuals are very common but there are few studies on antibody responses to different CoV species in animal, especially in bats.

When N-protein-based immunoassays were used, cross-reactivity leading to false positive results was always the challenges despite the advantages of easy production for recombinant N proteins.[21] Cross-reactivity to N proteins happened not only within subgroups of the same genus but also between different genera.[21,22] To rectify the problem of cross-reactivity, we constructed three fragments of N proteins as N1, N2, and N3 fragments according to Stockman's design.[23] From 31 serum samples collected from *S. kuhlii*, 11 samples reacted positive to only the N2 fragment, 3 samples reacted positive to only the N3 fragment, 4 samples reacted positive to both the N2 and N3 fragments, and 1 sample reacted positive to all three N fragments of *Scotophilus* bat CoV-512 (Table S1). Similar design was used to detect the antibodies to three N fragments of HCoV-OC43 in 46 serum samples: 16 samples were positive to the N2 and N3 fragments, 11 samples were positive to only the N2 fragment, and 11 samples were positive to all three fragments.[24] Stockman's group excluded one serum sample from positive antibody response to SARS-CoV because the serum sample only showed positive signals to the N2 fragment but not the N1 and N3 fragments of SARS-CoV.[24] The N3 fragment of CoV was chosen for the antigen of serological tests in this study due to location of antigenic epitopes in the central N2 and carboxyl N3 fragment[24–26] and the N2 fragment was excluded due to the presence of a highly conserved motif (FYYLGTGP) found in all known CoVs.[27]

M. fuliginosus and *R. monoceros* roost in the same irrigation culvert and usually rest side by side in a mixed group. Both bat species had specific antibodies to *Scotophilus* bat CoV-512 but *R. monoceros* (25/62, 40%) had higher detection rate than *M. fuliginosus* (1/18, 6%). While viral RNA and antibodies specific to *Scotophilus* bat CoV-512 were detected in both bat species, viral RNA and antibodies specific to SARS-related CoV can only be detected in *R. monoceros*. Some bat CoVs can only be detected in specific bat species in the same roosting site shared by many different bat species. *Miniopterus* bat CoV 1A and 1B can only be detected in *Miniopterus magnate* and *Miniopterus pusillus*, respectively, and *Miniopterus* bat CoV 1A, HKU-7, and HKU-8 were detected in *Miniopterus schreibersii* but not *Rhinolophus* and *Hipposideros* bats.[2,17] It suggested that interspecies transmission of bat CoVs required many factors other than co-inhabitant of different bat species.

Detection rates of CoV-specific antibodies in the serum samples and CoV RNA in the fecal samples of the same bat individuals were different because the targets and meanings of serological assays are different to molecular assays. Serological assays can provide the information whether animals have experience of infection while molecular assays provide the information of viral infection. There were only 3 fecal samples tested positive for CoV RdRp gene through RT-PCR but 7 and 11 serum samples tested positive for *Scotophilus* bat CoV-512 and SARS-CoV, respectively, from the same 30 *R. monoceros*. The studies in the Philippines and China showed the same conclusion. Antibodies reacted to the N protein of Bat CoV Philippines/Diliman 1525G2/2008 were found in 66.5% (119/179) of bat serum samples but CoV RdRp gene was detected in only 29.6% (53/179) of bats.[12] In Guangdong province of China, the detection rate of antibodies to *Rousettus* Bat CoV HKU9 was 43% (75/175) through Western blot (WB) and 64% (224/350) through ELISA, but the detection rate of viral RNA gene fragment was only 12% (42/350).[5] The antibody titers were significantly lower in the bats tested negative than those in the bats tested positive by RT-PCR.[5] The higher detection rate of CoV-specific antibodies may suggest that the CoV had circulated in the bat population for a period of time. Persistent infections of CoV have been observed in cell culture system, experimental infection system, and wild population.[28–31] Capture-mark-recapture data from longitudinal samples of individual bats will provide more information about the transmission dynamics of *Scotophilus* bat CoV 512 or SARS-like CoV in the populations of *S. kuhlii* and *R. monoceros*.

The detection rates of antibodies were similar to those of CoV RNA in the same 21 *S. kuhlii* individuals. That 6 out of 21 *Scotophilus* bats had only CoV RNA but no CoV-specific antibodies indicated the bats had early stage of viral infection and had not produced

sufficient antibody level for detection yet. That 5 out of 21 *Scotophilus* bats had only CoV-specific antibodies but no CoV RNA suggested the bats were exposed to CoV previously but had no current viral infection. For the bats younger than one year, the level of maternal antibodies and the introduction of new virus into the naïve population can determine whether we can detect CoV RNA and CoV-specific antibodies. Studies on the Henipavirus maternal antibodies in African fruit bats showed that the bats are susceptible to henipavirus infections once the titers of maternal antibodies waned.[32] Unlike the results of Lau's study,[5] the ELISA titers of CoV-specific antibodies were similar in the *Scotophilus* bats tested negative than those in the *Scotophilus* bats tested positive by RT-PCR (Table S2). More age-specific serological surveys are required to understand the role of maternal antibodies in the infection of *Scotophilus* bat CoV-512 and the production of antibodies.

The lactating female *S. kuhlii* and *R. monoceros* had higher detection rates of antibodies to *Scotophilus* bat CoV-512 and SARS-CoV than those of the non-lactating female bats in this study. The studies on the Henipavirus infection in African fruit bats showed that females in pregnancy or lactation are more likely to have higher antibody titers than non-pregnant lactation in order to provide adequate maternal antibodies to the young bat.[33,34] Increased horizontal virus transmissions associated with pregnancy and lactation period were observed from the Henipavirus infection in African fruit bats,[33] the CoV infection in the *Myotis myotis* population of Germany,[35] and the CoV amplification in maternity colonies.[36] The effects of lactation on the seroprevalence of CoV-specific antibodies were more significant in the population of *R. monoceros* than *S. kuhlii* because female *Rhinolophus* bats tend to roost together with their offspring in breeding season and female *Scotophilus* bats tend to roost together with not only their offspring but also male adult *Scotophilus* bats. There were no significant differences among the detection rates of CoV-specific antibodies and CoV RNA from adult and young *Scotophilus* bats.

This study provided valuable information about the antibody responses to *Scotophilus* bat CoV-512 in three insectivorous bat species and showed the coexistence of antibodies specific to *Scotophilus* bat CoV-512 and SARS-CoV in the same bat individuals. The data would assist the government to establish the strategy to control the bat-associated zoonosis diseases. Further longitudinal serological studies can provide more details on viral dynamics within different bat populations.

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CONFLICT OF INTEREST

None

REFERENCES

1. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, A novel coronavirus associated with severe acute respiratory syndrome, *New Engl J Med* **348**:1953–1966, 2003.
2. 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.
3. Chen YN, Phuong VN, Chen HC, Chou CH, Cheng HC, Wu CH, Detection of the severe acute respiratory syndrome-related coronavirus and alphacoronavirus in the bat population of Taiwan, *Zoonoses Public Health* **63**:608–615, 2016.
4. Drexler JF, Corman VM, Drosten C, Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS, *Antiviral Res* **101**:45–56, 2014.
5. Lau SKP, Poon RWS, Wong BHL, Wang M, Huang Y, Xu H, Guo R, Li KSM, Gao K, Chan K-H, Zheng B-J, Woo PCY, Yuen K-Y, 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.
6. 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.
7. 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.
8. Muller MA, Paweska JT, Leman PA, Drosten C, Grywna K, Kemp A, Braack L, Sonnenberg K, Niedrig M,

Swanepoel R, Coronavirus antibodies in African bat species, *Emerg Infect Dis* **13**:1367–1370, 2007.

9. 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.
10. Shin GC, Chung YS, Kim IS, Cho HW, Kang C, Antigenic characterization of severe acute respiratory syndrome-coronavirus nucleocapsid protein expressed in insect cells: The effect of phosphorylation on immunoreactivity and specificity, *Virus Res* **127**:71–80, 2007.
11. 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.
12. 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.
13. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF, Bats are natural reservoirs of SARS-like coronaviruses, *Science* **310**:676–679, 2005.
14. Su BG, Chen HC, Cheng HC, Chen YN, Detection of bat coronavirus and specific antibodies in chestnut bat (*Scotophilus kuhlii*) population in central Taiwan, *Taiwan Vet J* **42**:19–26, 2016.
15. 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.
16. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R_project.org/(2013).
17. Chu DK, Peiris JS, Chen MH, Guan Y, Poon LL, Genomic characterization of bat coronaviruses (1A, 1B and HKU8) and evidence for co-infections in *Miniopterus* bats, *J Gen Virol* **89**:1282–1287, 2008.
18. Gaunt ER, Hardie A, Claas EC, Simmonds P, Templeton KE, Epidemiology and clinical presentations of the four human coronavirus 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method, *J Clin Microbiol* **48**:2940–2947, 2010.
19. Shao X, Guo X, Esper F, Weibel C, Kahn JS, Seroepidemiology of group I human coronavirus in children, *J Clin Virol* **40**:207–213, 2007.
20. Zhou W, Wang W, Wang H, Lu R, Tan W, First infection by all four non-severe acute respiratory syndrome human coronaviruses takes place during childhood, *BMC Infect Dis* **13**:433, 2013.
21. 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.
22. 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.
23. 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.
24. Liang FY, Lin LC, Ying TH, Yao CW, Tang TK, Chen YW, Hou MH, Immunoreactivity characterisation of the three structural regions of the human coronavirus OC43 nucleocapsid protein by Western blot: Implications for the diagnosis of coronavirus infection, *J Virol Methods* **187**:413–420, 2013.
25. He Y, Zhou Y, Wu H, Kou Z, Liu S, Jiang S, Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus, *J Clin Microbiol* **42**:5309–5314, 2004.
26. Yu M, Stevens V, Berry JD, Crameri G, McEachern J, Tu C, Shi Z, Liang G, Weingartl H, Cardosa J, Eaton BT, Wang LF, Determination and application of immunodominant regions of SARS coronavirus spike and nucleocapsid proteins recognized by sera from different animal species, *J Immunol Methods* **331**:1–12, 2008.
27. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Peñaranda S, Bankamp B, Maher K, Chen MH, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman DD, Peret TC, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rasmussen M, Fouchier R, Günther S, Osterhaus AD, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ, Characterization of a novel coronavirus associated with severe acute respiratory syndrome, *Science* **300**:1394–1399, 2003.
28. Mizutani T, Fukushi S, Saijo M, Kurane I, Morikawa S, JNK and PI3k/Akt signaling pathways are required for establishing persistent SARS-CoV infection in Vero E6 cells, *Biochim Biophys Acta* **1741**:4–10, 2005.
29. Cai Y, Yu S, 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.
30. Subudhi S, Rapin N, Bollinger TK, Hill JE, Donaldson ME, Davy CM, Warnecke L, Turner JM, Kyle CJ, Willis CR, Misra V, A persistently infecting coronavirus in hibernating *Myotis lucifugus*, the North American little brown bat, *J Gen Virol* **98**:2297–2309, 2017.
31. Jeong J, Smith CS, Peel AJ, Ploweright RK, Kerlin DH, McBroom J, McCallum H, Persistent infections support maintenance of a coronavirus in a population of Australian bats (*Myotis macropus*), *Epidemiol Infect* **145**:2053–2061, 2017.
32. Peel AJ, Baker KS, Crameri G, Barr JA, Hayman DT, Wright E, Broder CC, Fernández-Loras A, Fooks AR,

Wang LF, Cunningham AA, Wood JL, Henipavirus neutralising antibodies in an isolated island population of African fruit bats, *PloS one* **7**:e30346, 2012.
33. Baker KS, Suu-Ire R, Barr J, Hayman DT, Broder CC, Horton DL, Durrant C, Murcia PR, Cunningham AA, Wood JL, Viral antibody dynamics in a chiropteran host, *J Anim Ecol* **83**:415–428, 2014.
34. Plowright RK, Field HE, Smith CS, Divljan A, Palmer C, Tabor G, Daszak P, Foley JE, Reproduction and nutritional stress are risk factors for Hendra virus infection in little red flying foxes (*Pteropus scapulatus*), *Proc Biol Sci* **275**:861–869, 2008.
35. 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.
36. Drexler JF, Corman VM, Wegner T, Tateno AF, Zerbinati RM, Gloza-Rausch F, Seebens A, Müller MA, Drosten C, Amplification of emerging viruses in a bat colony, *Emerg Infect Dis* **17**:449–456, 2011.