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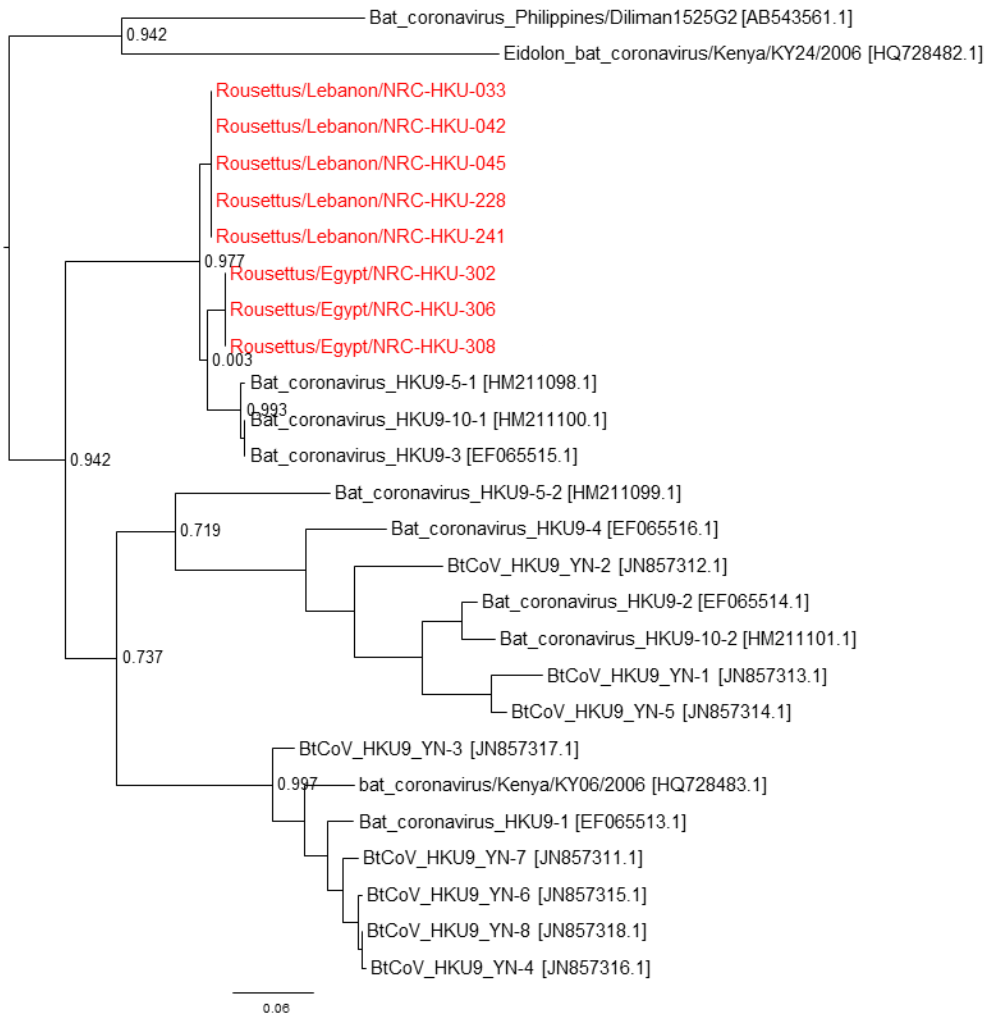
Technical Appendix 1 Table 1. Screening for coronaviruses in bats, Egypt, 2013–2015

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.