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For Infection, Genetics and Evolution

Genetic diversity of coronaviruses in bats in Lao PDR and Cambodia

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Highlights

- 1) Coronaviruses detected in bats from Lao PDR and Cambodia.
- 2) High diversity of α CoVs and β CoVs circulating in bats in Cambodia and Lao PDR.
- 3) One strain of β CoV, a new member of the MERS-CoV sister-clade, detected from *Pipistrellus coromandra*.
- 4) A α CoV strain genetically related to PEDV-CoV, detected from *Myotis horsfieldii*.
- 5) CoVs detected for the first time in diverse bats species and genus, *i.e.* *Megaerops niphanae*, *Myotis horsfieldii* and *Macroglossus* sp.

Abstract

South-East Asia is a hot spot for emerging zoonotic diseases, and bats have been recognized as hosts for a large number of zoonotic viruses such as Severe Acute Respiratory Syndrome (SARS), responsible for acute respiratory syndrome outbreaks. Thus, it is important to expand our knowledge of the presence of viruses in bats which could represent a risk to humans. Coronaviruses (CoVs) have been reported in bat species from Thailand, China, Indonesia, Taiwan and the Philippines. However no such work was conducted in Cambodia or Lao PDR. Between 2010 and 2013, 1965 bats were therefore sampled at interfaces with human populations in these two countries. They were tested for the presence of coronavirus by consensus reverse transcription-PCR assay. A total of 93 samples (4.7%) from 17 genera of bats tested positive. Sequence analysis revealed the presence of potentially 37 and 56 coronavirus belonging to alpha-coronavirus (α CoV) and beta-CoV (β CoV), respectively. The β CoVs group is known to include some coronaviruses highly pathogenic to human, such as SARS-CoV and MERS-CoV. All coronavirus sequences generated from frugivorous bats (family *Pteropodidae*) (n=55) clustered with other bat β CoVs of lineage D, whereas one coronavirus from *Pipistrellus coromandra* fell in the lineage C of β CoVs which also includes the MERS-CoV. α CoVs were all detected in various genera of insectivorous bats and clustered with diverse bat α CoV sequences previously published. A closely related strain of PEDV, responsible for severe diarrhea in pigs (PEDV-CoV), was detected in 2 *Myotis* bats. We highlighted the presence and the high diversity of coronaviruses circulating in bats from Cambodia and Lao PDR. Three new bat genera and species were newly identified as host of coronaviruses, namely *Macroglossus* sp., *Megaerops niphanae* and *Myotis horsfieldii*

1. Introduction

Coronaviruses (CoVs) are enveloped, single stranded positive-sense RNA viruses displaying a large genome of 26 kb to 32 kb (Masters, 2006). They are classified within 4 groups based on genotype and serological characteristics: *Alphacoronaviruses* (α CoVs), *Betacoronaviruses* (β CoVs), *Gammacoronaviruses*, and the recently created group of the *Deltacoronaviruses* (Woo *et al.*, 2009, 2012). Their host range is very wide and includes both mammalian and avian species. Coronaviruses can cause acute and chronic respiratory, enteric, neurological and hepatic diseases in their hosts (Weiss and Navas-Martin, 2005). Coronaviruses of animal origin were responsible for the Severe Acute Respiratory Syndrome (SARS) outbreak in 2003-2004, which was associated with deaths in Hong Kong, China, South East Asia and North America (Centers for Disease Control and Prevention (CDC), 2003; Peiris *et al.*, 2003) and the current epidemics of MERS in the Arabian Peninsula (Alsahafi and Cheng, 2016) and Korea (Choi, 2015).

Bats have been identified as natural reservoirs for several zoonotic viruses, such as henipaviruses (Young *et al.*, 1996; Chua *et al.*, 2000; Halpin *et al.*, 2000) lyssaviruses variants (Banyard *et al.*, 2014) and Ebola virus (Leroy *et al.*, 2005, 2009). Bats have been identified as the natural host of the SARS-CoV, (Wang *et al.*, 2006), and recently, the NeoCoV from the clade the Middle Eastern Respiratory Syndrome (MERS-CoV) was detected in a sub-Saharan bat (*Neoromicia capensis*) besides its camel host (Corman *et al.*, 2014). A growing number of coronaviruses have been detected in bats since the SARS-CoV outbreak (Chu *et al.*, 2006, 2008; Lau *et al.*, 2007, 2010, 2012; Watanabe *et al.*, 2010; Gouilh *et al.*, 2011; Tsuda *et al.*, 2012; Wacharapluesadee *et al.*, 2013, 2015; Anindita *et al.*, 2015; Xu *et al.*, 2016, Chen *et al.*, 2016,

Kim *et al.*, 2016) including a high diversity of coronaviruses, recently detected in five Thai provinces neighboring Cambodia (Wacharapluesadee *et al.*, 2013, 2015).

The order Chiroptera represents approximately 20% of all living mammal species (Teeling *et al.*, 2005). Over 25% of the world's bat diversity is found in South-East Asia, established in many natural, urban and suburban environments (Kingston, 2013). Seventy species of bats have been described so far in Cambodia whereas ninety species are known in Lao PDR, including *Yangochiroptera* and *Yinpterochiroptera* (Matveev, 2005; Sarak *et al.*, 2013). Apart from Singapore, bats are hunted for food or preparation of traditional medicines and are found in food markets throughout South-East Asia, despite bats are protected by the law in these countries (Lee *et al.*, 2014; Mildenstein *et al.*, 2016). Bat farms, where artificial roosts are erected to facilitate bat guano harvest to serve as agricultural fertilizer, are becoming common in South-East Asia, including in Cambodia (Thi *et al.*, 2014). In Thailand, coronaviruses belonging to the lineages B and C of betacoronaviruses were detected in bat guano (Gouilh *et al.*, 2011; Wacharapluesadee *et al.*, 2013). Moreover, several studies evidenced the presence of coronavirus belonging to the betacoronavirus group in close areas (He *et al.*, 2014; Anindita *et al.*, 2015; Wacharapluesadee *et al.*, 2015)

Due to evolving land-use such as deforestation, infrastructure development, urban development, and agricultural expansion, bat populations are settling in areas closer to human dwellings (Jung and Threlfall, 2016), increasing the likelihood of contact between bats and humans. Socio-economic-driven changes of the environment are also impacting the bats and thus may affect virus biodiversity (Looi and Chua, 2007; Turmelle and Olival, 2009; Brierley *et al.*,

2016). These factors may also contribute to an increased occurrence of contacts between bats and humans, which in turn, may increase the potential transmission of zoonotic pathogens, including a coronaviruses. Despite the prolificacy of such interfaces in the region, so far no study has been conducted on bat coronaviruses in Cambodia and Lao PDR. To our knowledge, this is the first study to investigate the presence and the diversity of coronaviruses in bats in Cambodia and Lao PDR.

2. Materials and methods

2.1. Ethics. The study was approved by the National Veterinary Research Institute and Forest Administration department of the Ministry of Agriculture Forestry and Fisheries in Cambodia, as well as by the National Animal Health Laboratory of the Ministry of Agriculture Forestry and Fisheries, in Lao PDR, and under the Institutional Animal Care and Use Committee at the University of California, Davis (protocol number: 16048). Since Cambodia and Lao PDR have no ethic committee overseeing animal experimentation, animals were treated in compliance with the guidelines of the American Society of Mammalogists and within the framework of the European Union legislation guidelines (Directive 86/609/EEC).

2.2. Collection of bat samples. Bat samples from various species were collected from different locations in Lao PDR and Cambodia, over a 3-year period (from November 2010 to December 2013). Bat species were identified to genus or species by trained biologists and veterinarians. The sampling was performed in two phases: Phase 1 was carried out in 2010 by the Institut Pasteur in Cambodia (IPC) and the *Muséum national d'Histoire naturelle* (MNHN; Paris, France) in cooperation with Cambodian government partners. Bats were captured and humanely euthanized in full compliance with local ethical and legal guidelines. The capture sites were located in Ratanakiri, Stung Treng and Preah Vihear provinces in Cambodia. They were chosen because of their easier accessibility to the collection team. Rectal swabs were stored in viral transport medium solution (VTM; containing tryptose phosphate Broth 2.95%, 145 mM of NaCl, 5% gelatin, 54 mM Amphotericin B, 106 U of penicillin-streptomycine per liter, 80 mg of gentamycine per liter [Sigma-Aldrich, Irvine, UK]). Tissue specimens (*i.e.* lung, liver, spleen,

kidney, heart) were placed in separate cryotubes. All specimens were immediately transferred into liquid nitrogen containers before being transported to the Institut Pasteur laboratory where they were stored at -80°C prior to testing. The second sampling phase was performed by the Wildlife Conservation Society (WCS) from 2011 to 2013. During this phase 2, no animals were killed and samples were collected at four key interfaces for increased contact (and potential disease transmission) between bats and humans: fresh-food markets; freshly hunted in rural communities for subsistence or sale; wild meat restaurants; and bat guano farms. In Lao PDR the majority of samples were obtained from freshly dead bats found in food markets, with a small number collected from bats captured by subsistence hunters; in Cambodia the majority of samples were collected at wild meat restaurants where bats were butchered, prepared and served, with additional samples obtained from freshly trapped bats (live and dead) held by hunters and middle-men in rural communities, and at a bat guano farm. In this study, sterile swabs were used to collect freshly voided fecal samples from tarpaulins placed under the guano farm roosts. Rectal and oral swabs and tissue samples were also collected from individual animals that had died of natural causes and were found fresh beneath bat guano farm roosts. Oral and rectal swabs were collected opportunistically from fresh dead and live bats at the market, hunting and restaurant locations. Swab and tissue samples were placed in separate cryovials in VTM and immediately stored on liquid nitrogen in dewars for transport to the laboratory where they were stored in -80°C freezers until testing.

2.3. RNA extraction and nested-RT-PCR. Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. Reverse transcription (RT) was performed using SuperScript III (Invitrogen, San Diego, CA). The PCR

mixture (final volume: 25 μ l) contained 2 μ l of cDNA, PCR buffer (50mM Tris-HCl (pH 9.0); 50 mM NaCl; 5mM MgCl₂), 200 μ M (each) deoxynucleoside triphosphates (dNTPs), 20 pmol of each primer targeting the RdRp gene (adapted from Watanabe *et al.*, 2010) (Table 1), and 1U of HOT FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia). The PCR mixture was incubated at 95°C for 12 min, followed by 35 cycles at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and by a final extension at 72°C for 5 min. A nested PCR amplification using forward primer CoV-Fwd2 and the same reverse primer, was performed on 1 μ l of the primary PCR products, using the same amplification conditions. The amplification of sequences specific to CoVs was attested by the visualization of a 440 bp and a 434 bp fragment after the first and second PCR round, respectively. To limit the risk of contamination, RNA extraction, reverse transcription-PCR (RT-PCR), nested-PCR and gel electrophoresis were carried out in separate rooms. In addition, negative controls (water) were included in each run of the nested-RT-PCR assay and results were validated only if these controls tested negative while the positive controls (plasmids prepared by cloning the gene of interest) had to test positive. Amplification of longer fragments of the RdRp gene (i.e. 1370bp) was performed on cDNA of 38 positive samples, by a nested RT-PCR using gene-specific primers designed by multiple alignments of known CoV sequences from the same clusters. Amplified product were sequenced in both directions by direct Sanger sequencing in commercial facilities (Macrogen, Inc., Seoul, Korea). Sequence data were deposited in GenBank and accession numbers ranging from KX284902 to KX520662 and KY010629 to KY010666 are provided in Supplementary Table 1. Since many coronaviruses from different bat genera were detected, and for the sake of clarity, a short name of the strain, the sample code, and the host classification were abbreviated to be used in the sequence nomenclature (Supplementary Table 2). For example, the sequence "Bat coronavirus

512/2005/PREDICT-KHP13-PTR1-0109", corresponding to the Bat coronavirus 512/2005 strain (BatCoV-512), detected in the sample KHP13-PTR1-0109 (P109), from a *Scotophilus kuhlii* (Sku) was abbreviated as: BatCoV-512_P109_Sku. The abbreviations used to code the bat classification, are listed in the Supplementary Table 2.

2.4. Sequence analysis. Sequences were assembled and analyzed using CLC Genomics Workbench version 3.6.1 and BioEdit, version 7.0.9.1. Sequences were aligned with a representative set of CoV sequences retrieved from GenBank using Seaview, version 4.5.4 (Gouy *et al.*, 2010). Phylogenetic trees based on RNA sequences were constructed using the Maximum Likelihood method with the GTR + G+ I model and bootstrap values (BP) were calculated after 1,000 replicates. The best evolutionary model was determined using MEGA version 6.06. Trees displaying protein sequences were constructed using the neighbor joining method and bootstrap values were calculated after 1,000 replicates. Phylogenetic and molecular evolutionary analyses were conducted using Seaview version 4.5.4.

2.5. Geographic data. Land cover data was obtained from GlobeLand30 service operated by the National Geomatics Center of China (NGCC, 2014). Initial data was produced in 2010 with an update in 2014. Images used for GlobeLand30 (GLC30) classification were multispectral images with a 30-meter resolution. Six classes of land cover were considered: cropland, forest, grassland, wetland, water bodies and human settlement area. Land cover structure for each sampling location of sampling is described in Supplementary Table 3. Data mapping was conducted with Quantum GIS, version 2.8.2.

3. Results

3.1. Sampling location, land cover use and hosts. A total of 1965 bats were sampled in 44 locations from nine provinces in Cambodia and eight provinces in Lao PDR (Supplementary Figure 1). The characteristics of the environments around sampling site are described in Supplementary Table 3. Bats originating from 5 districts (*i.e.* Kasi and Vang Vieng districts in Lao PDR and Choam Khsant, Kean Svay, and Moug Russei districts in Cambodia) represented 60% of all the bats collected. Sampled bats were wild, live captured during phase 1 (n=322), where as in phase 2 (n=1643) they were: hunted by villagers for local consumption (n=455); for sale in markets (n=791); being butchered and prepared for sale at wild meat restaurants (n= 392) or on a bat guano farm (n=5) (Supplementary Table 3). Apart from bats trapped during phase 1 or animals sampled on the guano farm (site reference C6) during phase 2, the exact location where the animals were captured remained imprecise. However, the chiroptera were always captured by hunters in areas close to the location where the animals were sampled, *i.e.* mostly in areas at the border of deep forests, in mixed agricultural zones with sparse forests, in suburban zones close to sparse forest, in natural protected forest areas, in places close to water surfaces or in limestone karst areas with mountain forests (Supplementary Figure 1, Supplementary Table 3). Landscape analysis around each sampling location led to a typology comprising four main groups of land cover composition (Supplementary Table 3, Supplementary Figure 1): 1) Deep forest area, well isolated from human settlements (sites: C3, C25, C29, C30, C32, C33, C34, C41, C42 and C43), 2) Isolated pockets of croplands surrounded by forest, very often forest is under fragmentation (sites: C2, C12, C16, C18, C19, C21, C22, C23, C24, C26, C27, C28, C30, C35, C36, C37, C38, C40 and C44), 3) Forest edge, mixed agricultural zones with sparse forests (sites: C5, C7, C13

and C14) and 4) typically agricultural zones, villages and suburban zones, often close to highly fragmented forests (sites: C1, C4, C6, C8, C9, C10, C11 and C20). For sites C1, C4, C6, C8 and C9 a key parameters was the presence of wetlands in the immediate vicinity.

3.2. Detection of coronaviruses. Coronavirus RNA was detected in 93 bats (4.7%) out of 1965 animals tested. Coronavirus RNA was detected in 21 of the 44 sites with detection rates varying depending upon sites (Table 2). However, it is not possible to run an in depth statistical analysis owing to the fact that sampling was not developed for that purpose and data are therefore biased. Detailed data are presented in Supplementary Table 3. Nevertheless, in highly transformed areas (mostly croplands and urbanized areas), bat biodiversity was lower while the detection rate of coronavirus was higher (Table 2, Supplementary Table 3). The phylogenetic analysis based on 320-bp nucleotides of the 93 sample sequences and 34 reference sequences from GenBank indicated that 37 sequences belonged to the α CoV genus while the 56 others fell into the β CoV genus (Figure 1). Out of the 56 bat- β CoVs, 55 belonged to the lineage D. The remaining strain detected (PREDICT-CoV-34_GT1-3_Pisp) belonged to the lineage C which comprises MERS-CoV and MERS-CoV-related viruses. The 55 lineage D bat- β CoVs clustered into four subclusters (D1, D2, D3 and D4) out of the five subclusters identified in this group D (Figure 1). The subcluster D1 (BP=100) comprised 7 different β CoVs, 2 sequences detected in pteropodid bats from Lao PDR and 5 from Cambodia. The subcluster D2 (BP= 80), contained 17 β CoVs detected from the Cambodian provinces of Preah Vihear, Stung Treng and Battambang. The subcluster D3 (BP= 45) contained 24 bat- β CoVs, which were mostly obtained from animals in Lao PDR (n=22) while only 2 were detected in bats from Cambodia. The subcluster D4 comprised the remaining bat- β CoVs (BP=78), detected in bats from Lao PDR only. Bat- α CoVs

were unevenly distributed within three subclusters (Figure 1). The α CoV subcluster 1 included 32 sequences from Cambodian bats. The α CoV subcluster 2 included two α CoVs from Cambodia, while the α CoV subcluster 4 cluster contained one sequence from Cambodia and two from Lao PDR. The same phylogenetic clustering was seen with analyses using the amino-acid sequences. A similar topology was obtained when analyzing the protein sequence (Supplementary Figure 2). The phylogenetic analysis based on 38 longer sequences of 1370 bp (Figure 2) and the corresponding 454 amino acid sequence (Supplementary Figure 3) confirmed the tree topology described on shorter sequences in Figure 1 and Supplementary Figure 2, respectively. Coronavirus RNA was detected in bats belonged to 10 distinct genera. The proportion of CoVs-positive bats significantly varied depending upon the bat genus (Pearson's Chi², $p < 0.0001$). The highest detection rates were found for the long-tongue fruit bats (*Macroglossus* sp.: 20%), the nectar cave bat (*Eonycteris spelaea*: 11.2%) and the lesser Asian bat (*Scotophilus* sp.: 9.3%). The 37 α CoV-positive bats belonged to five genera of the *Yangochiroptera* suborder, i.e. *Rhinolophus* (n=1), *Myotis* (n=2), *Scotophilus* (n=31), *Pipistrellus* (n=1) and *Hipposideros* (n=2). A total of 56 animal tested positive for a β CoV, out of which 55 belonged to five genera of *Yinpterochiroptera* suborder, family *Pteropodidae*: i.e. *Rousettus* (n=25), *Cynopterus* (n=15), *Eonycteris* (n=10), *Macroglossus* (n=4), *Megaerops* (n=1) whereas the bat infected by the PREDICT-CoV-34 strain was a *Yangochiroptera* of the genus *Pipistrellus* (Figure 2). All α CoV RNA were detected in insectivorous bats while most of the β CoV RNA were detected in fruit bats.

3.3. Phylogenetic clustering of CoVs according to host and location. 1965 bats were from 17 different genera and 5 families. Eighty percent of the samples belonged to two bat families only, *Vespertilionidae* (n=456) and *Pteropodidae* (n=1124). In Lao PDR, 95.7% of the samples

belonged to the *Yinpterochiroptera* suborder, including families *Pteropodidae* (78.1%), *Hipposideridae* (6.3%), and *Rhinolophidae* (11.3%) while only 4.3% were *Yangochiroptera* suborder from the family *Vespertilionidae*. In Cambodia, 35% of the bats collected belonged to suborder *Yinpterochiroptera*, and suborder *Yangochiroptera* accounted for the remainder of the collected samples (75%), including families *Emballuronidae* and *Vespertilionidae*. Only sequences from fruit bats from Cambodia and Lao PDR from the genera *Rousettus*, *Eonycteris*, *Macroglossus*, *Cynopterus* and *Megaerops* were found in the lineage D of β CoV. Subcluster D1 contained two sequences from *Macroglossus* sp. from Cambodia (PREDICT-CoV-22 strains) and five virus sequences from *Eonycteris spelaea* from Lao PDR and Cambodia (PREDICT-CoV-22, R91, R77, R74, R58). Seventeen sequences were found in *Cynopterus* (PREDICT-CoV-24 strains, R96, R75, R72, R65, R59, R71), *Rousettus* sp. and *Megaerops niphanae* (PREDICT-CoV-24) collected in Preah Vihear and Battambang in 2010 and 2013, respectively. They fell into subcluster β CoV-D2 and displayed 98% to 100% of amino acid similarity with CoV sequences previously detected in *Cynopterus sphinx* and *Hipposideros lekaguli* in South East Thailand and China, respectively (Wacharapluesadee *et al.*, 2015; Xu *et al.*, 2016). Similarly, the β CoV-D3 subcluster comprised 24 sequences found in *Rousettus*, *Eonycteris* and *Macroglossus* and CoVs previously identified in pteropodids from Hong Kong, Kenya, Thailand and Indonesia (genera *Rousettus* and *Dobsonia*) (Lau *et al.*, 2010; Tong *et al.*, 2009; Wacharapluesadee *et al.*, 2015; Anindita *et al.*, 2015). They displayed 96.9% to 100% of amino acid identity. Seven sequences detected in *Rousettus* bats from Lao PDR and displaying 100% of amino acid identity, formed their own branch in subcluster D4. Additionally, the PREDICT-CoV-34 strain, detected in *Pipistrellus coromandra* fell into lineage C of β CoV (BP=100) which contained sequence from MERS-CoV as well as sequences detected in *Pipistrellus* from Hong Kong, *Neoromicia* from

South Africa and *Myotis* from China (Annan *et al.*, 2013; Woo *et al.*, 2006; Corman *et al.*, 2013, 2015; Xu *et al.*, 2016). The PREDICT-CoV-34 strain showed the highest similarity to the bat coronavirus isolate JPDB144 recently described in *Myotis daubentonii* in China (89.5% nucleic acid identity and 95 % amino acid identity) (Xu *et al.*, 2016). Sequences from the subcluster 1 displayed similar traits and were only detected in Cambodian *Scotophilus* (n=31), with the exception of one sequence detected in Cambodian *Pipistrellus* (BatCoV512_SL2-9_Pisp). The α CoV subcluster 4 contained sequences related to the strain HKU10, detected in various bat genera in Thailand and Hong Kong (Gouilh *et al.*, 2011; Lau *et al.*, 2012; Wacharapluesadee *et al.*, 2015). In this subcluster, two sequences found in *Hipposideros larvatus* (PREDICT-CoV-53 strains) from Lao PDR formed their own branch (subcluster α CoV_4c), supported by a bootstrap of 100. They were related to the α CoV BatCoV Ratcha-67 (96.9% amino acid identity) previously detected in the same bat genus in Thailand (Gouilh *et al.*, 2011). Additionally, the sequence BatCoV-25 PA201 detected in *Rhinolophus shameli*, showed the highest similarity with a sequence detected in the same host species in Thailand (BatCoV B554) (Wacharapluesadee *et al.*, 2015). Interestingly, two α CoV sequences detected from *Myotis horsfieldii* in Cambodia, PEDV_Kch1-04 and PEDV_Kch1-13, fell with the CoV responsible for porcine epidemic diarrhea episodes in pigs (PEDV), in the α CoV subcluster 2. They displayed 93.4% to 97.2% nucleotide identity and 96.13% to 100% amino acid identity with the porcine CoV strain PEDV. All sequences of viruses identified in *Scotophilus* fell in the subcluster 1 of α CoVs, those from *Hipposideros* belonged to the subcluster α CoV_4c, the sequences found in *Myotis* were all from the subcluster 2 of α CoV and the sequences of the viruses identified in *Rousettus* were all grouped in the lineage β CoV_D. In *Pipistrellus* bats from Mondulkiri province caught in 2013, the viruses detected belonged to both the α CoV and the β CoV groups.

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4. Discussion

In this study, we report for the first time, the detection and description of coronaviruses in chiroptera from Cambodia and Lao PDR. This report is of importance for public health as these countries host a high biodiversity of bats and the interface at which humans and chiroptera are in close proximity. Bats are used for food and reared as a source of guano. Searching for bat-borne viruses known to be potentially pathogenic for humans such as coronaviruses is therefore of high importance. A significant diversity of CoVs was found in pteropodids both in Cambodia and Lao PDR. Overall, CoV detection rates (6.5% in Lao PDR and 4.85% in Cambodia) were in the same range as those found in Thailand (7%) (Wacharapluesadee *et al.*, 2015), but lower than those reported in Hong Kong (12%) or Philippines (29.6%) (Tsuda *et al.*, 2012; Woo *et al.*, 2006). Interestingly, for the first time, coronaviruses were detected in *Megaerops niphanae* and *Myotis horsfieldii*.

In this study, α CoVs were mostly associated with *Yangochiroptera* bats whereas β CoVs were found in *Yinpterochiroptera* bats, with the exception of three viruses, *i.e.*, 2 CoVs from the subcluster 4 of found in hipposiderids, and one β CoV from lineage C, detected in *Pipistrellus*. In Lao PDR, β CoVs from lineage D were all associated with pteropodids whereas α CoVs were detected from hipposiderids. In Cambodia, an association between host and virus clade was observed between bats from the genus *Myotis* and the α CoVs genetically related to PEDV strains. These results are in line with conclusions of previous studies: all CoVs detected in bats belonging to the *Myotis* genus were always α CoVs (Tang *et al.*, 2006; Woo *et al.*, 2006; Dominguez *et al.*, 2007; Gloza-Rausch *et al.*, 2008; Osborne *et al.*, 2011; August *et al.*, 2012;

Kemensi *et al.*, 2014; Fischer *et al.*, 2016). β CoVs from lineage D have frequently been found in frugivorous bat species from Madagascar, Kenya, Thailand, and Hong Kong (Woo *et al.*, 2007; Anindita *et al.*, 2015; Razanajatovo *et al.*, 2015; Wacharapluesadee *et al.*, 2015; Xu *et al.*, 2016). A similar trend was observed in this study, as β CoVs from the lineage D were only affiliated with frugivorous bats.

Although only part of the samples could yield a longer sequence, phylogeny based on these sequences was similar to that based on short sequences. This suggests that the phylogeny based on short sequences is reliable. Unfortunately sequencing of coronavirus can be challenging, due in part, to limited nucleic acid in field samples, as well as the high genetic diversity of the viruses (Drexler *et al.*, 2010; King *et al.*, 2012). Although some findings in this study suggest the possibility of host specificity, in particular for the genera *Hipposideros* and *Myotis* (associated to α CoVs from the subclusters 4c and 2 respectively), we also report shared hosts from different families for both α CoV and β CoV. For example, *Pipistrellus* bats from the same location were found to harbor α CoV from the subcluster 1 and lineage C β CoVs sequences. Coronaviruses from the latter lineage, which also includes the highly pathogenic MERS-CoV (Reusken *et al.*, 2016), have been detected in humans, camels, insectivorous bats (*Vespertilionidae*) and frugivorous bats (*Phyllostomidae*) (Reusken *et al.*, 2010; Annan *et al.*, 2013; Wacharapluesadee *et al.*, 2013; Corman *et al.*, 2014; Wang *et al.*, 2014, Munster *et al.*, 2016). There have also been found differences in the percentage of seropositive between *Rhinolophus ferrumequinum* and *Myotis myotis* in studies of lyssavirus of European bat colonies (Serra-Cobo *et al.*, 2013).

Pipistrellus coromandra is a synanthropic bat and the presence of CoVs in the same clade with pathogenic viruses raises the question of the potential risk for human health resulting from deforestation and urbanization that creates habitats for these bats. *Pipistrellus* bats, like *Myotis*, comprise species with differing habitats. However, in this work, *Pipistrellus coromandra* was found in deforested and agricultural regions, confirming thus its synanthropic behavior in the study area considered. Further risk-assessment studies should focus on the correlation between landscape change, land use and deforestation that may affect the distribution of human-dwelling bats and therefore the coronaviruses they harbor. This risk in Cambodia might be worsened by the fact that *Pipistrellus* bats are hunted for food. Another related risk might be the development of guano farms, also associated with agriculture (Stibig *et al.*, 2007; Broadhead and Izquierdo, 2010). Bats reared for guano in Thailand have shown to harbor lineage C β CoV (Wacharapluesadee *et al.*, 2013). This activity is also developing in Cambodia (Thi *et al.*, 2014) and the perception of risk from workers is weak. Furthermore, hunters and restaurant workers at sampling sites described being bitten by bats and were exposed to urine and feces (and in some cases blood). Bats on Cambodian guano farms are wild and free-ranging, with farmers constructing artificial roosts adjacent to their homes in order to attract bats. Farmers, who wear no protective equipment or clothing, collect guano voided onto tarpaulins or nets laid beneath the roosts and are regularly urinated and defecated upon and sometimes bitten. The risk of contamination by direct contact through urine and feces, or aerosols must therefore be considered.

The hypothesis of a potential case of horizontal transmission between livestock and bats can be raised with the PEDV-like viruses, detected in *Myotis horsfieldii*. Another α CoV closely

related to PEDV-CoV was recently detected in Brazil in Mexican free-tailed bats (*Tadarida brasiliensis*) (Simas *et al.*, 2015). Not only is this first report of the presence of this coronavirus in *Myotis horsfieldii*, but the strain detected seems genetically closely related to PEDV strains that infect swine and cattle (Song and Park, 2012). *Myotis* bats also belong to the *Vespertilionidae* family and dwell in dark places including houses, farms and barns. Investigating the presence of pig farms in areas where coronavirus are detected, and the circulation of *Myotis* bats in the surroundings, would provide data to explore potential relations between bats and livestock. The hypothesis of a possible origin of PEDV from bats as well as a potential cross-species transmission has been raised by previous studies (Huang *et al.*, 2013; Tang *et al.*, 2006) and would benefit from further investigation. To date, only a limited number of studies have investigated the capacity of bat coronaviruses to be infectious to other mammals, including humans. This is notably due to a lack of data on the spike protein of these bat viruses. Indeed, the spike protein is the primary determinant for the cell tropism and pathogenesis (Belouzard *et al.*, 2012).

Our study had some limitations related to sampling procedure that may have affected results. Many of the samples (n=1838) were collected from dead bats intended for human consumption. If rectal swab is the sample which represents the highest probability for detecting coronaviruses in bats (Watanabe *et al.*, 2010), it is possible that virus survival in dead animals might be affected. The health status of the animals sampled in our study was unknown but previous studies suggest that bats might not develop disease during coronavirus infections. RNA viruses seem to have little pathogenic effect on bat's life cycle (Li *et al.*, 2005) which may explain that bats are excellent reservoirs for zoonotic viruses including CoVs (Omatsu *et al.*,

2007; Brook and Dobson, 2015; Han *et al.*, 2015). Seasonality has been shown for some zoonotic virus infection rates in Chiroptera. Reproduction periods, female status and resource availabilities have been proven to affect the prevalence in bats infected by other RNA viruses (Middleton *et al.*, 2007; Plowright *et al.*, 2008; Wacharapluesadee *et al.*, 2010; George *et al.*, 2011; Amman *et al.*, 2012; Hayman, 2015; Amengual *et al.*, 2007). Greater shedding over such periods increase the probability of transmission of viruses to humans and the risk of emerging zoonoses (Serra-Cobo *et al.*, 2013). Seasonality was not explored in our study due to the heterogeneity of the sampling session over time, but it would be interesting to include these parameters in further investigations. Another limitation in this work is the lack of molecular identification of bats. The collection of bat samples for barcoding or Cyt identification was not part of the field procedure and thus no bat sample has been collected during the field work. The identification was therefore limited to the genus.

Almost all the bats which tested positive for coronaviruses were meant to be consumed by local human populations. The examples of Ebola or SARS-CoV outbreaks already suggested that wildlife hunting and consumption provided opportunities for human contamination (Bengis *et al.*, 2004 ; Xu *et al.*, 2004; Leroy *et al.*, 2009). In Cambodia and Lao PDR, bat consumption is widespread (Mickleburgh *et al.*, 2009; Lee *et al.*, 2014; Mildenstein *et al.*, 2016). These practices may increase the risk of human exposure to viruses through hosts that may be reservoirs for pathogens. Practices such as hunting, selling or cooking bats might represent efficient interfaces for virus transfer from bats to humans, and therefore need to be further investigated. It demonstrates the importance to develop guidance for rural communities exposed to bats, on how to deal with them and the potential virus threat.

The diversity of bat coronaviruses found circulating in Cambodia and Lao PDR suggests a correlation may exist between coronaviruses and host diversity which is at least for part the consequence of anthropogenic natural environment change, mostly deforestation. The natural landscapes surrounding the collection points correlated with the known biology and ecology of the bats sampled (Supplementary Table 3). Pteropodids and vespertilionids, known to roost in caves (*Eonycteris* or *Rousettus*, and *Ia* respectively), were collected close to karst areas in Lao PDR. In Cambodia, the *Yinpterochiroptera* bats belonged to 5 genera of pteropodids known to mostly live in trees (*Cynopterus*, *Macroglossus*, *Megaerops*, *Pteropus*, *Rousettus*). Forest coverage throughout Continental Southeast Asia has drastically evolved between 1990 and 2015 (Supplementary Figure 4). Cambodia is the country where forest loss has been the most intensive with 20% of the forest surface lost since 1990, while about 50% of the remaining surface is fragmented and 70% of the forest is in a “perforation” state, associated with agricultural development (WWF, 2013; World Bank, 2016). In Lao PDR, the forest coverage looks stable (73% in 1990, 81% in 2015), but the structure of the forest has changed. A typical feature is the fragmentation of the natural forest and its replacement by cultivated areas. However, compared to other countries in the region, the forest coverage remains the highest in Lao PDR and shows signs of resilience. This is mostly due to the geology of the country which is mainly karstic and thus less useful for agriculture. For both countries the demographic growth remains a key point for natural environment evolution, forest loss and dynamic of urban and suburban expansion. This environmental change is affecting the biodiversity of bats and therefore that of their coronaviruses. Continued deforestation, agricultural expansion and suburban growth might facilitate encounters between humans and coronaviruses from human-dwelling bats that could

potentially become harmful for humans, specific attention and studies should therefore be devoted to this aspect.

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Conflict of interest

Philippe Buchy is currently an employee of GSK vaccines.

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7. Figures legends

Figure 1. Phylogenetic analysis of coronavirus sequences based on a 320 nucleotides fragment on the RdRp gene

The tree was constructed using a Maximum Likelihood method with the GTR + G+ I model. Bootstrap values (BP) were calculated after 1,000 replicates. Tree is rooted to an avian coronavirus (FJ376622). The sequences detected in this study are shown with a bullet. Accession numbers related to the sequences are presented in Supplementary Table 1.

Figure 2. Sequence analysis of 1370 nucleic acid fragments of the RdRp gene of coronavirus sequences

The tree was constructed using a Maximum Likelihood method with the GTR + G+ I model. Bootstrap values (BP) were calculated after 1,000 replicates. Tree is rooted to an avian coronavirus (FJ376622). The sequences detected in this study are shown with a bullet. Accession numbers related to the sequences are presented in Supplementary Table 1.

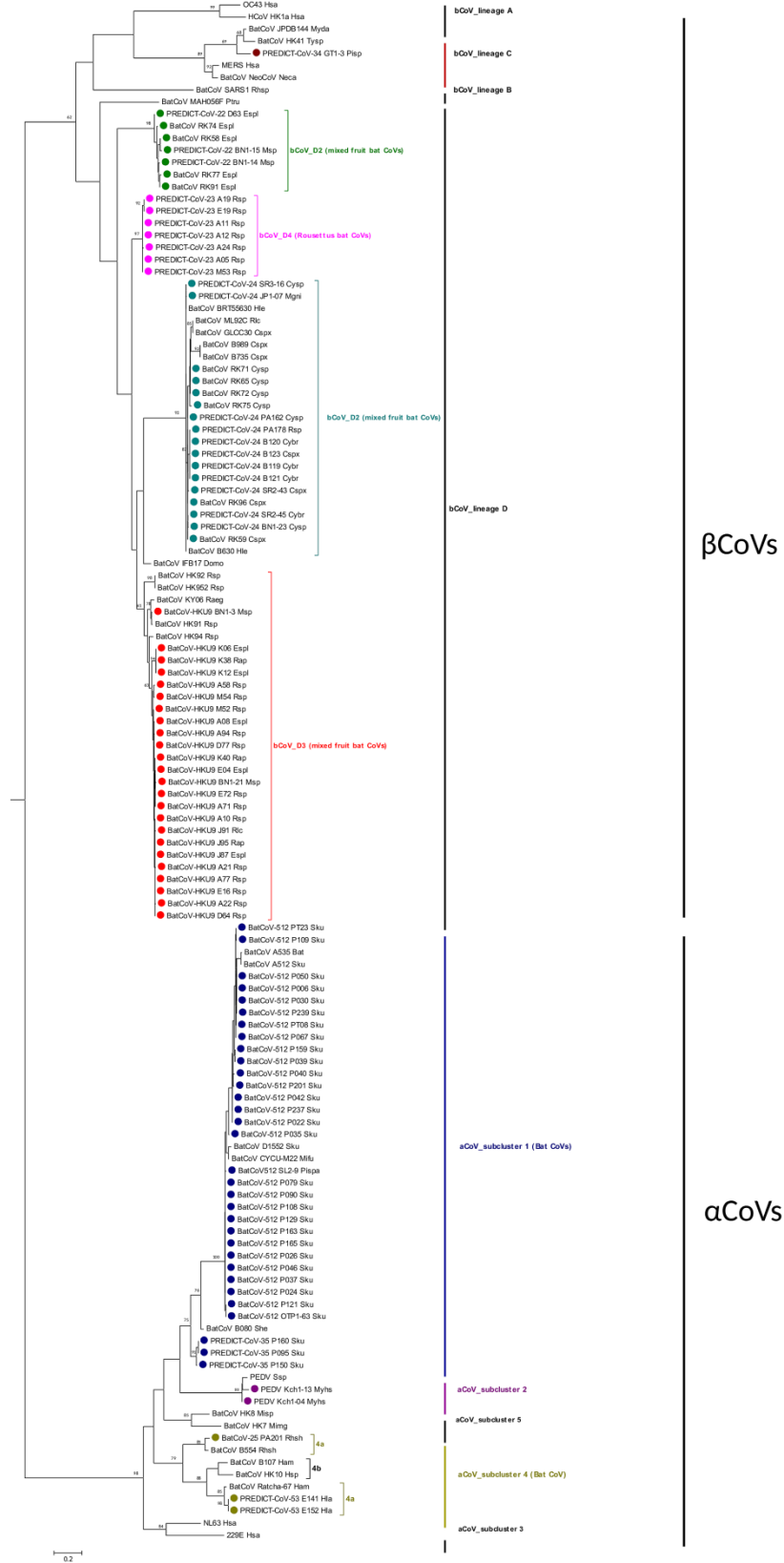


Fig. 1

ACCEPTED MANUSCRIPT

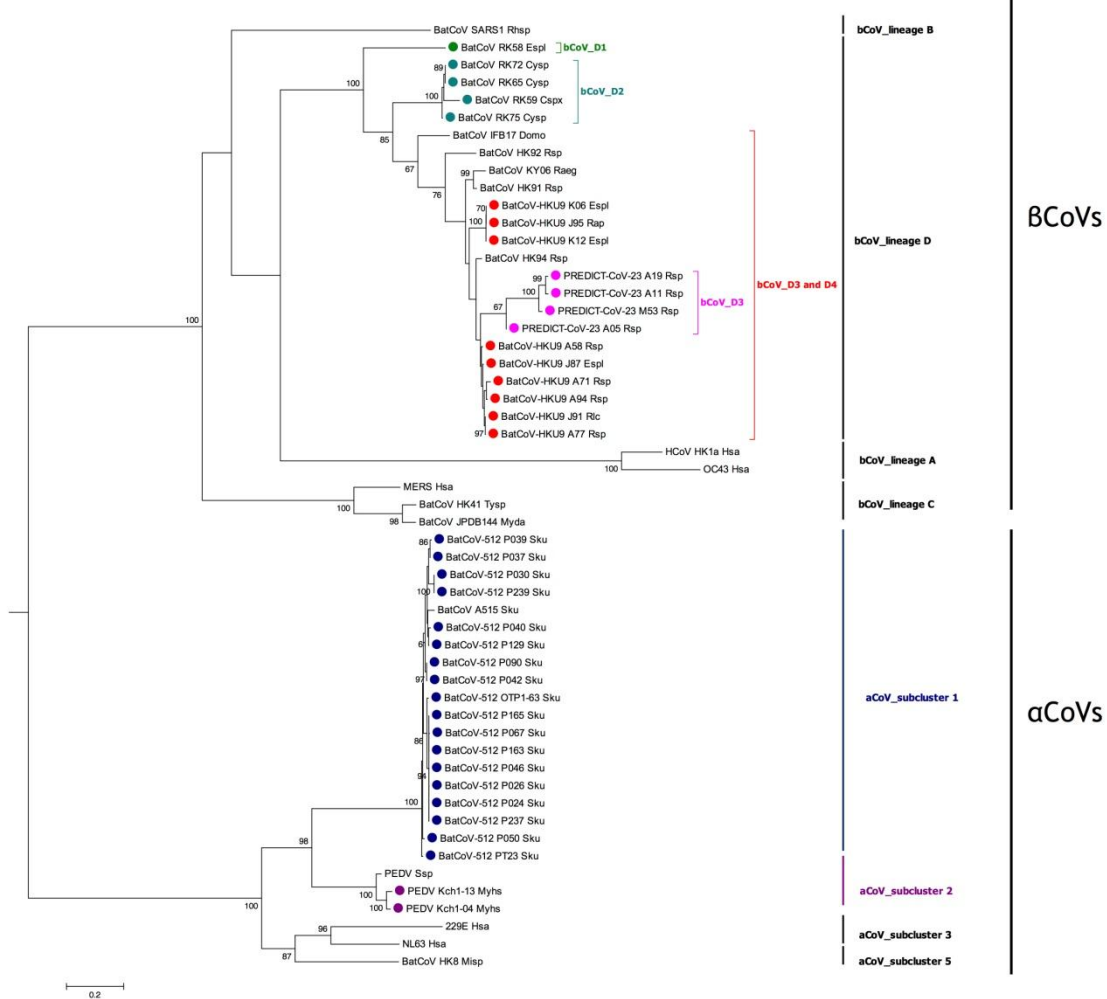


Fig. 2

Table 1: Primers used for the detection of CoVs

Gene	Round	Primer name	Sequence 5'-3'
RdRp	1st	CoV-Fwd1	GGTTGGGAYTAYCCHAARTGTGA
RdRp	1st and 2nd	CoV-Rvs2	CCATCATCASWYRAATCATCATA
RdRp	2nd	CoV-Fwd2	GAYTAYCCHAARTGTGAYAGAGC

Table 2. Detection rates of coronavirus RNA in bats and geographical origin of the positive animals

Bat Site <i>F/Family</i> positive) <i>Genus or Species</i>	Lao PDR		Origin of positive samples* ¹ Cambodia			
	Total No. of samples	No. of samples (no. of positive)	No. of samples (no. of positive)			No. of samples
	No. of samples (percentage (%) of positive)	(no. of positive)	H	W	G	(no. of
<i>Emballonuridae</i>	148 (0)	0 (0)				148 (0)
<i>Taphozous</i> sp. C32, C33	148 (0) 0 (0)		118(0)	30(0)-	-	148(0) C9, C1,
<i>Hipposideridae</i>	62 (3.2)	58 (2)				4 (0)
<i>Aselliscus</i> sp. 7 (0)	7 (0) C25		7(0)	-	-	0 (0)
<i>Hipposideros</i> sp. C31, C33	55 (3.6) 51 (2)			51(2)	4(0) -	4 (0) C19, C14(+), C21, C23, C24
<i>Megadermatidae</i>	21 (0)	0 (0)				21 (0)
<i>Megaderma</i> sp. C33, C41	21 (0) 0 (0)		-	21(0)	-	21 (0) C32,
<i>Pteropodidae</i>	1124 (4.9)	708 (30)				416 (25)
<i>Cynopterus</i> sp. C5(+), C7, C10(+), C12(+), C11,	341 (4.4) 23 (0)			183(8)	158(7) -	318 (15) C21, C23, C24, C26, C27,
C30, C32(+), C34, C35, C36,		C28				C29,
C38(+), C39(+), C40,						C37(+),
C42, C44						C41,

<i>Eonycteris spelaea</i>	89 (11.2)		61(6)	28(4)	-	28 (4)
C37(+), C38(+), C39(+), C40	61 (6)		16, C21(+), C23(+), C24(+)			
<i>Macroglossus</i> sp.	28 (14.3)		28(4)	-	-	21 (4)
C7, C10(+)	7 (0)	C21, C24				
<i>Megaerops niphanae</i>	130 (0.8)		122(1)	8(0)	-	16 (1)
C5, C7, C29, C30, C34, C39	114 (0)		C13, C14, C15, C16, C20			
<i>Megaerops</i> sp.	12 (0)	C21 C23, C24, C26, C27	-	12(0)	-	12 (0)
	0 (0)					C36, C37
<i>Pteropus</i> sp.	10 (0)		10(0)	-	-	10 (0)
	0 (0)					C11
<i>Rousettus</i> sp.	514 (4.9)		506(24)	8(1)	-	11(1)
C10, C11, C33(+), C34, C37	503 (24)		C14, C15(+), C16(+), C17,			
		C20, C21(+), C22, C23(+),				
		C24(+)				
Rhinolophidae	154 (0.7)					52 (1)
	102 (0)					
<i>Rhinolophus</i> sp.	154(0.7)		102(0)	52(1)	-	52(1)
C31, C33(+), C37, C41, C42, C43	102(0)		C14, C21, C23, C25			
Vespertilionidae	456 (7.7)					418 (35)
	38 (0)					
<i>Harpiocephalus</i> sp.	1 (0)		1(0)	-	-	0 (0)
	1 (0)	C20				
<i>Ia io</i>	32 (0)		32(0)	-	-	0 (0)
	32 (0)	C18				
<i>Myotis horsfieldii</i>	50 (4)		50(2)	-	-	50 (2)
C4(+)	0 (0)					
<i>Myotis ricketti</i>	5 (0)		5(0)	-	-	0 (0)
	5 (0)	C20				
<i>Pipistrellus coromandra</i>	29 (6.9)		29(2)	-	-	29 (2)
C2(+), C3(+)	0 (0)					

<i>Scotophilus</i> sp.	338 (9.3)		333(30)	-	5(1)	338 (31)
C1(+), C6(+), C8, C9(+)		0 (0)				
<i>Tylonycteris</i> sp.	1 (0)		-	1(0)	-	1 (0)
	0 (0)					C35
Total	1965 (4.7)		1638(79)	322(13)	5(1)	1059
(61)		906 (32)				

Bat families are shown in bold

Sites where bats tested positive for coronavirus are in bold and marked with (+)

*¹ H: Hunted bats, sold in markets of restaurants; W: wild bats, caught in their natural environment; G: bats collected in bat guano farms

Graphical abstract

