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## Co-Circulation and Persistence of Genetically Distinct Saffold Viruses, Denmark

**To the Editor:** Cardioviruses are positive-sense, single-stranded RNA viruses of the family *Picornaviridae*, genus *Cardiovirus*. Until recently, cardioviruses were primarily known for their ability to infect rodents. In 2007, findings of a retrospective study of undiagnosed enteric illnesses in the United States were published, including results from analysis of a fecal sample from an infant girl whose symptoms were diagnosed as fever of unknown origin in 1981. The novel human cardiovirus that was isolated was designated Saffold virus (SAFV) (1). Eight genotypes of SAFV have been described (1–4), and a ninth was recently isolated in Nigeria (O. Blinkova, unpub. data). Serologic studies indicate that infection with SAFV genotypes 2 and 3 generally occurs in early life (5), although the clinical significance of these findings remains unclear.

The first SAFV infection in Denmark was recorded in 2009 (6). To elucidate the molecular epidemiology of SAFV, we performed a 3-year surveillance study of SAFV in Denmark. During 2009–2011, we tested 1,393 fecal samples from 454 children. Surveillance included collection of fecal samples from children at 6, 10, and 15 months of age; additional fecal samples were

collected when the children had gastroenteritis. Most of the SAFV-positive samples reported in this study were obtained from a randomized trial in the pediatric department of University Hospital of Holbaek (Holbaek) on the effect of probiotic therapy on the incidence of infection during early childhood (M. Gyhrs, unpub. data). The study was approved by the local ethics committee; Den Regionale Videnskabetiske Komité for Region Sjælland, Denmark.

Nucleic acids were extracted from 200- $\mu$ L fecal suspension (10% in phosphate-buffered saline) by using the Cobas AmpliPrep Total Nucleic Acid Isolation Kit (Roche Diagnostics, Ltd., Mannheim, Germany) on the MagnaPure LC instrument (Roche Diagnostics). We used 5  $\mu$ L of extracted nucleic acid for reverse transcription PCR (RT-PCR) (total volume 25  $\mu$ L) using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). The samples were tested for SAFV by using real-time RT-PCR primer/probe, and all positive samples were genotyped by partial sequencing of the viral protein (VP) 1 gene (6). Overall, 38 (2.8%) of the clinical samples were positive for SAFV (online Technical Appendix, [wwwnc.cdc.gov/EID/pdfs/12-0793-Techapp.pdf](http://wwwnc.cdc.gov/EID/pdfs/12-0793-Techapp.pdf)), all of which fell into genotype 2 (SAFV-2), which is most prevalent in Western nations. Of these samples, 31 had sequence information of sufficient length for additional analyses. All SAFV-2 sequences were submitted to GenBank (accession nos. JX048000–JX048030).

To determine the evolutionary history of strains of SAFV identified in persons in Denmark, we combined the VP1 sequences collected here with all others available on GenBank. We aligned sequences as described using MUSCLE software (7), then checked the alignments using manual calculations. We performed phylogenetic analysis using the

maximum likelihood method as described in PhyML 3.0 (8), on the basis of the best-fit GTR+ $\Gamma$  nucleotide model as determined by jModelTest (9). Phylogenetic robustness was determined by using 1,000 bootstrap replicates.

Our phylogenetic analysis places the strains isolated in Denmark within the SAFV-2 group (online Technical Appendix). These SAFV-2 strains were further subdivided into 2 strongly supported clusters: DK-A, which comprised viruses isolated during 2009–2011, demonstrating probable persistence in Denmark during this period; and DK-B, a smaller group that included viruses from United States, Germany, and the Netherlands, indicating widespread viral gene flow (Figure). The DK-B strains were

identified in samples collected during 2009–2010, supporting probable sustained viral persistence within Denmark. Within DK-B, strain 115883 is phylogenetically distinct from the other DK-B viruses.

We next measured the selection pressures acting on these lineages through the mean number of nonsynonymous ( $d_N$ ) to synonymous ( $d_S$ ) nucleotide substitutions per site using the single-likelihood ancestor counting, fixed effects likelihood, and random effects likelihood methods available in the Datamonkey HyPhy package as described (10). The DK-A and DK-B groups differed significantly in selection pressure: DK-A,  $d_N/d_S$  ratio = 0.195 (95% CI 0.105–0.328); and DK-B,  $d_N/d_S$  ratio = 0.033 (95% CI 0.015–

0.062), which indicates stronger purifying selection on the DK-B group. Ancestral state reconstruction, performed by using Datamonkey (10), revealed that the ancestors of DK-A and DK-B differ only at aa 135 in VP1: Val in DK-A and Ala in DK-B. Notably, aa 135 was positively selected in DK-A (random effects likelihood:  $d_N/d_S = 3.53$ , Bayes factor = 50; fixed effects likelihood:  $d_N/d_S \gg 1$ ; cutoff  $p = 0.1$ ), with more tentative evidence for adaptation at aa 135 in DK-B:  $d_N/d_S$  ratio  $\gg 1$  by using fixed effects likelihood ( $p = 0.2$ ). The functions of aa 135 in VP1, and what it means for the fitness of SAFV, merit further consideration.

We conclude that SAFV-2 has been introduced into Denmark in 3 groups: DK-A, viral strain 115883 and strains of DK-B reported in Denmark; all have recently co-circulated in this country. We have demonstrated the entry and persistence of 3 phylogenetically distinct lineages of SAFV-2 in Denmark. That SAFV-2 can persist between years suggests that it might be common, yet underreported, in Denmark, which provides the opportunity for spread to additional localities. Increased awareness of improved laboratory protocols for SAFV detection is needed among clinicians in Denmark and neighboring countries.

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Figure. Phylogenetic analyses of Saffold viruses (SAFVs). Phylogenetic analysis of SAFV-2. Strains from Denmark are named by using the isolation numbers assigned for the study, then the country of origin and year of sampling in parentheses. The 2 subgroups (DK-A and -B) are shown. The tree is rooted by using SAFV-1 outgroup sequence accession no. EF165067. Bootstrap values >70% are shown. DK, Denmark; US, United States. Scale bars represent 0.2 nucleotide substitutions per site.

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## Pathogenic *Leptospira* spp. in Bats, Madagascar and Union of the Comoros

**To the Editor:** Leptospirosis is a zoonosis of global distribution; incidence rates are particularly high in tropical areas (1). Leptospirosis is a major public health problem on islands in the southwestern Indian Ocean, particularly La Réunion, Mayotte, and the Seychelles (where incidence rates are among the highest in the world) (1). In contrast, no human case has been reported on the nearby islands of Madagascar and Union of the Comoros. However, the recent demonstration of pathogenic *Leptospira* spp. in small mammals introduced to Madagascar suggests possible transmission from free-living animals to humans (2).

In addition to the fact that incidence rates vary among humans, clinical bacterial isolates from different islands belong to different serogroups and serovars and show diverse molecular features (3,4). This diversity might be correlated with that of the reservoir hosts; the islands in the southwestern Indian Ocean are a hot spot of biodiversity with extraordinary levels of vertebrate endemism. Most studies investigating wild-animal reservoirs of *Leptospira* spp. on the islands in the southwestern Indian Ocean have focused on small mammals that had been introduced to the islands (2,5), although bats infected with pathogenic *Leptospira* spp. have been identified in other regions (6). Whether bats are a reservoir of *Leptospira* spp. on these islands remains unknown. Therefore, we looked for this bacterium in bats from Madagascar and Union of the Comoros and characterized associated genetic diversity.

As part of an ongoing program aimed at identifying viral and

bacterial infectious agents in island wild fauna, 129 insectivorous and frugivorous bats were tested for *Leptospira* spp. The bats belonged to 12 species: 9 from Madagascar (*Mormopterus jugularis*, *Otomops madagascariensis*, *Triaenops furculus*, *T. menamena*, *Miniopterus gleni*, *Miniopterus griffithsi*, *Miniopterus mahafaliensis*, *Myotis goudoti*, *Hypsugo anchietae*) and 3 from Union of the Comoros (*Rousettus obliviosus*, *Chaerephon pusillus*, *Miniopterus griveaudi*). Bats were captured in mist nets or harp traps at 8 sites in Madagascar and 6 sites in Union of the Comoros. Organs were collected in the field and immediately stored in liquid nitrogen.

Total nucleic acids were extracted from a pool of kidney, spleen, and lung tissue by using the Biorobot EZ1 and EZ1 Virus Mini Kit version 2.0 (QIAGEN, Les Ulis, France). Reverse transcription was then performed with GoScript reverse transcriptase (Promega, Charbonnières-les-Bains, France) to obtain cDNA. We screened pathogenic *Leptospira* spp. with a probe-specific real-time PCR (7). The 25 positive samples were subsequently subjected to a PCR procedure that amplified fragments from 682 to 1,293 bp of the 16S rRNA gene (depending on the amplification success) by using published primers (8–10). Resulting PCR products from 7 samples were sequenced and compared with available sequences in GenBank by using phylogenetic construction with PhyML 3.0.

Of the 12 bat species tested, 11 were positive for *Leptospira* spp. (the only *H. anchietae* bat tested was negative). Among 52 bats from Madagascar, 18 (34.6%) were infected; detection rates were often high, e.g., 8 (80%) of 10 *T. menamena* bats. In contrast, among 77 bats from Union of the Comoros, only 9 (11.7%) were infected. *Leptospira* spp. seem to be ubiquitous in the study areas; infected bats were found at 7 of 8

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## Technical Appendix

Table. Epidemiologic and sequence information of Saffold virus isolated from specimens collected from children, Denmark, 2009–2011

Isolate name	Age/sex	Clinical symptom	Isolation date	GenBank accession no.*
T9707	16 mo/M	Ataxia	2009 Jun 6	JF693617
S20019	16 mo/F	Diarrhea	2009 Sep 11	JF693614
W48653	16 mo/M	Diarrhea	2009 Oct 6	JF693618
M68541	5 y,6 mo/M	Diarrhea	2009 Oct 1	JF693619
H66354	6 y,1 mo/F	Diarrhea	2010 Jan 1	JF693612
H66359	3 y,5 mo/M	Diarrhea	2010 Jan 1	JF693613
H44677	5 y,5 mo/M	Diarrhea	2009 Oct 14	JF693615
T52830	11 mo/M	Diarrhea	2010 Sep 27	JX048000
M19039	9 mo/F	Diarrhea	2010 Nov 14	JX048001
T69389	11 mo/M	Diarrhea	2010 Nov 27	JX048002
H17696	10 mo/F	Diarrhea	2011 Feb 9	JX048003
H17866	10 mo/F	Diarrhea	2011 Feb 9	JX048004
H65680	11 mo/F	Diarrhea	2010 Nov 11	JX048005
115751	6 mo/M	None	2009 Nov 5	JX048006
115883	6 mo/F	None	2010 Apr 20	JX048007
116142	15 mo/M	None	2010 Oct 5	JX048008
116150	6 mo/M	None	2010 Oct 19	JX048009
116177	11 mo/F	None	2010 Oct 27	JX048010
116220	6 mo/F	None	2010 Nov 11	JX048011
116235	6 mo/M	None	2010 Nov 15	JX048012
116236	16 mo/F	None	2010 Nov 14	JX048013
116310	10 mo/M	None	2010 Dec 29	JX048014

Table. Epidemiologic and sequence information of Saffold virus isolated from specimens collected from children, Denmark, 2009–2011

116325	16 mo/M	None	2011 Jan 11	JX048015
116366	11 mo/M	None	2011 Jan 25	JX048016
116370	15 mo/F	None	2011 Jan 28	JX048017
116398	15 mo/M	None	2011 Feb 15	JX048018
116051	11 mo/F	None	2010 Aug 23	JX048019
116067	11 mo/F	None	2010 Sep 1	JX048020
116599	14 mo/F	None	2011 Jun 21	JX048021
116709	16 mo/F	None	2011 Oct 6	JX048022
116712	16 mo/F	None	2011 Oct 11	JX048023
115765	6 mo/F	None	2009 Nov 21	JX048024
115779	5 mo/F	None	2009 Dec 9	JX048025
116237	15 mo/M	None	2010 Nov 14	JX048026
116255	5 mo/M	None	2010 Nov 23	JX048027
116256	6 mo/F	None	2010 Nov 24	JX048028
T1774	12 mo/F	None	2010 Dec 14	JX048029
T1785	12 mo/F	None	2010 Dec 12	JX048030
T24768†	2 y,3 mo/F	Sudden death	2009 Aug 1	JF693616

\*GenBank accession nos beginning with JF indicate data from previous study (6); all other data are from this study.

†Isolate derived from blood specimen; all other samples were from fecal specimens.