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No Evidence of Coronavirus Infection by Reverse Transcriptase-PCR in Bats in Belgium

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ABSTRACT: No coronavirus was detected by PCR in lung and intestine samples of 100 bats, mostly common pipistrelles (*Pipistrellus pipistrellus*), collected dead between 2008 and 2013 for rabies surveillance in Belgium. The negative results contrast with the high prevalence of coronaviruses detected in fecal pellets from live-captured bats in some European countries.

The Middle East respiratory syndrome (MERS) coronavirus (CoV) is an emerging betacoronavirus causing severe acute respiratory infection in the deep airways and lungs of humans (Milne-Price et al. 2014). The first cases were retrospectively identified in April 2012. Until 9 May 2014, the World Health Organization recorded 536 cases with 145 deaths (27% case-fatality). Patients were often elderly, with weakened immune systems, and in poor health. All cases originated from the Middle East. Sporadic cases continue, and family or hospital clusters occur, but there is no evidence of sustained transmission in humans. Mild or subclinical infections have been reported. The virus has virologic, epidemiologic, and clinical characteristics in common with the severe acute respiratory syndrome (SARS) CoV, which emerged in China in 2002 (Hui et al. 2014).

The epidemiologic pattern of human infections suggests zoonotic infection from an unknown reservoir host. Identification of that host would contribute to reduction of transmission to humans. Information on MERS CoV in animals is scarce. Cross-neutralizing antibodies have been detected in one-humped camels (*Camelus dromedarius*) in Egypt, Oman, and Spain (Perera et al. 2013). The role of Camelidae as a reservoir or intermediary host is unclear.

Bats are increasingly recognized as ancestral hosts of mammalian coronaviruses. SARS CoV has a bat reservoir (Cui et al. 2007), and the closest relative of MERS CoV (VM314/2008 betacoronavirus) was isolated from a *Pipistrellus* sp. bat in the Netherlands (Reusken et al. 2010). The MERS CoV and VM314/2008 share 98% nucleotide identity in an 816-base pair (bp) fragment of the RNA-dependent RNA polymerase (RdRp) gene and homologies in a 131-bp fragment of the spike receptor-binding domain (van Boheemen et al. 2012; Annan et al. 2013). VM314-related viruses were detected in 15% of fecal samples of *Pipistrellus* spp. in Europe, including Ukraine and Romania (Annan et al. 2013). Lau et al. (2013) suggest that MERS CoV has diverged from European bat coronaviruses. Memish et al. (2013) detected a coronavirus with 100% homology in a 181-bp fragment from feces of an Egyptian tomb bat (*Taphozous perforatus*) in Saudi Arabia. Further studies are needed to confirm the phylogenetic relationship between MERS CoV and bat coronaviruses and to determine the pathogenicity of this virus in bat hosts.

In Belgium, carcasses of sick or dead-found bats collected for rabies analysis are stored at the National Reference Centre of Rabies (WIV-ISP, Brussels, Belgium). We examined lungs and intestines from 100 lyssavirus-negative bats for coronaviruses. The bats were collected in 2008 ($n=11$), 2009 ($n=18$), 2010 ($n=41$), 2011 ($n=8$), 2012 ($n=2$), and 2013 ($n=20$). Most bats (80%) were found dead near homes,

sometimes caught or retrieved by house cats; 20% were collected from animal rescue centers. Bats that died in these centers were stored at −20 C prior to transport to the laboratory. Dead-found bats were stored as soon as possible at −80 C in the laboratory. After necropsy, tissue samples were stored at −80 C for ≤4 yr. Samples were submitted to no more than two freeze-thaw cycles prior to analysis. Lungs and intestines were homogenized in β-mercaptoethanol lysis buffer using stainless-steel beads and a tissue homogenizer (Bullet Blender®, Next Advance Bio-Connect, Huissen, the Netherlands). RNA was extracted with QIAmp Viral RNA Minikit (QIAGEN Benelux BV, Antwerp, Belgium) and examined with a specific MERS CoV real-time RT-PCR (Corman et al. 2012) and two pancoronavirus RT-PCRs (Ksiazek et al. 2003; Vijgen et al. 2008). The specific RT-PCR uses primers targeting elements upstream of the E gene of the MERS CoV; the pancoronavirus RT-PCRs use primers targeting a conserved region in the RdRp gene present in all coronaviruses. Presence of the 18s rRNA housekeeping gene was confirmed in all samples to guarantee quality of the material. For the 100 lung samples, all 18s rRNA cycle threshold (Ct) values were below 30 (mean 17.73 [13.28–29.33]), and we included only the 60 intestines for which the 18s rRNA Ct values were below 30 (mean Ct 23.3 [12.18–29.93]). The bats were insectivorous Microchiroptera. We analyzed lungs from all bats (100) and intestines from 40/57 common pipistrelles (*Pipistrellus pipistrellus*), 13/19 Nathusius's pipistrelles (*Pipistrellus nathusii*), 0/3 Daubenton's myotis (*Myotis daubentonii*), 3/5 brown long-eared bats (*Plecotus auritus*), 0/1 noctules (*Nyctalus noctula*), 0/1 common serotines (*Eptesicus serotinus*), and 4/14 undetermined species. The lungs and intestines of all bats were negative by the MERS CoV-specific RT-PCR. Similarly, neither pancoronavirus RT-PCR detected any coronavirus. The pancoronavirus RT-PCR of Vijgen et al. (2008) amplified nonspecific fragments of a size similar to the positive control, especially in lungs. The sequence of these fragments corresponded, however, with the signal recognition particle RNA.

Because we detected no coronavirus in the bats in this study, coronaviruses were likely not a cause of death or severe disease. The limited sample size does not allow strong conclusions regarding presence or absence of MERS-like coronaviruses in Belgian bats. Only six of about 20 Belgian species were available, and because most tissues were collected from bats that were found dead, the quality of the samples is uncertain. Nevertheless, only samples with a strong signal (Ct<30) for the 18s rRNA housekeeping gene were included, suggesting that the results are true negatives. In another study with rabies virus, viral RNA remained more stable than 18s rRNA over time in decomposing tissues (data not shown), indicating that a strong signal for 18s rRNA is a good indicator for test inclusion. Still, passive surveillance in dead-found animals has disadvantages for standardization of sample collection, and it cannot be excluded that viral RNA was compromised by sample handling.

Our results contrast with the 15% coronavirus-positive fecal samples from *Pipistrellus* spp. reported by Annan et al. (2013). In that study, 36.6% of Nathusius's pipistrelles (*Pipistrellus nathusii*) were positive for MERS CoV-related viruses, whereas only 2.4% of common pipistrelles were positive. We tested mainly common pipistrelles, which is one of the most prevalent bat species in Belgium, and individuals typically live near human dwellings. Bats are protected in Europe, and their disturbance should be avoided, but comparison of our results with those of Annan et al. (2013) suggests that fecal swabbing of live-captured animals might be more efficient for coronavirus screening.

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