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Detection of polyoma and corona viruses in bats of Canada

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Several instances of emerging diseases in humans appear to be caused by the spillover of viruses endemic to bats, either directly or through other animal intermediaries. The objective of this study was to detect, identify and characterize viruses in bats in the province of Manitoba and other regions of Canada. Bats were sampled from three sources: live-trapped *Myotis lucifugus* from Manitoba, rabies-negative *Eptesicus fuscus*, *M. lucifugus*, *M. yumanensis*, *M. septentrionalis*, *M. californicus*, *M. evotis*, *Lasionycteris* (*L.*) *noctivagans* and *Lasiurus* (*Las.*) *cinereus*, provided by the Centre of Expertise for Rabies of the Canadian Food Inspection Agency (CFIA), and *L. noctivagans*, *Las. cinereus and Las. borealis* collected from a wind farm in Manitoba. We attempted to isolate viruses from fresh tissue samples taken from trapped bats in cultured cells of bat, primate, rodent, porcine, ovine and avian origin. We also screened bat tissues by PCR using primers designed to amplify nucleic acids from members of certain families of viruses. We detected RNA of a group 1 coronavirus from *M. lucifugus* (3 of 31 animals) and DNA from an as-yet undescribed polyomavirus from female *M. lucifugus* (4 of 31 animals) and *M. californicus* (pooled tissues from two females).

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INTRODUCTION

In a recent article, Jones *et al.* (2008) examined over 330 events of emerging infectious disease in humans over a 60 year period from 1940 to 2004. They concluded that most of the events were zoonotic and that over 70 % of these could be attributed to wildlife. Other studies (Taylor *et al.*, 2001; Woolhouse & Gowtage-Sequeria, 2005) provide similar estimates. Several of the zoonotic viruses that have emerged in various regions of the world in the last two decades are enzootic in bats and are transmitted, albeit infrequently, to humans and domestic animals (reviewed by Calisher *et al.*, 2006; Dobson, 2005; Wong *et al.*, 2007). Economic or sociological conditions that lead to an increase in bat–human contact appear to predispose these cross-species transmissions. In some of the emergent viruses, such as Hendra and Nipah viruses, there is evidence of direct transmission of bat viruses to horses and swine and from them to humans. Nipah virus has also been transmitted directly from bats to humans (Luby *et al.*, 2006) and between humans (Gurley *et al.*, 2007). For other viruses such as severe acute respiratory syndrome (SARS) (Lau *et al.*, 2005), Marburg (Towner *et al.*, 2007) and Ebola (Leroy *et al.*, 2005), viruses similar to those causing disease in people have been isolated from or detected in bats. Melaka, a virus similar to Tioman virus previously isolated from bats (Chua *et al.*, 2007), was recovered from an

The GenBank/EMBL/DDBJ accession number of the complete nucleotide sequence of the Myotis polyomavirus and deduced amino acid sequence is FJ188392.

Three supplementary tables showing primer sequences, background information on bat specimens and GenBank accession numbers of the sequences analysed in this study are available with the online version of this paper.

individual with severe respiratory disease. In addition, bat lyssaviruses in the UK (Nathwani *et al.*, 2003) and Australia (Hanna *et al.*, 2000; Samaratunga *et al.*, 1998), both of which are considered to be free of terrestrial rabies, have caused fatal disease in people.

Increased human development in wilderness areas of Canada and encroachment into bat habitats could lead to similar inter-species transmission of potentially zoonotic viruses that may be present in bats, particularly via species which can roost in human-made structures. Additionally, widespread mortality of the so-called 'migratory tree bats', that has now been documented at industrial-scale wind energy facilities throughout North America (Arnett *et al.*, 2008; Baerwald *et al.*, 2008; Betts, 1998) has created the potential for direct virus transmission between formerly cryptic forest bat species and domestic pets or livestock and indirect transmission to wildlife species which scavenge bat carcasses at wind turbines (e.g. foxes, skunks and crows; Klug & Barclay, 2008). Identification of such viruses and characterization of their biology and epizootiology would be invaluable in anticipating and possibly preventing transmission to humans or, if it occurred, in controlling its spread and impact.

At least 17 species of bats have been detected in Canada. These include little brown bat (*Myotis lucifugus*), Yuma myotis (*M. yumanensis*), long-legged myotis (*M. volans*), fringed myotis (*M. thysanodes*), northern myotis (*M. septentrionalis*), eastern small-footed myotis (*M. leibii*), Keen's myotis (*M. keenii*), long-eared myotis (*M. evotis*), western small-footed myotis (*M. ciliolabrum*), California myotis (*M. californicus*), big brown bat (*Eptesicus fuscus*), silver-haired bat [*Lasionycteris* (*L.*) *noctivagans*], hoary bat [*Lasiurus* (*Las.*) *cinereus*], red bat (*Las. borealis*), tri-coloured bat (formerly the eastern pipstrelle, *Perimyotis subflavus*), spotted bat (*Euderma maculatum*) and Townsend's big-eared bat (*Corynorhinus townsendii*). Of these species, the little brown bat, silver-haired bat, hoary bat, northern myotis, big brown bat and eastern red bat have been observed in the province of Manitoba (details in Bilecki, 2003). During winter in Manitoba, the little and big brown bats and northern myotis bats hibernate, while the three other local species, the 'migratory tree bats' (silver-haired bat, hoary bat and eastern red bat), migrate south. The little brown and northern myotis bats hibernate in caves in the extensive limestone karst in the inter-lake and northern regions of Manitoba. Big brown bat hibernacula have not as-yet been identified. The little brown bats congregate at the caves in mid-August to late September, presumably to mate. Later in the year, they move to the caves where they habitually hibernate during winter. The bats leave the caves in mid-May to early June and females form maternity colonies in tree hollows and other structures, including buildings. Little brown bats, in particular, are common inhabitants of buildings throughout their range and are, therefore, the species most likely to be encountered by humans in Manitoba. In contrast, two of the three migratory tree bats (hoary and eastern red bats) have rarely been encountered by people historically, because they roost exclusively in the foliage of trees (Willis & Brigham, 2005). The third migratory species (the silver-haired bat) is also considered a tree-roosting species (Betts, 1998) but has been more often encountered by people when roosting in deadfall or woodpiles

If bats indeed harbour viruses that have the potential to cause severe disease in humans and other domestic animals, and if climatic and socio-economic changes are likely to lead to conditions that facilitate contact between bats and domestic animals, it might be useful to identify viruses that infect bats. Such surveys have been conducted elsewhere in the world with the identification of several previously unknown bat viruses. Because of the devastating effect of SARS on the economy of several countries, most of these studies have specifically targeted coronaviruses (Carrington *et al.*, 2008; Chu *et al.*, 2006, Dominguez *et al.*, 2007; Gloza-Rausch *et al.*, 2008; Lau *et al.*, 2005; Poon *et al.*, 2005). Our objective in this study was to carry out a preliminary screen of bats in Canada for the presence of viruses of several families. We attempted to isolate viruses from fresh tissue samples taken from trapped bats in cultured cells of bat, primate, rodent, porcine, ovine and avian origin. We also screened nucleic acids purified from these samples by PCR using low-specificity primers designed to amplify nucleic acids from all members of certain families of viruses. We detected RNA related to that of Rocky Mountain bat coronavirus (RMBCV) from little brown bats and DNA of a previously undescribed polyomavirus from female little brown bats and California myotis.

METHODS

Bats. Bats for the study were obtained from three sources.

(i) Little brown bats were trapped using a harp trap set up at a cave in northern Manitoba during a September 2007 mating swarm. Some hibernating bats were also collected from hibernacula in two other caves in May 2008. Bats were euthanized on site by an overdose of inhaled isoflurane (Aerrane) and placed on ice for transport to the National Microbiology Laboratory (NML) in Winnipeg. Bats were processed for virus isolation within 48 h of collection. A Wildlife Scientific Permit (WB 06638) to trap the bats was obtained from the Wildlife and Ecosystem Protection Branch of Manitoba Conservation. The bats were treated in accordance with procedures approved by the Animal Care Committee of the Canadian Science Centre for Human and Animal Health (H-07-001 Rev.1). All individuals handling bats had been immunized against rabies and, according to recent tests, had antibody levels considered protective.

(ii) The Centre of Expertise for Rabies at the Canadian Food Inspection Agency (CFIA) provided bats diagnosed as negative for rabies. These bats had been submitted to the CFIA laboratories in Lethbridge, Alberta and Ottawa, Ontario, and frozen at −20 °C after diagnosis. This group of bats included little and big brown bats, silver-haired bats, hoary bats, Yuma myotis and California myotis.

(iii) Dead bats were collected for a survey of bat mortality at a Manitoba wind farm. These included silver-haired, hoary and red bats.

Sampling tissues. All bats were processed in biosecurity level 3 facilities at either the NML or the CFIA Fallowfield laboratory in Ottawa. Carcasses were sprayed with 70 % ethanol and placed on absorbent paper for 5 min before dissection. One kidney and spleen, and portions of liver, lung, small intestine and brain were removed from each bat. All tissues from each live-trapped bat were pooled. For bats obtained from CFIA and the wind farm, tissues from up to four bats of the same species were pooled. BA [Medium 199 (Gibco), 50 mM Tris/HCl, pH 7.6, 1 % BSA and penicillin–streptomycin (Gibco)] was added to tissues from individual animals (2 ml) and pooled tissues (4 ml) and the tissues were homogenized either in a Polytron homogenizer or by shaking with a sterile ball bearing in a Retsch M300 oscillating homogenizer. The samples were then centrifuged at 16 000 ***g*** for 5 min and the supernatant was transferred to a fresh tube.

Inoculation of cultured cells. Lamb and pig kidney cells, baby hamster kidney cells (BHK-21), chicken fibroblasts and quail fibroblasts (QT-35) were obtained from Marta Sabara (CFIA) and mouse fibroblasts (NIH-3T3), African green monkey (Vero) cells and bat tracheal (BT) cells were obtained from James Strong (Public Health Agency of Canada, NML). All cells were grown in medium normally used to propagate the cells.

Tissue homogenates were diluted 1/10 and 1/100 in BA and 100 µl of undiluted or diluted homogenate was added to cultured cells in 24-well culture dishes. After incubating for 1 h at 37 °C, the inocula were removed and replaced with 1 ml medium containing 5 % fetal bovine serum. Cells were observed for cytopathic effect (CPE) every 2 days. Any morphological differences or differences in the colour of the growth medium from mock-infected cells were considered a CPE. After 3 days of incubation, cultures inoculated with the lowest dilution of homogenate that contained viable cells were dispersed by trypsinization and re-plated. Inoculated cells were passaged in this manner five times and were discarded if no obvious CPE was observed. Samples from supernatants from cultures that showed CPE were transferred to electron microscope grids and screened for virus particles by the NML electron microscopy laboratory.

Extraction of nucleic acids. Total nucleic acids were extracted from 140 µl tissue homogenate using the QIAamp viral RNA mini kit (Qiagen) following the manufacturer's instructions [this kit purifies viral DNA as well as RNA (Allan Grolla, personal communication)]. An additional washing step with buffer AW1 (Qiagen) was included. Before extraction, all samples were seeded with bacteriophage MS2 RNA as an internal control. Nucleic acid samples for PCR for DNA viruses and for the two-step real-time PCR for West Nile virus were used directly. For PCR for RNA viruses, RNA was converted to cDNA using the Qiagen Sensiscript RT kit with random nonamers (Gene Link) as primers. Control RNA from Hendra, Nipah, measles, SARS corona and rabies viruses were obtained from NML laboratories.

PCR. The primer sequences used for PCR and RT-PCR are provided in Supplementary Table S1 (available in JGV Online). PCRs for corona, lyssa, flavi, bunya, Cache Valley and morbilli viruses were performed with ABS AmpliTaq (Applied Biosystems) according to the manufacturer's instructions. The amplification process included holding the samples at 94 °C for 15 min; 18 'touch down' cycles of 94 °C for 15 s, 55–37 °C (with a 1 °C decrease in temperature per cycle) and 72 °C for 30 s; 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s; 72 °C for 7 min. Secondary amplifications for corona, lyssa and morbilli viruses were performed on 1 µl of the primary PCR product using the same amplification conditions. PCR products were analysed by electrophoresis on 2 % agarose gels. In some gels, PCR products from control viral cDNAs were included. PCR products of the expected size were submitted to the NML genomics core facility and sequences were analysed by BLAST to determine if they resembled known viral sequences. Bacteriophage MS2 RNA, seeded into raw samples as a control for RNA extraction, was detected using quantitative RT-PCR (Dreier *et al.*, 2005) by the virology facility at the NML.

Broad spectrum PCR for polyomavirus detected a conserved portion of the VP1 and VP3 genes (Johne *et al.*, 2005). The remainder of the genome of polyomaviruses from three bats was obtained by 'long-range' PCR using primers facing outwards from the initial product of the VP1 gene. The larger PCR product was cloned into pCRTOPO using a PCR cloning kit (Invitrogen). The sequence of the fragment was determined using primers bracketing the cloning site of pCRTOPO and additional primers were designed as sequencing progressed. Sequences were aligned into contigs using DNASTAR and represented complete sequences of both DNA strands. Open reading frames (ORFs) were deduced using MacVector 7. Deduced amino acid sequences of the Myotis polyomavirus genes for Large T-antigen, Small t-antigen, VP1 and VP2,3 were compared with those of other polyomaviruses using the PHYLIP 3.63 package. The complete nucleotide sequence of the Myotis polyomavirus and deduced amino acid sequences of the deduced proteins were submitted to GenBank and assigned the accession number FJ188392.

RESULTS

We attempted to detect viruses in Canadian bats by using two complementary strategies: to isolate virus in a variety of cultured cells and to screen samples by PCR using primers designed to amplify nucleic acids from members of selected families and genera of viruses and from individual viruses. The target viral families and genera were selected either because of a precedent for members spilling over into humans or because the tests were available in our laboratory, thus allowing us to undertake a broad generalized screening. In positive control reactions, the primers detected nucleic acids from representative target viruses.

Live-trapped *M. lucifugus*

We examined 31 live-trapped *M. lucifugus* (Table 1). Twenty-one bats were collected from a mating swarm in September 2007. Of these, thirteen were female and seven were male (one unknown). An additional five bats were collected from each of two cave hibernacula in May 2008. All of these bats were male. Various tissues from individual bats were pooled and attempts were made to isolate virus using cultured cells. However, we observed no virus particles by electron microscopy, even after repeated attempts to detect virus in tissue culture supernatants and concentrated material. We did, however, detect virus infection by PCR analysis of nucleic acids purified from tissue homogenates of seven of the 31 live-trapped *M. lucifugus* examined (Table 1). We determined the nucleotide sequence of the PCR products, which confirmed that four of the viruses were polyomavirus while three were coronavirus. All four bats from which we detected polyomavirus were females.

Bats from the Centre for Expertise in Rabies and bats collected from a wind energy facility

The Centre for Expertise in Rabies provided 84 bat carcasses representing four genera and six species

Table 1. Virus nucleic acids detected in live-trapped *M. lucifugus*

NVD, No virus was detected.

Bat no.	Sex*	Cave†	Virus detected‡
1	F	St G	NVD
2	F	St G	NVD
3	F	St G	NVD
4	U	St G	NVD
5	M	St G	NVD
6	M	St G	NVD
7	F	St G	NVD
8	M	St G	NVD
9	M	St G	NVD
10	F	St G	Polyoma
11	F	St G	Polyoma
12	F	St G	Polyoma
13	F	St G	NVD
14	M	St G	Corona
15	F	St G	NVD
16	F	St G	NVD
17	M	St G	NVD
18	F	St G	NVD
19	F	St G	NVD
20	M	St G	NVD
21	F	St G	Polyoma
22	M	Dale's	NVD
23	M	Dale's	NVD
24	M	Dale's	Corona
25	M	Dale's	NVD
26	M	Dale's	NVD
27	M	Abyss	NVD
28	M	Abyss	NVD
29	M	Abyss	NVD
30	M	Abyss	Corona
31	M	Abyss	NVD

*F, Female; M, male; U, unknown.

†Bats were collected at three caves: St George (St G), Dale's and Abyss. Bats from St George cave were trapped during a mating swarm in September 2007. Bats from Dale's and Abyss caves were collected in May 2008.

‡Polyoma or corona viruses were detected by PCR in tissue lysates of seven of 31 animals.

(Supplementary Table S2, available in JGV Online). These bats were submitted to the Centre for Rabies Testing and were found to be negative for rabies virus. The carcasses, which had been stored frozen at −20 °C, were thawed overnight at room temperature before dissection. We detected polyomavirus in a pool from two female *M. californicus* bats submitted to the Centre from British Columbia. We also obtained 15 bat carcasses representing the three migrating species (*Las. cinereus*, *L. noctivagans* and *Las. borealis*) from a wind plant near Winnipeg. The carcasses were collected in autumn 2007 and frozen at −20 °C until the tissues were analysed by PCR. An additional *Las. cinerus* bat was submitted by a local veterinarian. No viruses were detected in these animals (total of 100).

Myotis polyomavirus

Polyomavirus DNA was detected using primers designed from a conserved portion of the VP1 gene of polyomaviruses. Using primers designed from the nucleotide sequence of the VP1 fragment, we amplified the remainder of the viral genome from three bats. The sequence of the entire genome was determined and putative ORFs for major polyomavirus proteins (VP1, VP2 and 3, Large T antigen and Small t-antigen) were deduced. The derived amino acid sequences for these proteins were compared with those of all other known polyomaviruses. Only VP1 sequences were available for the goose polyomavirus.

Fig. 1 depicts phylogenetic trees of the polyomavirus viral proteins based on a parsimony analysis of the amino acid sequences of the proteins. While there were some variations in the groupings when the sequences of the different proteins were compared, all four proteins of the Myotis bat virus were closely related to those of the mouse pneumotropic (Kilham) and squirrel monkey viruses.

Myotis coronavirus

We amplified a 500 bp segment of RNA from a coronavirus in three *M. lucifugus* bats using primers designed to amplify a portion of the coronavirus RNA polymerase gene. Bat no. 14 was trapped in September 2007, while bats 24 and 30 were collected in May 2008; the three bats were from different caves (Table 1). The sequence of the amplified portion of the coronavirus gene most closely resembled those of the RMBCV sequence detected in *M. occultus* (Dominguez *et al.*, 2007) and *E. fuscus* in Colorado (Dominguez *et al.*, 2007). A comparison of the sequences (Fig. 2) using CLUSTAL_X showed that the sequences of viruses from *M. lucifugus* bats 14 and 24 were very similar. All three sequences were closely related to sequences amplified from *M. occultus* but had several differences from those amplified from *E. fuscus* (Dominguez *et al.*, 2007).

When the 500 bp segment from the Myotis coronavirus was compared with corresponding sequences from coronaviruses detected in bats in North America, Europe and South-East Asia (Fig. 3), the sequences of the *Myotis* coronavirus most closely resembled sequences from Group 1 coronaviruses, including viruses from the other North American bats and Asian bats *Rhinolophus sinicus*, *Myotis ricketti* and *Miniopterus australis* (Woo *et al.*, 2006). In our analysis, the sequences of coronaviruses detected in European bats (Gloza-Rausch *et al.*, 2008) formed a distinct clade within the group 1 coronaviruses.

DISCUSSION

The objective of the project was to screen bats in the province of Manitoba and other regions of Canada for viruses. Most of

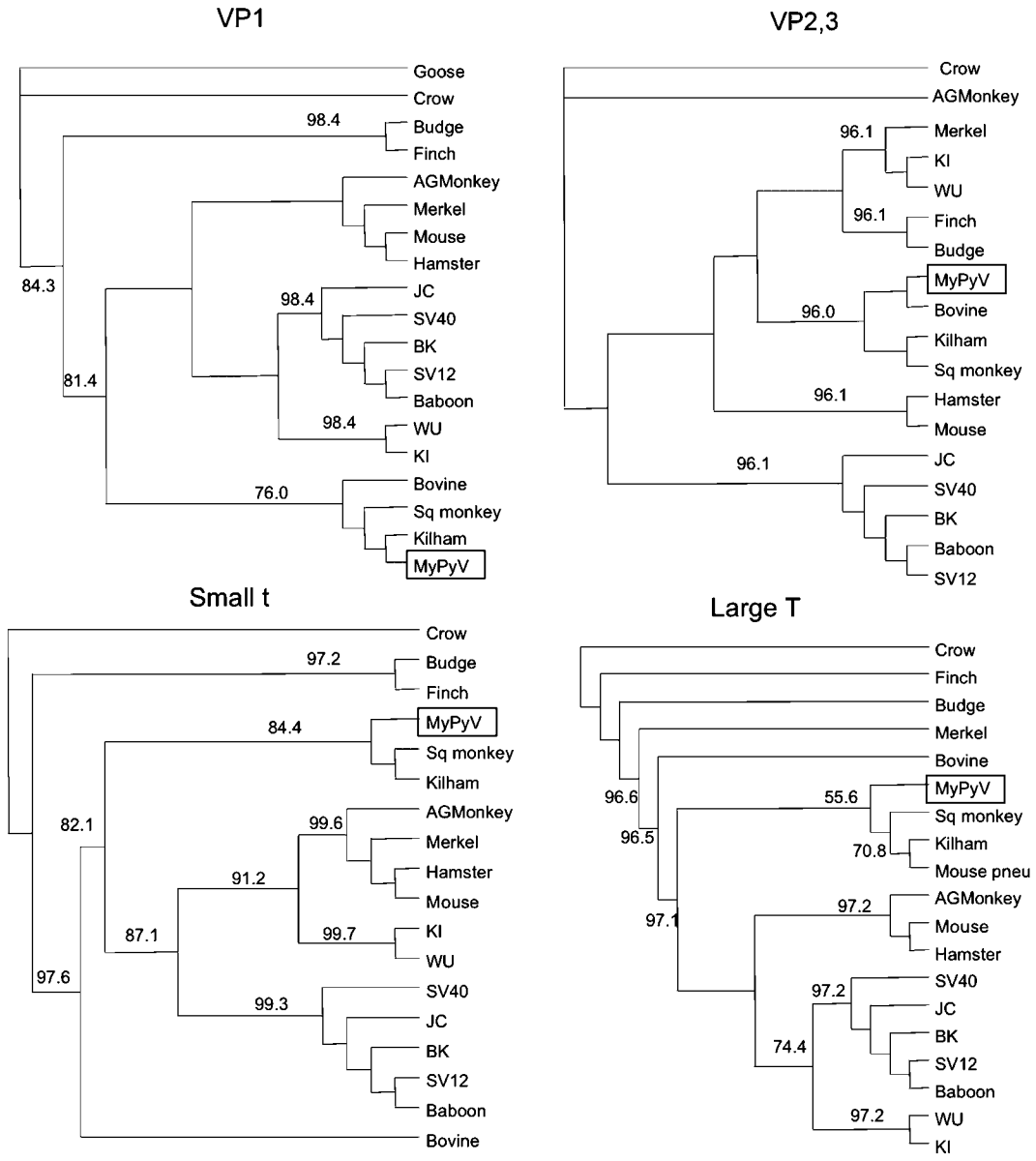

Fig. 1. Phylogenetic relationships between the amino acid sequences of VP1, VP2,3, Large T-Antigen and Small t-antigen of polyomaviruses. The sequences for Myotis polyomavirus (MyPyV, boxed) were derived as described in Methods, the others were obtained from GenBank (see Supplementary Table S3). The trees for each of the four proteins were generated using SEQBOOT, Protpars and CONSENSE. The consensus trees were based on a maximum-parsimony analysis using 1000 bootstrap replicates of the data. For each tree, the bootstrap value (of 1000 bootstrap replicates) for a specific branch is indicated either above or below the branch. Note that the values are given as a percentage. Values <70 % are generally not given except where the value is of some particular interest. AGMonkey, African green monkey polyomavirus; JC, JC virus; SV40/12, Simian virus 40/12; WU, WU polyomavirus; KI, KI polyomavirus.

the viruses were detected in live-trapped *M. lucifugus*. Seven of the 31 animals (over 22 %) examined yielded virus. With the exception of two *M. californicus*, no viruses were detected in any of the bats obtained from the rabies laboratory or the wind plant. These 100 bats represented eleven species. Our failure to detect viruses in these animals likely reflects the condition of the animals. All were thawed after being frozen for several months and it appeared that considerable time had elapsed between the death of these animals, many by trauma, and freezing. Bats from the wind plant may have been exposed to the elements for as long as 24 h before being found by searchers and collected. In contrast, the live-trapped bats were sampled within a day of euthanasia and maintained on ice during that period.

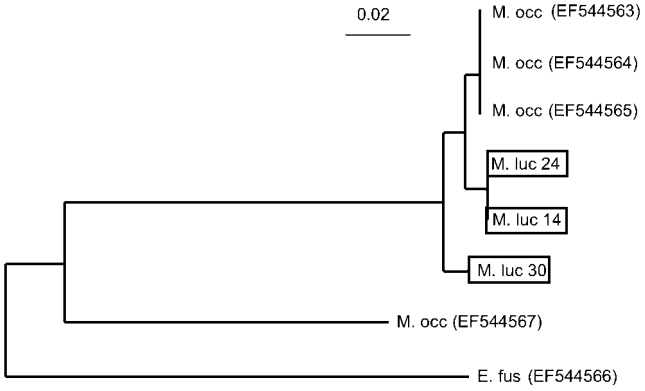

Fig. 2. Relationship of sequences from a conserved segment of the RNA polymerase gene of RMBCV detected in North American bats. The nucleotide sequences for virus detected in *M. lucifugus* (M. luc, boxed; these are MyPyV sequences) were derived as described in Methods, the others [*M. occultus* (M. occ) and *E. fuscus* (E. fus)] were obtained from GenBank (see Supplementary Table S3). GenBank accession numbers are given in parentheses. Only the corresponding sequences present in all samples were included in the CLUSTAL_X alignment. The unrooted trees were drawn in NJPlot using data from CLUSTAL_X.

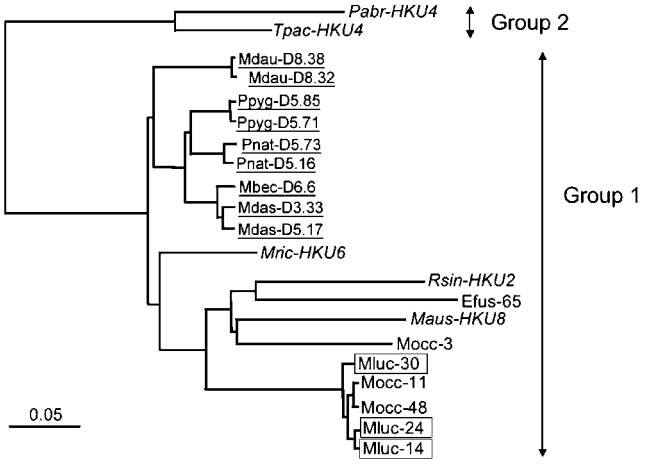

Fig. 3. Phylogenetic relationship between the nucleotide sequences of an approximately 250 bp segment of the RNA polymerase gene from several bat coronaviruses. The nucleotide sequences for virus detected in *M. lucifugus* (MyPyV strains; boxed) were derived as described in Methods, others were obtained from GenBank (see Supplementary Table S3). Sequences corresponding to those amplified by PCR were extracted from some sequences before alignment. Sequences of bat coronaviruses detected in North America (Roman type), Asia (italics) and Europe (underlined) are represented. The bat species in which the viruses were detected and their accession numbers are: Pabr, *Pipistrelles abramus*, DQ249216; Tpac, *Tylonycteris pachypus*, DQ075642; Mdau, *M. daubentonii*, D8.38, EU375874; D8.32, EU375875; Mdas, *M. dasycneme*, D3.33, EU375854; D5.17, EU375861; Pnat, *P. nathusii*, D5.16, EU375864; D5.73, EU375869; Mbec, *M. bechsteinii*, D6.6, EU375865; Ppyg, *P. pygmaeus*, D5.71, EU375868; D5.85, EU375870; Mric, *M. ricketti*, DQ249224; Maus, *M. australis*, DQ249228; Efus, *Eptesicus fuscus*, EF544566; Mocc, *M. occultus*, 3, EF544567; 11, EF544563; 48, EF544565. The unrooted trees were drawn in NJPlot using data from CLUSTAL_X.

Using a strategy that exploited the circular nature of polyomavirus genomes (Johne *et al.*, 2006), we recovered the entire genome of the virus detected in two *Myotis* species. Analysis of the complete sequence of the viral genomes recovered from three animals revealed an as-yet undescribed polyomavirus. We suggest that the virus should be called Myotis polyomavirus (MyPyV). The genome of MyPyV appeared to have the same arrangement of genes as other polyomaviruses, with potential ORFs for the capsid proteins VP1, VP2 and VP3. ORFs with potential splice sites, the non-structural proteins Small t and Large T antigens were also present. The genome contained other small ORFs, including one for a potential 'agno' protein. However, we could not detect significant similarity between the deduced amino acid sequence of these ORFs and peptides identified for the better-characterized polyomaviruses.

Some mammalian polyomaviruses cause tumours when susceptible rodents are infected experimentally and the newly discovered Merkel polyomavirus is associated with the aggressive Merkel cell skin cancer in humans (Feng *et al.*, 2008). In addition, both the mouse polyomavirus and pneumotropic virus can cause disease when inoculated into newborn mice. However, the mammalian polyomaviruses are not generally associated with acute disease in natural non-immunocompromised hosts. While the naturally occurring mouse virus does retain its ability to cause fatal disease in newborn mice, infection under natural conditions in feral mice does not result in disease (Carroll *et al.*, 2007), possibly because of the simultaneous transmission of virus and maternal immunity to newborn pups. In contrast with the mammalian viruses, at least two of the avian viruses cause acute, frequently fatal inflammatory disease in some species of psittacine birds and geese (Johne & Müller, 2007). The cycle of infection of most polyomaviruses in natural hosts has not been determined. However, the avian polyomavirus appears to have a unique form of reverse vertical transmission in the European pied flycatcher, where blowfly larvae transmit the virus to nestlings, which then infect the parents through the faecal–oral route (Potti *et al.*, 2007).

Like other mammalian polyomaviruses in natural hosts, MyPyV appears not to cause disease in adult *M. lucifugus*. We detected the virus in apparently healthy bats in a mating swarm. While the number of bats we examined was too low to reach a definitive conclusion, it is interesting that we only detected the virus in females of two *Myotis* species. *Myotis* females rear pups in maternity roosts with little input into offspring care by males. It is tempting to

speculate that a natural cycle of infection involving ectoparasites, similar to that of the avian polyomavirus in the European pied flycatcher (Potti *et al.*, 2007), might result in the infection of pups in crowded maternity roosts with subsequent infection of the females.

We detected RNA of a group 1 coronavirus in several of the *M. lucifugus* examined. Our analysis of the virus depends entirely on the nucleotide sequence of a 500 bp segment of the gene for the viral RNA polymerase. The information was sufficient to determine that we had detected a virus very closely related to RMBCV described by Dominguez *et al.* (2007). The *M. lucifugus* virus was most closely related (possibly a variant) to virus detected in *M. occultus*, but it was distinct from RMBCV from *E. fuscus*.

Since the discovery of SARS-like coronavirus in bats, several studies screening bats for coronaviruses have led to the discovery of many coronaviruses in bats of Asia, Europe and North America. These viruses appear to have a benign relationship with their Chiroptera hosts and display the diversity and constant population growth dynamics that are characteristic of long virus–host associations (Vijaykrishna *et al.*, 2007). This led some to speculate (Vijaykrishna *et al.*, 2007) that bats may be reservoir hosts for all coronaviruses and that rare spillover and adaptation in other species may have led to the development of coronaviruses of humans, domestic animals and other species.

Climatic and socio-economic changes in the coming years may lead to more frequent contact between bats and humans and domestic animals, increasing the chance of spillover of bat viruses and the emergence of new infectious diseases. While we failed to detect virus in bats killed at a wind energy facility, our sample size from this site was small. We suggest that this aspect of our survey should be repeated with larger numbers of bats, as wind plants are a potentially new source of transmission from cryptic, forest bat species which have historically had extremely low rates of contact with domestic animals or humans. Knowledge of viruses that parasitize bats and ecological factors that govern maintenance in bats may help us to reduce the chance of spillover infection or to ameliorate its effects should it occur. For instance, a catalogue of viruses parasitizing bats and molecular information on their gene products would make it easier to detect a virus in the early stages of an epidemic. It would also speed up the identification of reservoir species. Our preliminary study is a small step in that direction.

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