The persistent prevalence and evolution of cross-family recombinant coronavirus GCCDC1 among a bat population: a two-year follow-up

Joseph O. Obameso1,3†, Hong Li2†, Hao Jia4,5, Min Han1, Shiyuan Zhu4,5, Canping Huang5, Yuhui Zhao1, Min Zhao1,3, Yu Bai1,3, Fei Yuan1, Honglan Zhao5, Xia Peng2, Wen Xu2, Wenjie Tan5, Yingze Zhao5, Kwok-Yung Yuen6, William J. Liu4,5, Lin Lu* & George F. Gao1,3,5*

1 CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;
2 Yunnan Provincial Center for Disease Control and Prevention, Kunming 650022, China;
3 University of Chinese Academy of Sciences, Beijing 100049, China;
4 College of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035, China;
5 Key Laboratory of Medical Virology and Viral Diseases, Ministry of Health of People’s Republic of China, National Institute for Viral Disease Control and Prevention, Beijing 102206, China;
6 State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong Special Administrative Region, Hong Kong, China

Received September 11, 2017; accepted October 23, 2017; published online December 1, 2017

INTRODUCTION

Bats (Order Chiroptera) are the only group of mammals capable of flight with the ability to circumnavigate at night, i.e., nocturnal animals (Calisher et al., 2006). Bats have been the focus of particular interest not just because of their ecological interest, but also because of their medical importance in public health, as they play a significant role in evolving and transmission of zoonotic viral agents that...
stance a substantial danger to human and animal well-beings. Bats possess special peculiarities and features which make them host to different viruses, such features including structural diversity and complex lifestyle, diverse mode of feeding ranging from insectivores (feed on insects), frugivore (fruit eater), nectarivore (sucking nectar), palynivore (eating pollen), piscivore (eating fish) while others feed on arthropods, vertebrates or practice haematophagy (feed on blood). Bats are social animals that live in a densely populated roost, demographic and spatial structure of population with long life, which may significantly contribute to maintaining viruses as well as the high possibility of intra- and inter-species transmission of viral infections (Calisher et al., 2006). In addition, excreta during migration may be an important factor in the spread of viruses (López-Roig et al., 2014).

Bats are connected with the increasing numbers of emerging and re-emerging viruses including Hendra virus (HeV), Nipah virus (NiV) and Ebola virus (EBOV), and also coronaviruses, etc. (Li et al., 2016). The studies of bats as reservoirs for a number of mammalian coronaviruses came to the spotlight since the discovery of severe acute respiratory syndrome (SARS)-like coronaviruses in 2005 isolated from south China (Lau et al., 2005; Li et al., 2005), and subsequently, a SARS-CoV receptor ACE2-using coronavirus was isolated from bat (Ge et al., 2013). Sister to SARS-CoV was the middle east respiratory syndrome-associated coronavirus (MERS-CoV) emerged in 2012, the close-related viruses of which was also found in bats (Lelli et al., 2013; Zumla et al., 2016). Actually, a number of bat coronaviruses have been documented in recent years (Hu et al., 2017), and the MERS-CoV phylogenetically closely related bat coronaviruses (BatCoVs) HKU4 can also recognize human CD26 for cell entry, indicating origin of human-susceptible coronaviruses from bats (Wang et al., 2014).

Coronavirus encrypts a non-segmented, largest positive sense single-stranded RNA genome known ranging from 27 to 32 kb in length (Lai, 1996; Sokolova et al., 1996; Du et al., 2016). In the subfamily of Coronaviridae, Alphacoronavirus and Betacoronavirus infect and cause diseases in mammals, while Gammacoronavirus and Deltacoronavirus are mostly confined to avian infections and diseases (Jackwood et al., 2012; Woo et al., 2012). Report has shown that coronaviruses possess low proofreading capability that can also exhibit comparatively high mutation and recombinant rates, which make them one of the most diverse, genetically distinct, and recently emerging groups of viruses (Minskaia et al., 2006). The continuous mutation and recombination of the coronaviruses probably bridge the species barrier and enable them to spread into the human population (Baric et al., 1995).

Recently, we discovered a novel Rousettus bat coronavirus GCCDC1 (Ro-BatCoV GCCDC1) with full genome sequenced, which exhibits a putative heterologous recombination event between a bat coronavirus and a bat orthoreovirus (Huang et al., 2016). We found that the p10 gene of bat orthoreovirus in Ro-BatCoV GCCDC1 genome has no homologous gene from any parental strain of coronaviruses. However, there is dearth of information whether this virus with the recombinant gene can persist and evolve in their hosts.

Herein we carried out a longitudinal survey on one population of *Rousettus leschenaulti* bats in Yunnan Province, Mainland China, between 2014 and 2015. The persistence of the Ro-BatCoV GCCDC1 in the bat population and its potential evolution was investigated. Our study provides the first glimpse of the virus evolution in one prospectively and longitudinally observed bat population.

**RESULTS**

**The persistent prevalence of the Ro-BatCoV GCCDC1 in the bats**

A total of 568 rectal swab samples from *R. leschenaulti* bats were collected between May 2014 (*n*=118), October 2014 (*n*=270) and October 2015 (*n*=180) in one cave located in Xishuangbanna, Yunnan Province, China (Table 1). The anal swab samples were screened individually for the presence of coronavirus using RT-PCR detection method. Coronavirus positive ratios were 39.8% (47/118), 38.8% (70/270) and 35.6% (64/180) in bats sampled in May 2014, October 2014 and October 2015, respectively (Table 1). These results showed a persistent prevalence of Ro-BatCoV GCCDC1 in this bat population.

**The evolution of RdRp from Ro-Bat GCCDC1**

Based on the identified PCR CoV-positive bat samples, the PCR amplicons with the length of 228bp of Ro-BatCoV GCCDC1 RdRp were sequenced, which showed the conservation of this newly identified bat CoV. We analyzed the RdRp sequences which had the corresponding p10 sequences available, thus we have 97 pairs of RdRp and p10 from 97 bat specimens. The phylogenetic analysis of the RdRp sequences revealed two groups of Ro-BatCoV GCCDC1 designated Group I (RdRp-I) and Group II (RdRp-II) (Figure 1A). The result from our study revealed that all the Ro-BatCoV GCCDC1 RdRp from the first round sampling in May 2014 belonged to the RdRp-I, while subsequent sampling in October 2014 and 2015, respectively, showed the involvement of viruses featured with RdRp-II.

**The evolution of p10 gene in Ro-BatCoV GCCDC1**

Based on the result of PCR amplification using specific primers, the nucleic acid sequences of p10 genes (420 bp) of
Ro-BatCoV GCCDC1 were subjected to phylogenetic analysis, which displayed high level of sequence similarity to Ro-BatCoV GCCDC1 p10 genome segments (Huang et al., 2016). In pairwise comparison of nucleotide sequence, the nucleotide sequence identity was 99% compared to Ro-BatCoV GCCDC1. However, based on the phylogenetic analysis of all the available Ro-BatCoV GCCDC1 in public domain, the p10 genes were divided into two groups: Group (A) and Group (B) (Figure 1B). All the three times of sampling Ro-BatCoV GCCDC1 covered both Group A and Group B, which indicated the persistence of the groups of p10 in Ro-BatCoV GCCDC1.

When compared to p10 genes from traditional orthoreoviruses (Figure 2), the p10 from the typical six Ro-BatCoV GCCDC1: GCCDC1/2-53 and GCCDC1/2-110 sampled in October 2014, and GCCDC1/3-19 and GCCDC1/3-31 in October 2015 and our previously identified Ro-BatCoV GCCDC1 strains 346-KU762337 and Ro-BatCoV GCCDC1 strains 356-KU762338, are clustered into one independent branch indicating the uniqueness of the bat coronavirus-derived p10 gene. However, we can still observe that p10 genes in 2-53 and 3-31 belong to Group A and p10 of other four samples are in Group B.

The potential dynamic recombination of Ro-BatCoV GCCDC1

To further investigate the dynamic evolution of Ro-BatCoV GCCDC1 in the longitudinally collected bat samples, we further analyzed the potential recombination of the viruses by constructing a model that pairs of RdRp and p10 genes, each pair of which is sequenced from single Ro-BatCoV GCCDC1 sample. Five different nonsynonymous mutations were found in all available RdRp protein segments (Figure 3A). However, only two mutations were featured by the two groups of RdRp (T-648 and I-666 for RdRp-I and A-648 and M-666 for RdRp-II). Comparison of the translated amino acid sequences of p10 gene of both Group A and B of Ro-BatCoV GCCDC1 identified four nonsynonymous mutations at position 7, 14, 19 and 34, respectively (Figure 3B). Group A p10 was featured with the combination of M-7, T-14, A-19, and I-34, while Group B possesses substitutions V-7, T-14, N-19 and S-34 in the four positions. When we paired the corresponding RdRp and p10 genes groups with their featured substitutions, there were four different combinations of the putative assembled genomes in all the bat samples tested (Figure 4A). Ro-BatCoV GCCDC1 virus IA possesses Group I of RdRp gene and Group A of p10 gene. Ro-BatCoV GCCDC1 virus IB is featured with Group I of RdRp gene and Group B of p10 gene. Ro-BatCoV GCCDC1 virus IIA has Group II of RdRp gene and Group A of p10 gene. Ro-BatCoV GCCDC1 virus IIB characterizes by Group II of RdRp gene and Group B of p10 gene.

During the first round of sampling in May 2014, only viruses IA and IB were discovered in the bat population (Figure 4B). However, five months later in October 2014 IIA and IIB recombinants can be found in the bat swabs. Further, the third round sampling in October 2015 showed that while the four combinations were persistent in the bat population, the two viruses IIA and IIB became more dominant.

DISCUSSION

In our previous study, we demonstrated that a novel coronavirus Ro-BatCoV GCCDC1 encoded a putatively recombinant nonstructural protein p10 derived from reovirus, which induces the formation of syncytia in infected cells (Huang et al., 2016). Sequel to this, we now report that Ro-BatCoV GCCDC1 is continuously circulating in bats from Yunnan Province in China. We observed high prevalence of the Ro-BatCoV GCCDC1 on a consistent trend for a period
of 2 years that is relatively high in proportion to the populations under study. The cross-family recombinant \( p10 \) gene stably exists in the novel virus. Notably, we observed the dynamic evolution of this virus in the special bat population. Our study provides beneficial recommendation for the surveillance and pre-warning of potential interspecies transmittable viruses in bats.

The recombination events in coronavirus involving interchange of intra-family genetic materials has been reported (Baric et al., 1995). In our previous study, a traditional reovirus gene \( p10 \) located at the upstream of N protein and downstream of NS7a of Ro-BatCoV GCCDC1 genome showing the ability of coronavirus to undergo a heterologous inter-family recombination (Huang et al., 2016). In our

---

**Figure 2**  Phylogenetic relationships of Ro-BatCoV GCCDC1 compared to other viruses. Phylogenetic trees of the \( p10 \) sequence of Ro-BatCoV GCCDC1 together with the \( p10 \) genes from other reoviruses. The phylograms were constructed by using MEGA6 software package to determine the maximum-likelihood tree for the sequence.

**Figure 3**  Multiple sequence alignment of Ro-BatCoV GCCDC1 genes based on samples from three sampling periods. Sequence alignment of deduced amino acid of RdRp (A) and \( p10 \) proteins (B). Consensus and sequence logo for conserved amino acid residues are shown on top of the alignment. The residues with color indicate the position of mutation.
current study, we observed a persistent prevalence Ro-BatCoV GCCDC1 in the bat population, and we also found that the recombinant p10 is a constitutive gene in this virus. We did not get all the p10 sequences from the RdRp positive samples. This may be due to a lower amplification efficiency of the p10 primers. Thus, the deep sequencing of the whole genomes and also the virus isolation from the original samples are still needed to elucidate more features of Ro-BatCoV GCCDC1.

The emergence of recombinant p10 gene in Ro-BatCoV GCCDC1 could be due to unhindered access of reoviruses in bat, being a prehistoric mammal which might have favoured viral integration and persistence. p10 persistence may have utilized intrinsic factors mimicking ability to incorporate its genome into coronavirus or of the host’s cells. p10 has been reported to share similarities with viroporins, which has the ability to form hydrophilic channels that allow low molecular weight hydrophilic molecules to cross the membrane thereby interrupt membrane integrity, breakdown ionic gradients and eventual release of essential compounds from the cell (Carrasco et al., 1989; Carrasco et al., 1993). This could lead to series of events that favor viral replication, encourage viral transmission and persistence of Ro-BatCoV GCCDC1 in bat population. Interestingly, the phylogenetic analysis revealed that two distinct groups of p10 with featured amino acids persistently exist in Ro-BatCoV GCCDC1. However, the comparison of the two p10 in the pathogenesis of bat coronavirus need further investigations.

Summarily, the new Ro-BatCoV GCCDC1 virus is not only persistently prevalent in the bat population, but also exhibits continuous evolution. Probability also exists that Ro-BatCoV GCCDC1 might be under diverse selective pressures. The evolving bat coronaviruses could have seized the advantage of conserved mammalian receptors for cellular replication and other biological pathways that go through radiation over a long time which could boost the capability for conveyance of bat-related viruses to other animals (Calisher et al., 2006). Our study benefits the understanding of dynamic virus evolution in special bat population and underlines the surveillance of potential interspecies viruses in bats.

MATERIALS AND METHODS

Sample collection, storage and transportation

Anal swab samples were collected from Rousettus leschenaulti (R. leschenaulti) bats captured on three occasions, May 2014, October 2014 and October 2015, in one cave located in Xishuangbanna, Yunnan Province, China. Rectal swabs were collected from each bat and placed in viral transport medium (VTM) in screw cap tubes, kept on cold ice (at 4°C to 10°C) and transported to the laboratory at local CDC where they were frozen in liquid nitrogen. The VTM containing Earle’s Balanced Salt Solution, 5% bovine albumin, 50,000 μg mL⁻¹ vancomycin, 50,000 μg mL⁻¹ amikacin, 10,000 units mL⁻¹ nystatin as described in our previous study (Huang et al., 2016). The samples were later transported to the laboratory in dry ice and stored at −80°C until further analysis.

RNA extraction

VTM containing swab samples were manipulated by centrifugation at 8,000 r min⁻¹ for 5 min at 4°C using a Thermal centrifuge. Total RNA was extracted from 140 μL of the supernatants using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer’s protocol. The RNA was eluted in 60 μL AVE buffer and stored at −80°C for subsequent use.

Viral detection by nested-PCR

The extracted total RNA was transcribed to cDNA using reverse transcriptase enzyme (Thermo, USA) and then screened for the presence of coronavirus RNA using pan-coronavirus RT-PCR degenerate primers. The primers were designed from a highly conserved RdRp region of coronaviruses (Table 2). Theoretically, this pan-coronavirus RT-PCR primers should amplify a 299 bp fragment of the polymerase gene of all coronaviruses in the first round and 228 bp in a semi-nested PCR for the second round. If the samples were RT-PCR positive to coronavirus screening, specific p10 primers (Table 2) that covered the whole p10 gene were used to screen for the presence of reovirus p10.
gene in Ro-BatCoV GCCDC1. The amplified PCR products were purified following electrophoresis in 1% agarose gels that revealed the expected amplicon in agarose gels.

Virus isolation

RT-PCR positive samples for coronavirus were cultured in human epithelial colorectal adenocarcinoma cells (CaCo-2), human lung carcinoma cells (A549), human epithelial type 2 HeLa derivative cells (HeP-2), baby hamster kidney (BHK-21) and rhesus monkey kidney cells (LLC-mk2). Cells were grown in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) supplemented with 10% or 20% fetal bovine serum (FBS) and incubated at 37°C with 5% CO₂. The cell lines were inoculated with the supernatant of PCR positive samples in 24-well plates, incubated for 2 h for virus adsorption, 300 μL fresh cell culture medium with 2% FBS was added and incubated at 37°C in 5% CO₂ incubator. The plates were observed daily for viral cytopathic effect (CPE).

Table 2 The primers for coronaviridae gene identification and amplification

| Primer name | Target gene | Sequence (5′→3′) | G+C (%) | Tₘ (°C) | Length of the amplification fragments |
|-------------|-------------|-----------------|---------|---------|---------------------------------------|
| RdRp-OF     | RdRp        | TGTTAATTGGAACCAAGCAGAGTAYYGNGNGNTG | 42.1    | 51.3    | OF+OR=299 bp                           |
| RdRp-IF     | RdRp        | GGGTTGTTCTTATGGGTAGGTTGGATAYCCNAARTGYGA | 42.1    | 52.3    | IF+OR=228 bp                           |
| RdRp-OR     | RdRp        | TAGTAGCATCTCGCTGCTAGTNCNCNCNGGYTT | 52.6    | 53.4    |                                       |
| CoV-p10-OF  | p10         | GAAGGGGAGACTAAGAAGA | 42.1    | 50.8    | OF+OR=715 bp                           |
| CoV-p10-OR  | p10         | ACAGGCTTATACTGTAAATG | 35.0    | 49.2    |                                       |
| CoV-p10-IF  | p10         | GCAGATGAGAAGTACAAACCA  | 43.5    | 56.0    | IF+IR=447 bp                           |
| CoV-p10-IR  | p10         | GCAGCAAGCAAGCAGACCA | 55.0    | 57.4    |                                       |

Molecular and sequencing analysis

The used reference sequences were downloaded from GenBank. Multiple sequence alignment of nucleic acid sequences was performed using ClustalW and the Geneious program (http://www.geneious.com). For RdRp and p10 phylogenetic analysis, maximum-likelihood trees were constructed with MEGA 6 (http://www.megasoftware.net), and 1,000 bootstrap replicates were run.

Compliance and ethics  The author(s) declare that they have no conflict of interest.

Acknowledgements  We appreciate the great efforts of Drs. Yongming Zhou, Honghua Wen, Huaxing Liu, who participated in the collection of the bat samples. This work was supported by the National Key Research and Development Program of China (2017YFC1200202), the National Natural Science Foundation of China (81290342, 81461168030), the Major Special Projects for Infectious Disease Research of China (2016ZX10004-222-003), and China National Grand S&T Special Project (2014ZX10004-001-006). Joseph O. Obameso was supported by CAS-TWAS President's Fellowship of the University of Chinese Academy of Sciences (UCAS) and The World Academy of Sciences (TWAS). George F. Gao is a leading principle investigator of the NSFC Innovative Research Group (81621091). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Crameri, G., Hu, Z., Zhang, H., Zhang, J., McEachern, J., Field, H., Daszak, P., Eaton, B.T., Zhang, S., and Wang, L.F. (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science* 310, 676–679.

Li, Z., Liu, D., Ran, X., Liu, C., Guo, D., Hu, X., Tian, J., Zhang, X., Shao, Y., Liu, S., and Qu, L. (2016). Characterization and pathogenicity of a novel mammalian orthoreovirus from wild short-nosed fruit bats. *Infect Genet Evol* 43, 347–353.

López-Roig, M., Bourhy, H., Lavenir, R., and Serra-Cobo, J. (2014). Seroprevalence dynamics of European bat lyssavirus type 1 in a multi-species bat colony. *Viruses* 6, 3386–3399.

Minskaia, E., Hertzig, T., Gorbalenya, A.E., Campanacci, V., Cambillau, C., Canard, B., and Ziebuhr, J. (2006). Discovery of an RNA virus 3′→5′ exoribonuclease that is critically involved in coronavirus RNA synthesis. *Proc Natl Acad Sci USA* 103, 5108–5113.

Sokolova, T.M., Uryvaev, L.V., Selivanova, T.K., Bobrova, O.V., Lebedev, A., and Bystrov, N.S. (1996). Recombination of alpha-viruses during mixed infection in cultured cells. Formation of hybrid forms of RNA of Venezuelan equine encephalomyelitis virus and Karelian fever virus in the region of genes for nucleocapsid and envelope proteins. *Vopr Virusol* 41, 245–252.

Wang, Q., Qi, J., Yuan, Y., Xuan, Y., Han, P., Wan, Y., Ji, W., Li, Y., Wu, Y., Wang, J., Iwamoto, A., Woo, P.C., Yuen, K.Y., Yan, J., Lu, G., and Gao, G.F. (2014). Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. *Cell Host Microbe* 16, 328–337.

Woo, P.C.Y., Lau, S.K.P., Lam, C.S.F., Lau, C.C.Y., Tsang, A.K.L., Lau, J.H.N., Bai, R., Teng, J.L.L., Tsang, C.C.C., Wang, M., Zheng, B.J., Chan, K.H., and Yuen, K.Y. (2012). 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.

Zumla, A., Chan, J.F.W., Azhar, E.I., Hui, D.S.C., and Yuen, K.Y. (2016). Coronaviruses—drug discovery and therapeutic options. *Nat Rev Drug Discov* 15, 327–347.