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Surveillance for Coronaviruses in Bats, Lebanon and Egypt, 2013–2015

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To the Editor: Coronaviruses (CoVs) in bats are genetically diverse, and evidence suggests they are ancestors of Middle East respiratory virus CoV (MERS-CoV), severe acute respiratory syndrome CoV, and human CoVs 229E and NL63 (*1–4*). We tested several bat species in Lebanon and Egypt to understand the diversity of bat CoVs there.

Samples were collected during February 2013–April 2015. A total of 821 bats were captured live in their caves; sampled (oral swab, rectal swab, serum); and released, except for 72 bats that died or were euthanized upon capture. Lungs and livers of euthanized bats were harvested and homogenized. Caves were in proximity to human-inhabited area but not in proximity to camels.

In Egypt, we sampled 3 bat species (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/22/1/15-1397-Techapp1.pdf). Eighty-two Egyptian tomb bats (*Taphozous perforatus*) tested negative for CoV. We also sampled 31 desert pipistrelle bats (*Pipistrellus deserti*) and detected an HKU9-like betacoronavirus (b-CoV) in the liver of 1 bat (prevalence 3.2%). From 257 specimens from Egyptian fruit bats (*Rousettus aegyptiacus*), we detected b-CoV in 18 samples from 18 different bats (prevalence 7%). A murine hepatitis virus–like CoV was detected in the lung of 1 bat. HKU9-like viruses were detected in 5 oral, 2 lung, 5 liver, and 5 rectal samples. Overall, 5.1% of the bats tested positive.

In Lebanon, we sampled 4 bat species. Four *Rhinolophus hipposideros* bats and 6 *Miniopterus schribersii* bats tested negative. One of 3 *Rhinolophus ferrumequinum* bats sampled was positive. We sampled 438 *Rousettus aegyptiacus* bats from 10 different locations and detected HKU9-like viruses in 24 rectal swab specimens (prevalence 5.5%). Overall, 5.5% of the bats tested positive.

A subset of the samples (696 samples: 516 from Egypt, 180 from Lebanon) were tested for MERS-CoV by using the specific upstream of E quantitative reverse transcription PCR; all tested negative. Serum samples from 814 bats tested negative for MERS-CoV antibodies.

Phylogenetic analysis revealed that the RNA-dependent RNA polymerase (*RdRp*) genes of viruses detected in *R. aegyptiacus* bats in Lebanon and Egypt were closely related to the *RdRp* gene of HKU9 CoV (Figure). Our viruses clustered in 3 groups: A, B, and C. Group A viruses were closely related to HKU9-10-2 virus and included viruses from Egypt. Group B included viruses from both countries and were closely related to HKU9-1 and HKU9-4 viruses. Group C also included viruses from both countries that were related to HKU9-3 and HKU9-5 viruses. The *RdRp* fragments sequenced had <90% nt similarity among groups A, B, and C. Within-group nucleotide similarity was >90%, and amino acid variability was 2%–4% (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/22/1/15-1397-Techapp2.xlsx). The phylogenetic tree of the N gene also showed proximity of the viruses detected in our study to HKU9 viruses (online Technical Appendix 1). Viruses from Lebanon clustered together as did the viruses from Egypt.

Most of the positive samples were detected in Egyptian fruit bats. These are cave-dwelling species that inhabit regions of East Africa, Egypt, the Eastern Mediterranean, Cyprus, and Turkey (*5*). This species is a reservoir for several viruses, including Marburg, Kasokero, and Sosuga

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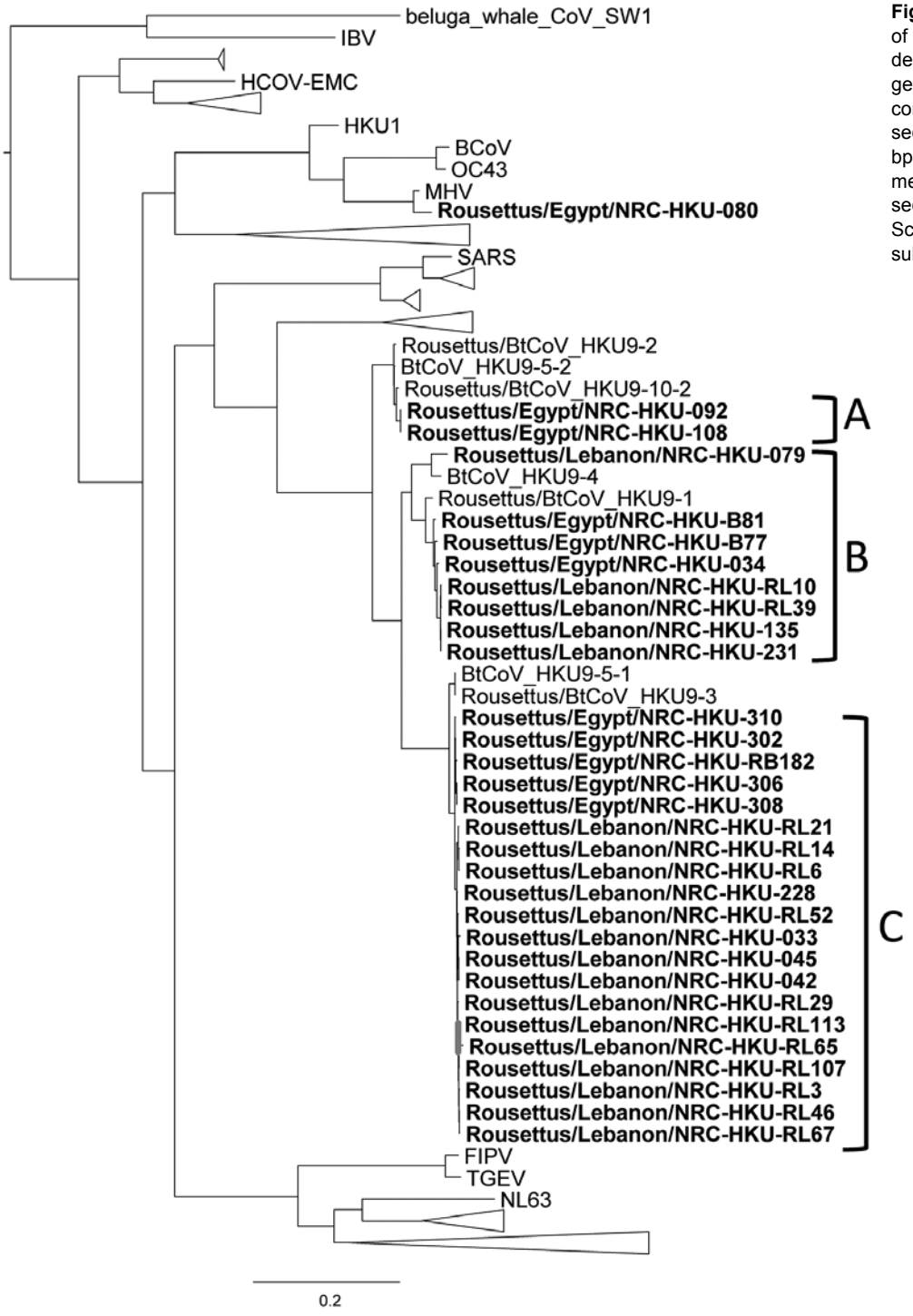

Figure. Phylogenetic tree of the coronavirus RNA-dependent RNA polymerase gene. This tree was constructed on the basis of a sequence alignment of 330 bp using the neighbor-joining method. Bold text indicates sequences found in this study. Scale bar indicates nucleotide substitutions per site.

viruses (*6*–*8*). The b-CoVs HKU9 and HKU10 were detected in Chinese fruit bats (*9*). All but 1 of the detected viruses were HKU9-like. However, there was enough genetic variability within the sequenced *RdRp* fragments to suggest the circulation of at least 3 diverse groups comprising 3 different CoV species.

Our detection of CoVs in oral, rectal, lung, and liver samples suggests that CoV infection in those bats was systemic, although the bats were apparently healthy. One bat had a murine hepatitis virus–like infection. This bat was captured from a brood that inhabited the windowsills of a historic building in urban Cairo. This infection might

have been a cross-species infection from mice to bats in the same habitat.

Although bats rarely come in direct contact with humans, humans can come into more frequent contact with bat urine and feces and, in the case of fruit bats, bat saliva through partially eaten fruits. Bats in the Middle East are not eaten for food but are occasionally hunted. In this study, HKU9-related viruses were detected in apparently healthy fruit bat species from Egypt and Lebanon and appear to cause systemic infection. HKU9-related viruses are not known to cause human disease. MERS-CoV was not detected in bats sampled in this study. More surveillance for bat CoVs in the Middle East is needed, and the zoonotic potential for bat-CoVs requires further study.

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Ebola Virus Disease Complicated by Late-Onset Encephalitis and Polyarthritis, Sierra Leone

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To the Editor: Ebola virus (EBOV) disease is usually an acute illness, but increasing evidence exists of persistent infections and post-Ebola syndromes. We report a case of EBOV encephalitis.

A 30-year-old woman with no known EBOV contact sought treatment at an Ebola isolation unit in Freetown, Sierra Leone, on January 1, 2015 (day 7 of illness). She was afebrile and weak, but ambulatory, with a history of fever, vomiting, diarrhea, headache, and muscle and joint pain. According to local protocol, she was given oral antimalarial, antimicrobial, and antiemetic drugs and oral rehydration therapy. On day 8 of illness, after testing EBOV PCR–positive (cycle threshold [C_t] value of 23.5) (*1*), she was given intravenous ceftriaxone (2 g) for 7 days, artesunate (180 mg) for 3 days, and Ringer's lactate (4–6 L) with supplemental KCl for 5 days.

During days 13–15, the patient improved, moving independently and talking. On day 16, she became confused; by day 20, she was unresponsive to voices. Intravenous ceftriaxone (2 g) and artesunate (180 mg) were administered for an additional 7 and 3 days, respectively. On days 28 and 29, she was still unconscious; serum PCR test results on both days were negative for EBOV. On day 29, she was transferred to Connaught Hospital in Freetown, where she had a Glasgow Coma Scale score of 9/15 (E3, V1, M5) but no localizing or focal signs. She was

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Surveillance for Coronaviruses in Bats, Lebanon and Egypt, 2013–2015

Technical Appendix 1

Laboratory Methods

Screening

Viral RNA was extracted by using the Qiaamp viral RNA minikit (QIAGEN, Hilden, Germany). The RNA was eluted in 60 µL AVE buffer and was used as a template for further detection by a pan-coronavirus nested PCR targeting the *RNA-dependent RNA polymerase* (*RdRp*) gene. First-round reverse transcription PCR (RT-PCR) was conducted by using forward primer 5-GGKTG–GGAYTAYCCKAARTG-3 and reverse primer 5-TGYTGTSWRCA-RAAYTCRTG-3 and QIAGEN 1-step RT-PCR kit. A 25-µL reaction mixture contained 5 µL of 5X reaction buffer, 1 µL dNTPs, 1 µL enzyme mix, 1.5 µL (10 Pmole) forward primer, 1.5 µL (10 Pmole) reverse primer, 10 µL ddH_2O, and 5 µL of sample RNA. The PCR cycler conditions for the amplification were 50°C for 30 min (reverse transcription) then 95°C for 15 min, 45 cycle of 94°C for 15 s (denaturation), 48°C for 30 s (annealing), 72°C for 40 s (extension), then 72°C for 10 min (final extension). The PCR product was then put through a second round PCR by using a new set of primers (forward primer 5-GGTTGG-GACTATCCTAAGTGTGA-3, reverse primer 5-CCATCATCAGATAG-AATCATCAT-3) which amplify a final PCR product of 440 bp. Using Phusion High Fidelity PCR Master Mix Kit (Thermo Scientific, Waltham, MA, USA), a 25-µL reaction contained 12.5 µL of 2X phusion master mix, 1.5 µL (10 Pmole) forward primer, 1.5 µL (10 Pmole) reverse primer, 7.5 µL H_2O, and 2 µL of PCR product. The PCR cycler conditions were 98°C for 2 min then 45 amplification cycles (98°C for 15 s, 48°C for 15 s, 72°C for 30 s), then 72°°C for 2 min. The final PCR amplicons were gel purified using the

QIAquick gel purification kit (QIAGEN) and analyzed by sequencing (*1*). The *upE* quantitative reverse transcription PCR was performed as previously described (*2*).

Sequencing

The second round forward and reverse primers were to sequence the purified DNA amplicons using a BigDyeR Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions and were further amplified for 26 cycles at 95°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The reaction product was purified by exclusion chromatography in CentriSep columns (Princeton Separations, Adelphia, NJ, USA). The recovered materials were sequenced by using a 96-capillary 3730xl DNA Analyzer (Applied Biosystems). Sequences were assembled by using SeqMan DNA Lasergene 7 software (DNASTAR, Madison, WI, USA). Sequence analysis was performed by using BioEdit 7.0 and MEGA 6.0 for multiple sequence alignment and phylogenetic tree construction of applying the neighbor-joining method with Kimura's 2-parameter distance model and 1,000 bootstrap replicates (*3*,*4*).

An RT nested PCR designed specifically for this study was used for amplifying N gene sequences of HKU9-like viruses. Reaction conditions of this assay were same as the *RdRp* assay above except for the 1-step RT-PCR with outer forward primer 5-ATGTCTGGAMGGAATAAGCCCCG-3 and inner reverse primer 5-TTATTAGGATTACGDGTGCCCAT-3, and nested PCR with inner forward primer 5-GTTCAAGCAAGAATCTGACGGTT-3 and inner reverse primer 5-ACCTTCTTCACCCACCCAGTATA-3. The expect size of the second PCR product was 400 bp.

GenBank accession nos.: KT220528–KT220562, KT368821, KT581588–KT581603.

Serology

A pseudo-particle neutralization assay was used to test bat serum against MERS-CoV as previously described (*5*).

Ethical Statement

Ethics approval was obtained from St. Jude Children's Research Hospital Institutional Animal Use and Care Committee (Memphis, TN, USA). Swabs and tissues were tested by RT-PCR, and positive samples were sequenced.

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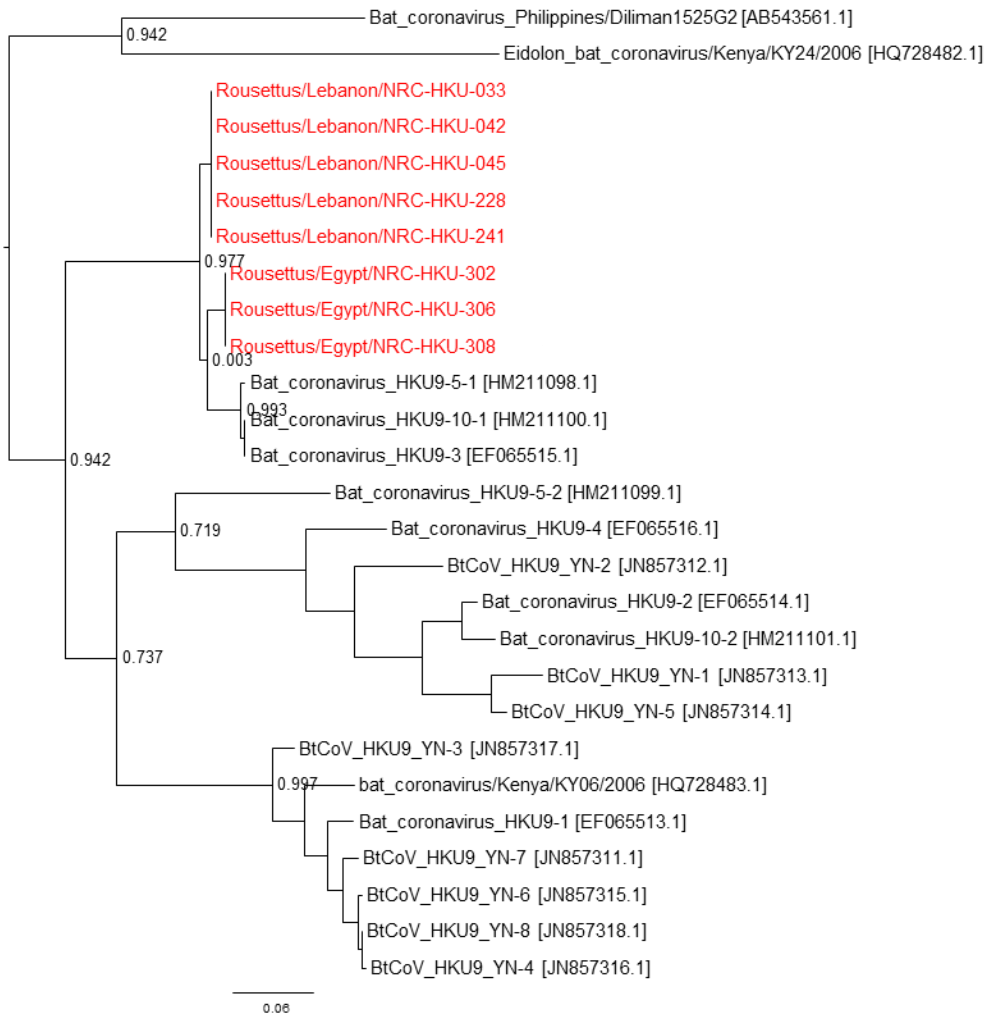
Technical Appendix 1 Table 1. Screening for coronaviruses in bats, Egypt, 2013–2015

Species	No bats	No. samples., type	Results	Location	Date
Taphozous perforatus	5	5 Serum 5 Oral 5 Lung 5 Liver		Abu-Rawwash, 13 km west of Cairo	Feb 2013
T. perforatus	52	52 Serum 52 Oral 52 Rectal		Abu-Rawwash, 13 km west of Cairo	Aug 2013
T. perforatus	25	24 Serum 25 Oral 25 Rectal		Abu-Rawwash, 13 km west of Cairo	Oct 2013
Pipistrellus deserti	29	29 Serum 29 Oral 29 Lung 29 Liver	1 (HKU9-like)	Abu-Rawwash, 13 km west of Cairo	Feb 2013
P. deserti	2	1 Serum 2 Oral 2 Rectal		Abu-Rawwash, 13 km west of Cairo	Oct 2013
Rousettus aegyptiacus	14	14 Serum 14 Oral 14 Lung	1 (murine hepatitis viru–like)	Cairo	Feb 2013
R. aegyptiacus	24	24 Serum 24 Oral 24 Lung 24 Liver	3 (HKU9-like) 2 (HKU9-like) 5 (HKU9-like)	Abu-Rawwash, 13 km west of Cairo	Feb 2013
R. aegyptiacus	5	4 Serum 5 Oral 5 Rectal	4 (HKU9-like)	Abu-Rawwash, 13 km west of Cairo	Oct 2013
R. aegyptiacus	102	101 Serum 102 Oral	2 (HKU9-like)	Abu-Rawwash, 13 km west of Cairo	Oct 2014
R. aegyptiacus	112	112 Serum	1 (HKU9-like)	Abu-Rawwash, 13 km west of Cairo	Nov 2014

Species	No bats	No. samples., type	Results	Location	Date
		112 Oral 112 Rectal			
Total	370		19 positive samples (5.14%)		

Technical Appendix 1 Table 2. Screening for coronaviruses in bats, Lebanon, 2013–2015

Species	No bats	Sample no. ,type	Result	Location	Date
Rhinolophus hipposideros	4	4 Serum 4 Rectal		Zgharta, North Lebanon	Oct 2013
Rhinolophus ferrumequinum	1	1 Serum 1 Rectal	1 (HKU9-like)	Aley, Mount Lebanon	Oct 2013
Rhinolophus ferrumequinum	2	2 Serum 2 Rectal		Amchit, North Lebanon	Oct 2013
Miniopterus schribersii	6	6 Serum 6 Rectal		Amchit, North Lebanon	Oct 2013
Rousettus aegyptiacus	50	50 Serum 50 Rectal	4 (HKU9-like)	Akkar, North Lebanon	Oct 2013
Rousettus aegyptiacus	1	1 Serum 1 Rectal	1 (HKU9-like)	Amchit, North Lebanon	Oct 2013
Rousettus aegyptiacus	51	51 Serum 51 Rectal	1 (HKU9-like)	Bisri, South Lebanon	Oct 2013
Rousettus aegyptiacus	34	34 Serum 34 Rectal	3 (HKU9-like)	Antelias, Mount Lebanon	Oct 2013
Rousettus aegyptiacus	21	21 Serum 21 Rectal	5 (HKU9-like)	Ras Keefa, North Lebanon	Apr 2014
Rousettus aegyptiacus	5	5 Serum 5 Rectal		Berqayel, North Lebanon	Jun 2014
Rousettus aegyptiacus	75	72 Serum 75 Rectal	7 (HKU9-like)	Tripoli, North Lebanon	Jun 2014
Rousettus aegyptiacus	34	34 Serum 34 Rectal	3 (HKU9-like)	Antelias, Mount Lebanon	Sep 2014
Rousettus aegyptiacus	101	101 Serum 101 Rectal		Bisri, South Lebanon	Jun 2014
Rousettus aegyptiacus	30	30 Serum 30 Rectal		Jbeil, Mount Lebanon	Jun 2014
Rousettus aegyptiacus	32	32 Serum 32 Rectal		Edde, Mount Lebanon	Oct 2014
Rousettus aegyptiacus	4	4 Serum 4 Rectal		Karm Saddeh, North Lebanon	Apr 2015
Total	451		25 positive samples (5.54%)		



Technical Appendix 1 Figure. Phylogenetic tree of the coronavirus N gene. This tree was constructed on the basis of sequence alignment of 400 bp of the N gene and neighbor-joining method. Sequences in red are those found in this study. Scale bar indicates nucleotide substitutions per site.