Coronaviruses in bats from Mexico

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Abstract:

Bats are reservoirs for a wide range of human pathogens including Nipah, Hendra, rabies, Ebola, Marburg and SARS coronavirus. The recent implication of a novel β-coronavirus (CoV) as the cause of fatal respiratory disease in the Middle East emphasizes the importance of surveillance for CoVs that have potential to move from bats into the human population. In a screen of 606 bats from 42 different species in Campeche, Chiapas and Mexico City we identified 13 distinct CoVs. Nine were α-CoVs; four were β-CoVs. Twelve were novel. Analyses of these viruses in the context of their hosts and ecological habitat indicated that host species is a strong selective driver in CoV evolution, even in allopatric populations separated by significant geographical distance; and that a single species/genus of bat can contain multiple CoVs. A β-CoV with 96.5% amino acid identity to the β-CoV associated with human disease in the Middle East was found in a Nyctinomops laticaudatus bat, suggesting that efforts to identify the viral reservoir should include surveillance of the families Molossidae/Vespertilionidae, or the closely related Nycteridae/Emballonuridae. While it is important to investigate unknown viral diversity in bats, it is also important to remember that the majority of viruses they carry will not pose any clinical risk, and bats should not be ubiquitously stigmatized as significant threats to public health.
Introduction:

Coronaviruses (subfamily: Coronavirinae) are enveloped, single-stranded positive sense RNA viruses with spherical virions of 120-160 nm (King et al., 2012). They are among the largest RNA viruses, with complex polyadenylated genomes of 26-32 kb, and are divided into four genera; alpha (α) and beta (β) coronaviruses (infecting mainly mammals), and gamma (γ) and delta (δ) coronaviruses (infecting mainly birds) (King et al., 2012; Woo et al., 2012). Infection with coronaviruses (CoVs) is often asymptomatic, however they can be responsible for a range of respiratory and enteric diseases of medical and veterinary importance. Chief among these is the SARS-CoV, which caused a pandemic in 2002-2003. This outbreak lasted for eight months, infected 8,096 people and resulted in 774 deaths (WHO, 2004). Since then, a renewed public health interest in these viruses has been stimulated by the emergence of a novel β-CoV in nine people from the Middle East. In these cases the patients suffered from acute, serious respiratory illness, presenting with fever, cough, shortness of breath, and difficulty breathing (Zaki et al., 2012). Five cases later died. It is currently unclear where this particular virus came from, though genomic analyses have shown similarity to bat coronaviruses (Zaki et al., 2012). Given that the majority of emerging pathogens are known to originate in animals (Jones et al., 2008), the concern is that this current outbreak may represent a further example of zoonotic transmission from wildlife to people, though further ecological, immunological and evolutionary information is still required to confirm this.

Knowledge about CoV diversity has increased significantly since the SARS pandemic, with the description of several novel viruses from a wide range of
mammalian and avian hosts (Cavanagh, 2005; Chu et al., 2011; Dong et al., 2007; Felippe et al., 2010; Guan et al., 2003; Jackwood et al., 2012; Lau et al., 2012b; Woo et al., 2009a; Woo et al., 2009b; Woo et al., 2012). Bats in particular seem to be important reservoirs for CoVs, and discovery efforts have been increasingly focused on them since the recognition of SARS-like CoVs in rhinolophid species (Lau et al., 2005; Li et al., 2005) and because bats appear to be reservoirs for a large number of other viruses (Calisher et al., 2006; Drexler et al., 2012; Jia et al., 2003; Leroy et al., 2005; Rahman et al., 2010; Towner et al., 2007). Several of the novel CoVs described in the last decade were identified in bats of various species and demonstrate a strong association between bats and CoVs. (August et al., 2012; Carrington et al., 2008; Chu et al., 2009; Dominguez et al., 2007; Drexler et al., 2011; Drexler et al., 2010; Falcon et al., 2011; Ge et al., 2012b; Gloza-Rausch et al., 2008; Li et al., 2005; Misra et al., 2009; Osborne et al., 2011; Pfefferle et al., 2009; Quan et al., 2010; Reusken et al., 2010; Tong et al., 2009b; Woo et al., 2006; Yuan et al., 2010).

The large number of CoVs that continue to be described in bats suggest that many (if not most) bat species might be associated with at least one CoV. Given that there are ~1200 extant bat species known, the existence of an equally large diversity of CoVs must be considered likely. Initially, most discovery effort was targeted towards bats from China (Ge et al., 2012a; Lau et al., 2010b; Li et al., 2005; Tang et al., 2006; Woo et al., 2006), followed by limited surveillance in other Southeast Asian countries including Japan, the Philippines and Thailand (Gouilh et al., 2011; Shirato et al., 2012; Watanabe et al., 2010). In the Old World, novel CoVs have been found in both Europe and Africa (August et al., 2012; Drexler et al., 2011; Drexler et al., 2010; Gloza-
In contrast, very few investigations have been conducted in the New World and little is known about the diversity of CoVs found here. Dominguez et al. (2007) were the first to test bats in the New World for CoV, followed by Donaldson et al. (2010) and Osborne et al. (2011). These groups tested bats captured in Colorado and Maryland and found α-CoVs from five different species of evening bats (*Eptesicus fuscus, Myotis evotis, Myotis lucifugus, Myotis occultus* and *Myotis volans*). All were unique compared to CoVs found in Asia. Misra et al. (2009) then tested *Myotis lucifugus* samples from Canada and detected a similar α-CoV to those found in myotis bats from Colorado. In South America, Carrington et al. (2008) identified an α-CoV in two species of leaf-nosed bats, *Carollia perspicillata* and *Glossophaga soricina*, which clustered most closely with CoVs from North American and European bats.

Nothing is known about the diversity of CoVs in Mexico. Many of the bats studied in Canada and the U.S are also found in Mexico, yet it is unknown whether similar viruses are found here. It is also unknown whether β-CoVs exist in the Americas, or whether the α-CoVs predominate. This is a substantive gap in our knowledge of CoV ecology because one-third of all bat species (and 75% of all known bat genera) are found in the neotropics, which includes southern Mexico (Osborne et al., 2011; Wilson & Reeder, 2005). It seems probable that the high ecological, trophic and taxonomic diversity found in Neotropical and Nearctic bats in Mexico (Arita & Ortega, 1998) would be matched by an equally diverse population of novel CoVs. In this study we examined 42 species of bats using broadly reactive consensus PCR for
the discovery of novel CoVs, and found an additional 13 viral lineages/clades, clustering in both the α-CoV and β-CoV genera. Phylogenetic analysis of these new viruses has provided insight into the molecular epidemiology of CoVs, and shows that host speciation is a significant driver in CoV evolution.

Results and Discussion:

The goal of this study was to increase our knowledge of CoV diversity in bats from southern Mexico. Three sites were included in the study: Campeche, Chiapas and Mexico City (Mexico Distrito Federal; D.F.). At two of the sites (Chiapas and Campeche) bats were captured in disturbed (D) and undisturbed (UD) habitat to investigate how anthropogenic activity may affect host and viral diversity. Such habitat gradients do not exist in D.F, which is a highly urbanized site. A total of 1046 samples were collected from 606 individuals, of 42 different bat species (Table 1).

Host (Bat) Diversity:

Host diversity was examined at all sites. In Chiapas, a species richness of 32 was recorded, and the calculated Shannon-Wiener diversity index (H') was 2.81 (Table 2). A comparison of UD and D habitat in Chiapas (t-test) revealed no significant difference in richness and diversity ($p=0.11$). In Campeche the overall species richness was 16 and the diversity index H' = 2.167 (Table 2). Again, no significant difference in host diversity was seen between the UD and D habitats ($p=0.44$). Previous work has shown that bat diversity often reflects the level of disturbance for a given habitat, with lower
diversity recorded in disturbed areas (Medellín et al., 2000). No such distinction was observed here between D and UD sites. This may reflect the dominance of bats from the genera *Artibeus* and *Carollia* (Table 1), both of which contain species that are known to be more adaptable and resistant to the effects of habitat fragmentation (Medellín et al., 2000). An increased sampling effort including larger spatial and temporal scales will be needed to assess whether the abundance and richness of less-well represented species alter the overall bat diversity in each fragment. In D.F. (Mexico City), eight species were captured and the diversity index $H'=1.69$. Sampling effort was not consistent among the three sites, precluding any direct comparisons of diversity between Chiapas, Campeche and D.F.

*Coronavirus Diversity:*

Broadly reactive consensus PCR revealed CoV sequences in 32/606 (5.3%) bats (Table 1). Sequence analyses indicated high phylogenetic diversity and the presence of 13 distinct clades at the nucleotide (NT) level (Figure 2). Clades 5a/5b and 11a/11b had high NT sequence identity and collapsed into a single group when analysed at the amino acid level (not shown). Nine of the viruses clustered with known α-CoVs, and four clustered with β-CoVs (Figure 2). One of the α-CoVs (Mex_Cov-6) was closely related to a virus identified previously in an *Eptesicus fuscus* bat, sampled on the Appalachian Trail in Maryland, USA (Donaldson et al., 2010). We therefore extend the known geographical range of this virus to southeastern Mexico and present the discovery of a further 12 novel CoVs.
Prior to this study, very little was known about the diversity of CoVs in the Neotropics, despite the high diversity of bat species found here (Wilson & Reeder, 2005). Here we demonstrate that several additional viruses from both the *Alphacoronavirus* and *Betacoronavirus* genera exist in Mexico. This particular study was limited to the analysis of a 329bp fragment of the RNA-dependent RNA polymerase (RdRp), however it was sufficient for the identification of these novel strains and therefore satisfied our primary goal of discovery.

CoV-positive sample types included 27 rectal swabs, 4 oral swabs and 1 blood sample (annotated on Figure 2). The high number of positive rectal samples agrees with previous studies, which showed CoV detection in bats to be almost exclusively restricted to faeces (Lau et al., 2005; Li et al., 2005; Pfefferle et al., 2009; Tang et al., 2006). Detection in oral swabs has also been demonstrated, but much less frequently (Carrington et al., 2008).

Phylogenetic analyses of this short fragment show that CoVs cluster based on the relatedness of host species. Figure 2 shows that all of the α-CoVs detected in phyllostomid bats cluster together; as do all α-CoVs discovered in miniopterid bats. The families *Vespertilionidae* and *Molossidae* are closely related (Agnarsson et al., 2011; Teeling et al., 2005), and viruses from these bats also cluster together, though the additional presence of CoV HKU2 (from a rhinolophid bat, (Woo et al., 2006)) in this group is currently unexplained. In the β-CoV genus a similar pattern is observed. All viruses identified in rhinolophid bats cluster together, as do viruses from the vespertilionid/molossid group, and equally so in the related mormoopid/phyllostomid group. These results suggest purifying selection, which is apparently effective at the
level of host species or genus. For example, the α-CoV Mex_CoV-1 was only found in
Carollia spp. bats, but could be present variably in the species Carollia sowelli or
Carollia perspicillata. The same is true of the β-CoVs Mex_CoV-11a and Mex_CoV-11b,
both of which were only found in Artibeus spp. bats, but which could be present in
either Artibeus lituratus or Artibeus phaeotis. Mex_CoV-6 was found in an Eptesicus sp.
bat and clustered very closely with the previously identified Eptesicus-associated CoV
(accession HQ585086). Finally, the close association of Mex-CoV-7 and 8 with Myotis
velifer and Tadarida brasiliensis respectively, also suggest strong host-specificity.
These results agree with previous studies that show individual CoVs are associated
with a single species or genus, even among co-roosting species - including Miniopterus,
Rousettus, Rhinolophus and Hipposideros bats (Chu et al., 2006; Drexler et al., 2010;
Gouilh et al., 2011; Pfefferle et al., 2009; Quan et al., 2010; Tang et al., 2006).
Phylogenetic association of CoVs with host species/genus is particularly evident
in allopatric populations separated by significant geographical distances; such as
Mex_CoV-6 and the previously identified HQ585086 virus from Maryland, both of
which were found in Eptesicus fuscus and shared a very high sequence identity despite
being separated by >2,500 km. Misra et al (2009) reported a similar observation in
North America, noting highly similar viruses from Myotis spp. bats in Canada and
Colorado. And the same appears to be true in Myotis ricketti in Asia (Tang et al., 2006),
in Chaerephon and Rousettus in Africa (Tong et al., 2009b), and in Nyctalus and Myotis
spp. bats in Europe (August et al., 2012; Drexler et al., 2010; Gloza-Rausch et al., 2008).
In all cases it was concluded that even if populations of these species were thousands
of kilometers apart, highly similar CoVs could be detected. It is important to qualify
that our results are based on a short sequence, and additional studies will be required
to assess whether our observations are consistent when additional sequence from
other genes/proteins is considered.

Mex_CoV-5b was the only virus to be found in two distinct (but related)
genera, having been detected in both *Artibeus* and *Carollia* bats (Figure 2). Such
findings have been reported previously, albeit rarely (Lau *et al.*, 2012a; Osborne *et
al.*, 2011; Tong *et al.*, 2009a), and demonstrate that CoVs can infect individuals from
different genera/suborders. It is interesting to note that this particular bat (*Carollia
sowelli*, PMX-1232) was captured in a disturbed habitat. Increased efforts for viral
discovery in this region will be required to investigate whether disturbed habitats
provide increased risk or opportunity for viruses to spillover into new species, as
previously suggested (Cottontail *et al.*, 2009; Keesing *et al.*, 2010; Suzán G *et al.*,
2012). That said, that the health risk to people likely remains low, and bats should
not be viewed as a liability, especially given the vital ecosystem functions they serve
(Medellín, 2009).

Strong associations of CoVs with host species/genus could prove to be
extremely useful in identifying potential reservoirs for viruses that do spillover into
other species, assuming that an emergent virus still shares sufficient similarity to those
circulating in the original host. A phylogenetic analysis of the new human β -CoV that
recently emerged in Saudi Arabia showed that the virus clusters with viruses from bats
in the vespertilionid/molossid families, and that the closest relative is the Mex_CoV-9
virus that was identified in a *Nyctinomops laticaudatus* bat from this study (Figure 3).
Sequence identity between these two viruses is 86.5% at the nucleotide level, but
96.5% at the amino acid level. When only the 1st and 2nd nucleotide positions are considered, nucleotide identity jumps to 97.1%, demonstrating both that there is strong purifying selection acting on these viruses, and that Mex_CoV-9 and human β-CoV have likely been evolving separately for quite some time. These results do not mean the Saudi Arabian CoV originates in Nyctinomops spp. bat, but do suggest that any search for the original reservoir of this virus should perhaps focus on bats in the molossid/vespertilionid families, or the related nycterid/emballonurid families.

Artibeus was the only genus shown to contain more than one CoV, with the detection of the α-CoVs Mex_CoV-5a and 5b, Mex_CoV-4, and the β-CoVs Mex_CoV-11a/b. However, bats in this genus were also the most frequently sampled (Table 1), which likely explains why more viruses were identified. Other studies have also identified multiple CoVs within a single species, including Rhinolophus sinicus (Yuan et al., 2010) and Rousettus leschenaulti in China (Lau et al., 2010a), and Miniopterus spp. and Rhinolophus spp. from Europe (Drexler et al., 2010); all of which were shown to be doubly and triply infected with different CoVs. Further studies focusing on rarely represented species would be needed to investigate whether population size determines the ability for bats to carry more than one CoV, or whether all genera are capable of supporting multiple CoVs independently of population size. In this study, when multiple CoVs were discovered in a given bat species/genus they were often closely related - for example α-CoVs Mex_CoV-5a/b and β-CoVs Mex_CoV-11a/b. When examined at the amino acid level, these clades collapsed into single groups, yet they maintained separate clades at the NT level, suggesting the contemporary evolution of new strains in these bats. Together, these results highlight the
importance of screening sufficient numbers of individuals per species when attempting to describe viral diversity in a given population/region.

Methods used to assess the diversity of host species were also used to measure CoV diversity at UD and D sites in Chiapas and Campeche. No significant difference was seen in CoV diversity across gradients (Chiapas = \( p = 0.10 \), Campeche = \( p = 0.47 \)), mirroring the non-significant difference also observed in the host (described above).

**Materials and Methods**

**Sites and Sampling:** Bats were captured at three different sites in Mexico, the Reserva de la Biosfera Montes Azules (Chiapas), the Reserva de la Biosfera Calakmul (Campeche), and Mexico City (Distrito Federal, Figure 1). The first two sites, located in southeastern Mexico represent regions of high species diversity, and are characterised by large tracts of continuous primary vegetation, while Mexico City represents a highly urbanized site. In Chiapas and Campeche bats were collected in two landscape gradients, assigned as (1) ‘Undisturbed’ forest (UD), where any sign of human impact is largely absent, and (2) ‘Disturbed’ (D), defined as the transition zone between areas of primary vegetation and agriculture/livestock, and by the presence of urban areas. Landscape units were separated by at least 10 km. Capture effort included two nights of trapping using 5x9m mist nets by roosts or foraging sites. Nets were opened at dusk and remained open for consecutive 4 hours. Identification of animals was made using field guides (Medellín et al., 2008). Oral and rectal swabs, and blood were collected (when possible) from each animal. For blood samples < 10% of
the blood volume was collected and for small bats blood was taken using protocols previously described (Smith et al., 2010). A veterinarian was present for all sampling and all animals were released safely at the site of capture. Samples were collected directly into lysis buffer and preserved at -80 °C until transfer to the Center for Infection and Immunity for CoV screening. Capture and sample collection was approved by the Institutional Animal Care and Use Committee at the University of California, Davis (protocol number: 16048).

**Laboratory Testing:** Total nucleic acid (TNA) was extracted from all samples using the EasyMag® (bioMérieux, Inc) platform, and cDNA synthesis performed using SuperScript® III first strand synthesis supermix (Invitrogen), all according the manufacturer’s instructions. CoV discovery was performed using broadly reactive consensus PCR primers, targeting the RdRp (Quan et al., 2010). PCR products of the expected size were cloned into Strataclone™ PCR cloning vector and sequenced using standard M13R primers. If an individual tested positive for CoV, the species of the bat was secondarily confirmed with genetic barcoding, targeting both COI and Cyt-b mitochondrial genes, as described previously (Townzen et al., 2008).

**Analysis:** Sequences were edited using Geneious Pro (5.6.4). Alignments were constructed using ClustalW, executed through Geneious, and refined manually. Neighbour-Joining (NJ) and Maximum-Likelihood trees were built in Mega (5.0), and bootstrapped using 1000 repetitions. Nucleotide trees that represent a consensus of both methods are presented. Evaluations of host and viral diversity at each site/habitat were made using the Shannon-Wiener diversity index (H’) using the Past 1.81 software. This index takes into account the number of individuals as well as the
number of taxa. A 0 value means that community has only a single taxon. Comparison of the Shannon-Wiener diversities (entropies) were calculated between habitat types for each regions using the Shannon t-test, described by Poole.

References:


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Deltacoronavirus supports bat coronaviruses as the gene source of Alphacoronavirus and Betacoronavirus and avian coronaviruses as the gene source of Gammacoronavirus and Deltacoronavirus. Journal of Virology 86, 3995-4008.


Figure Legends:

Figure 1:
Panel A: Map of sampling sites, a) Distrito Federal, Mexico City, b) Reserva de la Biosfera Montes Azules, Chiapas, c) the Reserva de la Biosfera Calakmul, Campeche.
Panel B: Picture of CoV-positive bat, spp Lonchorrina aurita. This individual (PMX-505) was positive for the novel α-CoV Mex_CoV-3.

Figure 2:
Maximum likelihood tree of a 329 bp fragment of the RdRp from bat CoVs only. Red = α-CoVs, blue = β-CoVs. All 32 positive animals from this study are presented on the tree, and begin with a PMX number that refers to the animal ID. The species of all PMX animals was confirmed by Cytb barcoding. CoVs identified in this study split into 13 clades at the nucleotide level, though Mex_CoV-5a/b and Mex_CoV-11a/b collapse into single clades when analysed at the amino acid level. Each clade is indicated by a blue
circle, and the total number of positive animals for each clade indicated within.

D=Disturbed habitat, UD=Undisturbed habitat, U=Urban habitat.

Figure 3:

Maximum likelihood tree of a 329 bp fragment of the RdRp from all CoVs. Red = α-CoVs, blue = β-CoVs, yellow = uiWW-CoVs. Viruses discovered in this study indicated by blue circles. The 2012 human β-CoV is indicated by an arrow, and clusters most closely to PMX-1247/Nyctinomops laticaudatus.

Table 1:

Summary of all bats captured at each site/fragment. A total of 606 bats were sampled across three sites. Number of CoV PCR positives indicated, together with the number of CoV clades. *= individual PMX-505/Lonchorhina aurita (see image of this animal in Figure 1). All bat species captured are endemic to the Americas.

Table 2:

Evaluations of host and viral diversity at each site/habitat using the Shannon diversity index. This index takes into account the number of individuals as well as the number of taxa. A 0 value means that community has only a single taxon.

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<td><strong>Lonchorhina aurita</strong></td>
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**Mormoopidae**

|              | **Mormops megalohyla**   | Insectivorous | 9            |             | 2             |       | 11    |
|              | **Pteronotus davyi**     | Insectivorous | 1            |             | 1             |       | 2     |
|              | **Pteronotus narinari**  | Insectivorous | 1 (1) [10]   | 1           | 10            | 7     | 19    |

**Molossidae**

|              | **Nyctinomops macrotis** | Insectivorous |             |             | 10            |       | 10    |
|              | **Nyctinomops lactealatus** | Insectivorous | 5 (1) [9]   |             |                |       | 5     |
|              | **Tadarida brasiliensis** | Insectivorous |             |             | 10 (3) [8]    |       | 10    |

**Vespertilionidae**

|              | **Myotis velifer**       | Insectivorous |             |             | 7 (3) [7]     |       | 7     |
|              | **Myotis occultus**      | Insectivorous |             |             | 1             |       | 1     |
|              | **Myotis keayi**         | Insectivorous |             |             | 2             |       | 2     |
|              | **Myotis nigriceps**     | Insectivorous |             |             | 1             |       | 1     |
|              | **Eptesicus fuscus**     | Insectivorous |             |             | 1 (1) [6]     | 2     | 3     |
|              | **Corynorhinus mexicanus** | Insectivorous |             |             | 1             |       | 1     |
|              | **Lasiurus intermedius** | Insectivorous |             |             | 1             |       | 1     |
|              | **Baueraeus dubiaquercus** | Insectivorous |             |             | 17            | 1     | 18    |

**Emballonuridae**

|              | **Rhynchonycteris naso** | Insectivorous |             |             | 1             |       | 1     |
|              | **Saccopteryx bilineata** | Insectivorous |             |             | 1             |       | 1     |

| Total Species/Families | 13 [9, 10, 5b, 11b] | 13 [1, 2, 4, 5b, 11a] | 26 [3, 5a, 11a, 11b] | 22 [1, 6] | 8 [7, 8] | 606 |
| Total Animals Captured | 144 | 96 | 130 | 202 | 34 |

DF: Disturbed to Undisturbed
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