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Influenza A Virus Infections in Dromedary Camels, Nigeria and Ethiopia, 2015–2017

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We examined nasal swabs and serum samples acquired from dromedary camels in Nigeria and Ethiopia during 2015–2017 for evidence of influenza virus infection. We detected antibodies against influenza A(H1N1) and A(H3N2) viruses and isolated an influenza A(H1N1) pdm09–like virus from a camel in Nigeria. Influenza surveillance in dromedary camels is needed.

Aquatic wild birds are the natural reservoir of influenza A virus, which comprises 16 hemagglutinin and 9 neuraminidase subtypes. Influenza A virus subtypes H1N1, H2N2, and H3N2 have caused pandemics in humans, and subtypes H1N1 and H3N2 circulate in humans as seasonal influenza. Pandemic influenza arises when an animal influenza virus evolves through the reassortment of animal and human virus gene segments (antigenic shift) to sustainably transmit in humans. Avian and swine influenza viruses have caused zoonotic infections, some resulting in fatal disease. Thus, influenza virus surveillance in animals is needed for pandemic preparedness (1).

Dromedary camel populations, estimated to be 30 million globally, can be found in parts of Africa, the Middle East, and Central Asia, often in close proximity to humans. An equine influenza A(H3N8) virus (2) and human influenza A/USSR/90/77(H1N1)–like viruses (which were associated with fatal disease in 1980–1983) (3) have been isolated from Bactrian camels in Mongolia. However, little is known of influenza A virus infections in dromedary populations.

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Figure. Maximum-likelihood phylogenetic tree showing relationship of influenza A(H1N1)pdm09 virus from dromedary camel, Nigeria, January 2016 (red circle), relative to other influenza A(H1N1)pdm09 viruses from humans worldwide on the basis of the hemagglutinin gene. Tree was constructed by using a general time-reversible model with FastTree (<https://www.geneious.com/plugins/fasttree-plugin>) and PhyML (<http://www.atgc-montpellier.fr/phyml>) (Appendix, <https://wwwnc.cdc.gov/EID/article/26/01/19-1165-App1.pdf>). Tree is rooted with an influenza A(H1N1)pdm09 virus collected in 2009. Bootstrap support values for the major branches are shown. Scale bar indicates number of nucleotide changes per base pair.

Therefore, we carried out a study to determine the prevalence of influenza A virus infection in dromedary camels.

As part of an investigation of Middle East respiratory syndrome coronavirus conducted during October 2015–February 2016 (4), we collected 2,166 nasal swabs and 150 serum samples from dromedaries at an abattoir in Kano, Nigeria. We also collected 102 nasal swabs and 100 serum samples from nomadic dromedary herds in Amibara, Ethiopia (in January 2017); 109 nasal swabs from herds in Asayita, Ethiopia (in July 2017); and 83 nasal swabs from herds in Dubti, Ethiopia (in July 2017). We put nasal swabs in virus transport medium, stored samples frozen at -80°C , and shipped them to Hong Kong, China, on dry ice for laboratory tests. We performed RNA extraction and detected the influenza A virus matrix gene using molecular methods (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/19-1165-App1.pdf>) (5).

Twelve nasal swabs from camels in Nigeria collected at different times (October [$n = 5$] and November [$n = 5$] 2015 and January [$n = 1$] and February [$n = 1$] 2016) were positive for the influenza A virus matrix gene; cycle thresholds for these samples were 33.7–38.9 (Appendix Table). One of the nasal swabs collected in Amibara was also positive (cycle threshold 37.8). We inoculated quantitative reverse transcription PCR–positive samples onto Madin Darby canine kidney cells grown in 24-well plates in minimum essential medium with tosylsulfonyl phenylalanyl chloromethyl ketone–treated trypsin (2 $\mu\text{g}/\text{mL}$) (Appendix) (6). We examined inoculated cells for cytopathic effect and passaged each culture twice. We defined virus detection as the appearance of cytopathic effect or the hemagglutination of turkey erythrocytes. We were able to isolate 1 virus, which we designated A/dromedary/NV1337/2016 (H1N1), from a swab collected in Nigeria on January 22, 2016 (Appendix Table).

We performed full-genome sequencing of A/dromedary/NV1337/2016 (Appendix) as previously described (6). We achieved sequencing read coverages of ≥ 100 for each nucleotide and were able to deduce the full virus genome (GenBank accession nos. MN453859–66). All 8 gene segments showed their highest identity (≥ 99.8) to contemporary influenza A(H1N1)pdm09 viruses (data not shown).

In a phylogenetic analysis, we compared the hemagglutinin gene of A/dromedary/NV1337/2016 with that of other influenza A(H1N1)pdm09 viruses available from GenBank and GISAID (<https://platform.gisaid.org>) (Figure). The dromedary influenza A virus

isolated in Nigeria in January 2016 was similar to other influenza viruses circulating in humans at the same time. The sampling dates for the influenza viruses detected in camels in Nigeria overlapped with the human influenza virus season, which typically occurs during October–March (Appendix Table) (7). This finding suggests reverse zoonosis of influenza viruses from humans to dromedaries. Whether these viruses were subsequently maintained in dromedary populations via camel-to-camel transmission is not clear. Further studies are needed to address this question. Transmission of influenza A virus also occurs from humans to swine, and these viruses can be maintained in swine populations for variable periods, sometimes decades (8).

We tested serum samples from dromedary camels for hemagglutination inhibition (HI) antibody against A/dromedary/NV1337/2016(H1N1) using standard methods (Appendix) (5); 4 serum samples from camels in Amibara had HI antibody (titers 1:40, 1:80, 1:160, and 1:160). The dromedaries that had influenza A virus RNA–positive nasal swabs were negative for HI antibody. Dromedaries recently infected with a virus are expected to be seronegative for that virus because antibody responses against viruses take around a week to develop (9), by which time nasal swab specimens are often negative for that virus’s genomic material. Microneutralization tests are more appropriate for testing antibody to contemporary H3N2 viruses (10); hence, we also tested serum samples for antibody to A/Hong Kong/4801/2014(H3N2) virus using the microneutralization test (Appendix). In total, 1 serum sample from a camel in Nigeria was positive at a titer of 1:80.

In conclusion, we provide evidence of influenza A virus infection in dromedaries. Our findings indicate that influenza virus surveillance in dromedary camel populations is needed.

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Bovine Kobuvirus in Calves with Diarrhea, United States

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We detected bovine kobuvirus (BKV) in calves with diarrhea in the United States. The strain identified is related genetically to BKVs detected in other countries. Histopathologic findings also confirmed viral infection in 2 BKV cases. Our data show BKV is a potential causative agent for diarrhea in calves.

Bovine kobuvirus (BKV; species *Aichivirus B*, genus *Kobuvirus*, family *Picornaviridae*) was identified initially as a cytopathic contaminant in a culture medium of HeLa cells in Japan in 2003 (1). Since then, BKV has been reported in Thailand, Hungary, the Netherlands, Korea, Italy, Brazil, China, and Egypt (2–9). However, circulation of BKV in North America remains unclear. We report detection of BKV in calves in the United States.

In April 2019, a fecal sample from a 10–14-day-old calf was submitted to University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA) for testing for enteric pathogens. Results of tests for rotavirus, coronavirus, cryptosporidium, and *Escherichia coli* were positive; results for *Salmonella* were negative.

We extracted nucleic acid from the fecal sample and conducted a sequence-independent single-primer amplification and library preparation by using Nextera XT DNA Library Preparation Kit (Illumina, <https://www.illumina.com>). We conducted sequencing on a MiSeq (Illumina) using MiSeq Reagent Kit V2 (Illumina) at 500 cycles, as previously described (10). We conducted a taxonomic analysis of raw FASTQ files using Kraken version 1 and MiniKraken DB (<https://ccb.jhu.edu/software/kraken>), which showed 15,582 kobuvirus sequence reads in addition to sequences for *E. coli*, coronavirus, and rotavirus. We assembled the complete genome of BKV IL35164 (GenBank accession no. MN336260) with a genome size of 8,337 nt. We used BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search the IL35164 genome and found it is closely related to and shares 89%–91% identities with 4 BKV strains, U-1, EGY-1, SC1, and CHZ. It shares only 77%–82% identity with sheep and ferret kobuviruses.

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Appendix

Methods

Reverse Transcription PCR (RT-PCR) for Detection of Influenza Viruses

RNA extraction from swabs samples or cell culture isolates was carried out by Biomerieux EasyMag or Roche MagNA pure automated extraction systems with protocols recommended by the manufacturers. Extracted RNA was tested by a real time quantitative RT-PCR (RT-qPCR) assay targeting a conserved region in influenza A matrix gene (*M*). A negative control was included for every 10 specimens tested and a positive control included in each RT-qPCR run.

For detection of influenza A virus, 4 μ L of RNA was amplified in a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) with a total reaction-mix volume of 20 μ L in 1x Taqman Fast Virus 1-step master mix (Applied Biosystems) containing 0.5mM of forward primer (5'-CTTCTAACCGAGGTCGAAACGTA-3'), reverse primer (5'-GGTGACAGGATTGGTCTTGTCTTTA-3) and hydrolysis probe (5'-FAM-TCAGGCCCCCTCAAAGCCGAG-BHQ1-3') for amplification of the matrix gene of influenza A virus. Cycling conditions were as follows: an initial reverse transcription step at 50°C for 5 minutes and initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, 72°C for 12 seconds with default ramp rates of the apparatus in fast reaction mode. Reactions with serial dilutions of known copy number control sample were included in each run for monitoring detection sensitivity and estimating gene copy numbers in samples.

Full-Length Influenza A Virus Genome Sequencing

Full-length cDNAs were synthesized using the uni-12 primer followed by amplification of full-length gene segments with primers targeting both ends of the gene segments (2). PCR

products were analyzed with 1% agarose gel electrophoresis. Products with expected segment sizes were sequenced using Illumina HiSeq 2500 system with Nextera XT library preparation method (Illumina). The virus genome was deduced by taking the consensus of sequencing raw reads mapped to a selected pdm09H1N1 reference genome. Sequencing read coverages of at least 100 were achieved for each nucleotide.

Phylogenetic Analysis

Phylogenetic tree of hemagglutinin of influenza A H1 pdm09H1N1 lineage viruses by the maximum likelihood method using IQTree with auto substitution model selection (3) and ultrafast bootstrap approximation (4). The tree was rooted to a pdm09H1N1 virus collected in year 2009. Support values for the major branches were shown. The length of the scale bar denotes the number of nucleotide changes along the horizontal branches of the tree.

Viral Culture

Madin-Darby canine kidney (MDCK) cells were plated in 24-well plates (TPP® tissue culture plates, Sigma-Aldrich) at 1×10^5 cells per well in minimum essential medium (MEM) (GIBCO, New York) with 10% fetal bovine serum (2). When the cells were semi-confluent (usually within 24 hours) the cells were washed three times with serum free MEM with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (2µg/ml) (Sigma, St. Louis, Missouri). Each well was inoculated with 100ul of the swab supernatant in MEM and incubated for one hour at 37°C in a CO₂ incubator. Each inoculated well was separated by an uninoculated cell control well to avoid cross-contamination and provide cell controls. The inoculum was replaced with MEM with trypsin (2µg/ml) and incubated for 5 days. The cells were observed for cytopathic effect (CPE) each day. When CPE appeared or on day 5 if no CPE was seen, aliquots of the cell supernatant was tested for hemagglutination with turkey red blood cells in U-bottomed plates. Culture supernatants with CPE or hemagglutination activity were also tested by RT-PCR for influenza matrix (M) gene and serially passaged in MDCK cells to obtain stock virus for aliquoting and freezing at -80°C.

Serology

Hemagglutination Inhibition (HI) Test

The sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co Ltd, Tokyo) at 37°C overnight to remove nonspecific inhibitors. Next morning residual RDE was

destroyed by heat inactivation at 56°C for 30 minutes and then diluted with six volumes of 0.9% NaCl solution before carrying out HI tests. Serial 2-fold dilutions of each RDE treated serum was prepared in duplicate in 96-well microtiter plates. Equal volumes (25µL) each serum dilution was mixed with virus antigen with a hemagglutination titer of 8 in each well. After a 1h incubation, 50µL of a 0.5% solution in PBS of washed turkey red blood cells (TRBC) was added to each well and incubated for 30min. The plates were inspected with tilting. The HI titer of each serum was defined as the highest serum dilution that inhibited hemagglutination and formed a clear button of TRBC at the bottom of the well with a tear-drop appearance being seen when the plate was tilted. Positive and negative control sera were included with each set of titrations. Antigen back titrations were done to ensure that the correct HA dose was used in each run (5).

Microneutralization Test

The 3 day microneutralization test as described in reference (6) was carried out in 96-well microtiter plates using neutralization of virus cytopathogenic effect (CPE) in Madin-Darby Canine Kidney (MDCK) cells and detectable hemagglutination in the culture supernatant as the read-out for evidence of virus replication. Serial serum dilutions in quadruplicate were mixed with 100 tissue culture infectious dose 50 (TCID₅₀) of A/Hong Kong/4801/2014 (H3N2) for 1 hour and then the virus-serum mixture was transferred to pre-formed MDCK cell monolayers. One hour after inoculation, serum-virus mixtures were removed and serum free MEM with 2 ug/ml TPCK trypsin was added to each well. The plates were incubated at 37°C in a CO₂ incubator and cytopathic effect was observed to determine the highest serum dilution that neutralized CPE in ≥50% of the wells. On day 3, an aliquot of each culture supernatants were tested for hemagglutination with turkey red blood cells. A virus back titration and positive and negative control sera were included in each assay.

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Appendix Table. Summary of influenza A M gene RT-qPCR positive samples*

Country	Lab ID	Sampling date, day/month/year	Influenza A M gene Ct	Hemagglutination titer passage 1	Hemagglutination titer passage 2
Nigeria	NV0282	22/10/2015	37.25	neg	neg
Nigeria	NV0325	24/10/2015	37.69	neg	neg
Nigeria	NV0334	24/10/2015	38.30	neg	neg
Nigeria	NV0467	20/10/2015	37.71	neg	neg
Nigeria	NV0480	30/10/2015	36.85	neg	neg
Nigeria	NV0619	04/11/2015	37.56	neg	neg
Nigeria	NV0714	09/11/2015	34.96	neg	neg
Nigeria	NV0823	13/11/2015	33.67	neg	neg
Nigeria	NV0974	19/11/2015	38.83	neg	neg
Nigeria	NV1250	30/11–2015	38.87	neg	neg
Nigeria	NV1337	22/1/2016	37.60	Positive HA titer 32	Positive HA titer 64
Nigeria	NV1875	2/11/2016	37.53	neg	neg
Ethiopia	CAC4377	1/12/2017	37.77	neg	neg

*Ct, cycle threshold; HA, hemagglutination; ID, identification; M, matrix; neg, negative; RT-qPCR, quantitative reverse transcription PCR.