Clinical Infectious Diseases

BRIEF REPORT

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Isolation of a Novel Recombinant Canine Coronavirus From a Visitor to Haiti: Further Evidence of Transmission of Coronaviruses of Zoonotic Origin to Humans

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We isolated a novel coronavirus from a medical team member presenting with fever and malaise after travel to Haiti. The virus showed 99.4% similarity with a recombinant canine coronavirus recently identified in a pneumonia patient in Malaysia, suggesting that infection with this virus and/or recombinant variants occurs in multiple locations.

Keywords. coronavirus; coronavirus: canine; coronavirus: zoonotic; coronavirus: recombinant; human coronavirus infection.

In March 2017, members of a medical team from University of Florida who had recently returned from a "mission trip" to Haiti presented with mild fever and malaise. Zika virus (ZIKV) was circulating in Haiti at the time, and because of concerns that their illnesses might represent ZIKV infection, freshly collected urine samples were obtained from team members and screened for ZIKV, in keeping with previously described methods [1]. All samples tested negative by reverse transcription polymerase chain reaction (RT-PCR) for ZIKV. However, at that time our routine procedure included efforts to isolate ZIKV from all diagnostic specimens, and consequently deidentified aliquots of the urine samples were subsequently inoculated onto Vero E6 and LLC-MK2 cells, which are susceptible and permissive for ZIKV.

Twenty urine samples from team members were screened. Samples from six patients produced cytopathic effects (CPE) in cell culture within 14 days of inoculation of cell lines; an example is shown for LLC-MK2 cells inoculated with urine sample Z19 (Figure 1). When aliquots of cell culture media from Vero E6 cells at 16 days post-inoculation were inoculated onto MDCK cells, CPE were noted the following day (Supplementary Figure 1). To determine whether the viruses causing CPE were of possible respiratory origin, cell culture media was tested using a GenMark eSensor XT-8 RVP system (eSensor RVP; GenMark Diagnostics, Inc., Carlsbad, California, USA) instrument [2]. Unexpectedly, the 6 samples tested showed mixed low signals for 3 of the 4 seasonal endemic human coronaviruses (threshold signal [nA] value above 3, the generally accepted positive cutoff, seen for Betacoronavirus OC43 [4 of 6 samples tested] and Alphacoronaviruses 229E [4 of 6 samples tested] and NL65 [2 of 6 samples]) (Supplementary Table 1). After follow-up RT-PCR tests of the cell culture media using species-specific coronavirus RT-PCR tests failed to establish an identity, an unbiased amplification and sequencing approach was attempted [3, 4].

As material extracted from Madin-Darby canine kidney (MDCK) cells culture media corresponding to sample Z19 appeared to have the highest virus yield based on the extent of CPE formed, RNA from this sample was purified and subjected to Sanger sequencing. Initial sequence analyses of a 2558 bp amplicon (Supplementary Figure 2) generated using an unbiased RT-PCR amplification method [5] indicated 97% (2475/2561) nucleotide (nt) identity to a porcine coronavirus, transmissible gastroenteritis virus (TGEV) strain Purdue P115 (Genbank Accession no. DQ811788.1), leading to the assumption that the virus was TGEV. However, primers based on TGEV did not effectively amplify or failed to amplify other sections of the virus' genome, suggesting that it was a different coronavirus. After the publication of Vlasova et al [6], primers that targeted parts of the RdRp gene and spike protein on the genome sequence they discovered (canine coronavirus isolate CCoV-HuPn-2018, GenBank MW591993.2) were tested and were found to produce PCR amplicons. This prompted us to focus efforts on amplifying the virus sequence of our isolate using canine coronavirus primers.

Ultimately, 39 primer pairs covering the whole virus genome were designed for complete genome sequencing (Supplementary Table 2). Three additional primers for 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) were also designed for this work; that work was accomplished using the RACE System (Invitrogen) used according to the manufacturer's

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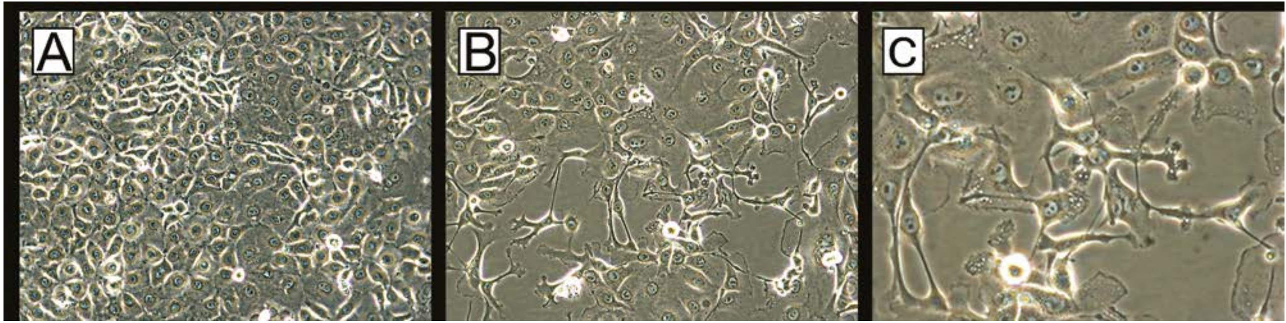

Figure 1. Virus-induced cytopathic effects in LLC-MK2 cells 16 days post-inoculation with urine specimen Z19. *A*, Mock-inoculated cells, original magnification 200×. *B*, Cells inoculated with urine, original magnification 200×. *C*, Detail from image *B*, original magnification 400×.

manual. By using the primers of Supplementary Table 2, virus genomic RNA (vgRNA) was reverse-transcribed into cDNA using an AccuScript High fidelity 1st Strand cDNA Synthesis Kit (Agilent, Santa Clara, California, USA), and PCR performed with Q5 high-fidelity DNA polymerase (New England Biolabs). Sanger sequencing was performed using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA). The strain has been designated HuCCoV_Z19Haiti; GenBank accession number is MZ420153. The five other samples showing CPE effects (Z03, Z04, Z11, Z12, and Z14) were only partly characterized (for verification purposes) after Z19 was fully sequenced. Primers 36F and 36R (Supplementary Table 2) were used to RT-PCR amplify 850 nt amplicons from the other 5, and after sequencing these amplicons were found to be identical to the sequence of Z19. The remaining 14 patient urine samples were negative on screening with multiple primers. Neither this virus—or other canine coronaviruses—were present in our laboratory prior to this study.

Further recombination and phylogenetic analyses were conducted as previously described in Lednicky et al [4]; details of methods are provided in Supplementary Materials. A maximum likelihood (ML) tree inferred on the full genome alignment (Figure 2A), regardless of potential recombinant genomic fragments, confirmed the close relationship between HuCCoV_Z19Haiti and CCoV-HuPn-2018, with 99.4% identity between the 2 virus strains. The second half of the HuCCoV_Z19Haiti genome, starting from gene E, showed greater divergence from CCoV-HuPn-2018 (similarity plot, Supplementary Figure 3, Supplementary Table 3). Furthermore, the HuCCoV_Z19Haiti isolate did not have the 36 nt deletion in gene N and the 228 nt deletion in ORF7b, both characteristic of the Malaysian strain. A further Blast search [7] on the NCBI database, conducted only with the genes E, M, N, and the ORF7 segment, did show a match with the Chinese canine coronavirus strain CCoV B639_ZJ_2019 [8] (Supplementary Figure 3). Fragmenting the genome of HuCCoV_Z19Haiti alignment by gene, as was done by Vlaslova et al [6], further confirmed the chimeric nature of the virus isolated in Haiti (Supplementary Figure 4). Both Spike S1 and S2 ML trees clustered HuCCoV_Z19Haiti with CCoV-HuPn-2018, although in the gene M ML tree the closest relative was the Chinese CCoV B639_ZI_2019. In the gene N phylogeny, the Haitian strain clusters with TGEV, although the bootstrap values might be too low to make a strong inference.

We identified the same pattern of recombination events reported by Vlasova et al [6] in the spike and ORF1 of the Haitian genome (Supplementary Table 4) suggesting that recombination occurred ancestrally to CCoV-HuPn-2018 and HuCCoV_Z19Haiti. The Haitian isolate, however, further diverged from the Malaysian strain through additional and multiple recombination events across the genome, notably affecting the gene E – ORF7 segment, which closely relates to CCoV B639_ZJ_2019. Further recombination events with other CCoVs overlapped to the segment originated from CCoV B639_ZJ_2019. To corroborate the recombination analysis, 5 subsets of genomic fragments were analyzed: the larger one constituted by most of the genome, minus the recombinant fragments, and 4 smaller ones constituted by the segments involved in the inferred recombination events involving HuCCoV_Z19Haiti. Recombinant segments common to CCoV-HuPn-2018 and HuCCoV_Z19Haiti were removed, as they were considered to have occurred prior to divergence; other recombinant segments, involving taxa other than the Haitian strain, were also removed from recombinant sequences. Following assessment of phylogenetic signal (Supplementary Figure 5), ML trees for each nonrecombinant fragment (Figure 2B–F) confirmed CCoV-HuPn-2018 as the major parent of the Haitian strain, and the chimeric nature of the other fragments, involving other CCoVs, as well as possibly unsampled ancestors of TGEVs.

COMMENT

Coronaviruses are known to infect a wide range of mammalian and bird species [9]. They have also long been recognized as one of the causes of the "common cold" in humans, associated with what have been termed the seasonal endemic human coronaviruses: HCoV 229E and NL63 in the genus *Alphacoronavirus*; and HCoV HKU1 and OC43 in the genus *Betacoronavirus* [10].

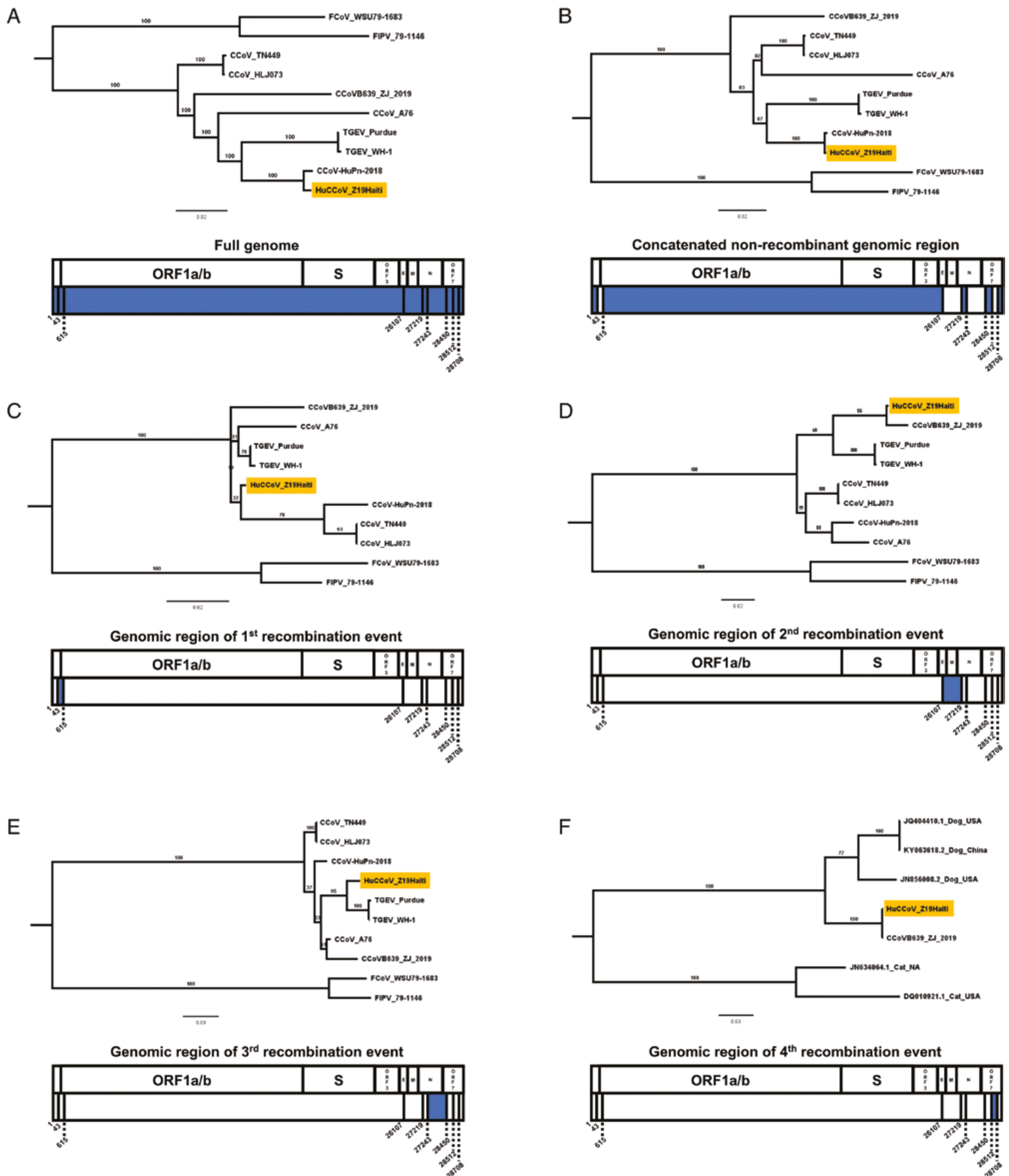

Figure 2. Maximum likelihood (ML) tree of alphacoronavirus strains. ML trees were inferred from 10 genome sequences, including CCoV-HuPn-2018 and CCoVB639_ZJ_2019, using the best fitting nucleotide substitution models as detected by Bayesian information criterion. Branches are scaled in number of nucleotide substitutions per site according to the bar below each tree. Nonparametric bootstrap values (1000 replicates) are indicated along supported branches. Haitian strain HuCCoV Z19 is highlighted. *A*, ML tree calculated using full genomes, prior to any recombination analysis. Panels *B–F* show trees inferred based on non-recombinant genomic fragments, indicated in blue in the schematic genome below each tree for clarity. Genome coordinates are based on HuCCoV Z19. *B*, ML tree calculated using non-recombinant segments of the genome. *C*, ML tree calculated using HuCCoV Z19 recombinant segment 43-615. *D*, ML tree calculated using HuCCoV Z19 recombinant segment 26107-27219. *E*, ML tree calculated using HuCCoV Z19 recombinant segment 27243-28450. *F*, ML tree calculated using HuCCoV Z19 recombinant segment 28512-28708. Segments involved in recombination events of genomes other than HuCCoV Z19 and CCoV-HuPn-2018 were replaced by gaps in the affected sequences in trees *B–F*.

However, over the past 2 decades we have seen the emergence of three coronavirus species that are highly pathogenic for humans, and which appear in each instance to have arisen from a zoonotic origin: severe acute respiratory syndrome coronavirus (SARS-CoV-1), Middle East respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome 2 (SARS-CoV-2), all in the genus *Betacoronavirus*.

Our group has recently reported isolation of a porcine deltacoronavirus (PDCoV) from children in Haiti presenting with fever and gastrointestinal complaints, with genomic and evolutionary analyses suggesting that human infections were the result of at least 2 independent zoonoses of distinct viral lineages that acquired a common mutational signature in the *nsp15* and the *spike* glycoprotein genes by convergent evolution [4]. As noted above, Vlasova et al reported isolation of an *Alphacoronavirus* of apparent canine origin, with evidence of recombination with a feline coronavirus, from patients with pneumonia in Malaysia [6]. We report here identification of a coronavirus of canine origin which is closely related to the Malaysian virus reported by Vlasova et al, albeit isolated in this instance from a visitor to Haiti, and with a further recombinational history. Samples were deidentified after initial screening by RT-PCR for Zika, limiting our ability to obtain detailed clinical and epidemiological information on specific infected individuals; however, all members of the group reported mild fever and malaise, and all recovered uneventfully. Our data highlight the potential among coronaviruses for rapid evolution combined with frequent recombination events, leading to periodic emergence of strains capable of crossing species barriers into human populations. In many instances such strains would appear to be of low virulence for humans, as reflected in our work with PDCoV and now CCoV-Haiti; however, the potential for such strains to carry or acquire genes capable of causing severe disease in humans remains of clear concern.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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