Host barriers to SARS-CoV-2 demonstrated by ferrets in a high-exposure domestic setting

Kaitlin Sawatzki†, Nichola J. Hill, Wendy B. Puryear, Alexa D. Fossa, Jonathon J. Stone, and Jonathan A. Runstadler

*Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine at Tufts University, North Grafton, MA 01536

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Ferrets (Mustela putorius furo) are mustelids of special relevance to laboratory studies of respiratory viruses and have been shown to be susceptible to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and onward transmission. Here, we report the results of a natural experiment where 29 ferrets in one home had prolonged, direct contact and constant environmental exposure to two humans with symptomatic disease, one of whom was confirmed positive for SARS-CoV-2. We observed no evidence of SARS-CoV-2 transmission from humans to ferrets based on viral and antibody assays. To better understand this discrepancy in experimental and natural infection in ferrets, we compared SARS-CoV-2 sequences from natural and experimental mustelid infections and identified two surface glycoprotein Spike (S) mutations associated with mustelids. While we found evidence that angiotensin-converting enzyme II provides a weak host barrier, one mutation only seen in ferrets is located in the novel S1/S2 cleavage site and is computationally predicted to decrease furin cleavage efficiency. These data support the hypothesis that furin is not the only host factor interacting with the novel S1/S2 cleavage site predicted to decrease furin cleavage efficiency. These data suggest that ferret infection may require additional investigation.

SARS-CoV-2 | virology | transmission | genetics | coronavirus

Significance

Ferrets have been demonstrated to be susceptible to laboratory infection of SARS-CoV-2, raising the possibility of natural transmission from humans into their pets in domestic settings. We demonstrate that ferrets may have host barriers that limit natural infection and transmission. First, we find no evidence of infection in 29 ferrets from a home with constant exposure to two adults with one confirmed and one suspected case of symptomatic COVID-19. Second, we analyze genetic sequences from viruses and hosts and demonstrate that ferrets have genetic factors that confer resistance to natural SARS-CoV-2 infection. These data suggest that ferret infection may require viral adaptation, and therefore ferrets may only be semi-permissive models of SARS-CoV-2 disease or transmission.

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†To whom correspondence may be addressed. Email: kaitlin.sawatzki@tufts.edu.

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Results
Absence of Natural SARS-CoV-2 Human-to-Ferret Transmission in a High-Exposure Setting. A household with 29 free-roaming ferrets cared for by two related, adult housemates was enrolled in the CoVERS study. Individual 1 experienced fever and fatigue from March 25 to April 6, and individual 2 experienced a sore throat, anosmia, migraine, and fatigue from March 28 to April 13 (Fig. 1A). Individual 2 tested positive for SARS-CoV-2/COVID-19 infection by nasopharyngeal swab and RT-PCR on April 1. Individual 1 is a suspected positive due to the timing and symptoms but was not tested. Neither person was hospitalized, and both cared for the ferrets during the entirety of their disease courses.

A 2-wk, in-home sample collection scheme was designed to begin during the household quarantine period (Fig. 1B). The ferrets were free to move in all spaces of the home during this period and handled as usual, including daily petting, feeding, and grooming. Individual 1 described daily close contact with holding, laying, and/or cuddling with all ferrets, as well as daily cage cleaning. The ferrets ranged in age from 8 mo to 7.5 y over 21 females and 8 males. A home sampling kit was sent to the participants, including material to safely collect and store ferret oral swabs. One participant had significant animal handling experience and performed all sample collection to standardize sampling procedures. Thirty oral swabs were collected and held in viral transport media in the participants’ freezer until the end of the study period. Frozen samples were directly transferred to a laboratory member and processed.

All samples were confirmed to have viable RNA, by a preliminary screen for constitutively expressed β-actin (Table 1). Each sample was then tested for evidence of active or recent SARS-CoV-2 infection, with three established primer sets: Open Reading Frame 1b nonstructural protein 14 (ORF1b) (20), Nucleocapsid (N) (14), and RNA-dependent RNA polymerase (RdRP) (21). All were below the limit of detection and determined to be negative for active or recent infection (Table 1).

We further took advantage of salivary immunoglobulin G (IgG), which has been shown to be highly sensitive and specific for SARS-CoV-2 testing (22). We tested samples for evidence of antibodies against SARS-CoV-2 surface glycoprotein RBD. Twenty-two ferrets (23 total samples) were confirmed to have measurable total IgG via binding to recombinant protein A/G but were all negative for binding to RBD (Table 1). Therefore, there is no evidence of viral infection or seroconversion in 29 ferrets living with two people with COVID-19.

Identification of Two Mustelid-Associated Mutations in SARS-CoV-2 Surface Glycoprotein. Our observed household data support the idea that there may be important barriers to natural infection in ferrets; however, ferrets have been shown to be susceptible to infection and onward transmission in experimental laboratory infections (9, 10, 18, 19). To further investigate this, we analyzed available genomic sequences of SARS-CoV-2 viruses of naturally infected American minks and experimentally infected ferrets (32 sequences representing 24 animals, accessed 1 August 2020). There

Fig. 1. COVID-19 disease course and ferret sample collection timeline. A household with two adults and 29 free-roaming ferrets was enrolled in the CoVERS study. (A) Both adults exhibited symptoms of SARS-CoV-2 infection in late March to early April of 2020, and one tested positive by RT-PCR on 1 April. (B) Oral swabs were collected from all ferrets in the home over a 2-wk period, beginning April 10, concurrent with symptomatic disease in Individual 2. One ferret (subject 3) was sampled twice. Two 7-y-old ferrets (subjects 12 and 16) died during the study period, one by euthanasia due to chronic disease, the other cause is unknown.
ever, N501T is seen in 5/17 pangolin-derived SARS-CoV-2 S genes, none exhibit the N501T mutation (Fig. 2). This mutation is of special interest, as this cleavage site partially distinguishes SARS-CoV-2 from other SARS-like viruses and allows immune evasion prior to receptor binding (23–25). Like N501T, S686G was observed in 11/11 ferrets, a minority variant in the donor inoculum, and increased proportional representation in the virome over time, suggesting positive selection (19). We found that no other human-derived viral sequences has been observed with this mutation (Fig. 2B). S686G has also not been observed in SARS-CoV-2-like viruses from other Carnivora (naturally infected felines and canines), all of which retained the complete cleavage site and adjacent P1’ serine. All mustelid-derived viruses retained the second, downstream S1/S2 cleavage site motif (IAY/TMS), as well as the S2’ TMPRSS2-processed cleavage site for fusion.

Host furin and furin-like proteases have been shown to cleave the S1/S2 polybasic cleavage site (3, 25, 26). P1’ residues are strongly favored to be serine in furin cleavage, and alternate residues are restricted by size and hydrophilicity due to their location in the furin binding pocket (27). Glycine is small but hydrophobic. We performed in silico analysis of the cleavage site to compare identical sequences that differed only at position 686, using PiTou 2.0 (28). PiTou scores are biologically meaningful prediction values of furin cleavage derived from binding strength and solvent accessibility. S686 results in a PiTou score of 9.19633, while S686G results in a score of 6.92387. While both are predicted to be cleaved by furin, S686 is estimated to have stronger interactions in the binding pocket (P6 to P2’). Therefore, S686G is an unfavorable substitution for furin cleavage.

We further performed phylogenetic analysis of the proprotein convertase family that cleaves polybasic sites (PCSK1 to 7), including furin, and Cathepsin L in a number of mammals, including ferret and the well-annotated ermine. However, we found no clear differences between ferrets, ermines, and other members of Carnivora (SI Appendix, Figs. S1–S8).

Discussion

Multiple studies have now demonstrated that ferrets may be directly infected by human-derived SARS-CoV-2 and, following infection, exhibit a nearly 100% transmission rate via direct contact (9, 10, 18, 19, 29). Recent reports also describe sporadic cases of natural infection (30, 31). However, our data suggest that the initial barrier of human-to-ferret transmission may be higher than relevant to most household pets. We calculated that a sample size of 10 animals was sufficient to test the hypothesis that at least one ferret was infected, given an observed attack rate of 87% in mink farms (95% CI, 0.05) (32). In this natural experiment, all 29 ferrets had significant opportunities for direct contact with all other ferrets and had direct exposure to at least one infected person.

Based on current knowledge of SARS-CoV-2 transmission and shedding in ferrets, we determined that our collection time points fell within the timeframe to obtain measurable viral RNA, even if transmission occurred on March 22, prior to any symptom onset in the household. However, it was important to perform additional antibody testing to address two concerns: first, that transmission could have occurred prior to March 22 and, second, that the level of infection and viral shedding was so low as to be below collection and screening sensitivity. In either scenario, we still expected a robust antibody presence within days of initial infection but found no evidence of RBD-specific antibodies. Despite significant and prolonged exposure in the home, we have

Table 1. No evidence of SARS-CoV-2 infection in ferrets

| Ferret | ACTB | ORF1b | N | RdRP | Total IgG | αRBD IgG |
|--------|------|-------|---|------|----------|----------|
| 1      | 33.036 | LOD   | LOD | LOD  | P       | N        |
| 2      | 28.120 | LOD   | LOD | LOD  | P       | N        |
| 3a     | 27.954 | LOD   | LOD | LOD  | P       | N        |
| 3b     | 28.945 | LOD   | LOD | LOD  | P       | N        |
| 4      | 26.230 | LOD   | LOD | LOD  | P       | N        |
| 5      | 29.067 | LOD   | LOD | LOD  | P       | N        |
| 6      | 29.729 | LOD   | LOD | LOD  | P       | N        |
| 7      | 29.360 | LOD   | LOD | LOD  | P       | N        |
| 8      | 26.755 | LOD   | LOD | LOD  | P       | N        |
| 9      | 33.049 | LOD   | LOD | LOD  | P       | N        |
| 10     | 32.820 | LOD   | LOD | N    | NA      |
| 11     | 29.781 | LOD   | LOD | LOD  | P       | N        |
| 12     | 29.010 | LOD   | LOD | LOD  | P       | N        |
| 13     | 27.730 | LOD   | LOD | N    | NA      |
| 14     | 32.163 | LOD   | LOD | P    | N        |
| 15     | 30.230 | LOD   | LOD | P    | N        |
| 16     | 27.861 | LOD   | LOD | P    | N        |
| 17     | 27.701 | LOD   | LOD | P    | N        |
| 18     | 27.687 | LOD   | LOD | N    | NA      |
| 19     | 30.832 | LOD   | LOD | N    | NA      |
| 20     | 31.758 | LOD   | LOD | P    | N        |
| 21     | 31.758 | LOD   | LOD | N    | NA      |
| 22     | 32.635 | LOD   | LOD | P    | N        |
| 23     | 27.098 | LOD   | LOD | P    | N        |
| 24     | 29.290 | LOD   | LOD | P    | N        |
| 25     | 29.806 | LOD   | LOD | N    | NA      |
| 26     | 35.042 | LOD   | LOD | N    | NA      |
| 27     | 30.032 | LOD   | LOD | P    | N        |
| 28     | 31.464 | LOD   | LOD | P    | N        |
| 29     | 29.476 | LOD   | LOD | P    | N        |

Ferret oral swabs were tested by semiquantitative real time RT-PCR and ELISA. Sample and RNA viability was confirmed by β-actin (ACTB). Three separate primers sets were used to test for SARS-CoV-2: ORF1b, N, and RdRP; LOD denotes under the limit of detection. ELISA was performed twice for total IgG antibodies against recombinant protein A/G (Total IgG) and purified SARS-CoV-2 RBD (αRBD IgG). ELISA results presented are negative (N), positive (P), or not applicable (NA) if there is insufficient total IgG.
concluded that there is no evidence of SARS-CoV-2/COVID-19 human-to-ferret transmission in this household.

An important caveat of this transmission study is the question of infectiousness in humans. We were unable to collect human samples in this work, and therefore we suspect, but cannot prove, that both adults had an infectious period. Symptomatic infectious correlate with contagiousness, and both cases had moderate symptomatic disease (Fig. 1A) (33). Based on symptom onset, we suspect that individual 1 may have been infectious while in the home and transmitted SARS-CoV-2 to individual 2 (34, 35).

Notably, ferret 12 (7 y old) was euthanized on April 16, and had a history of adrenal disease, and ferret 16 (7 y old) died unexpectedly on April 20. Both were swabbed within 4 d of their deaths and, we expect, would have been RT-PCR or antibody positive had their deaths been related to SARS-CoV-2 infection.

We found no evidence of SARS-CoV-2 transmission to ferrets, a finding at odds with the high transmission rates observed in laboratory and mink farm settings. Further evidence of natural transmission to household ferrets has exhibited a lower prevalence than those seen in dogs and cats, with only one currently confirmed case worldwide (15, 30, 31). To investigate whether viral or host genetics played a role in this discrepancy, we utilized available data for analysis and biologically relevant hypothesis generation using computational tools.

Fig. 2. Mustelid-associated mutations in SARS-CoV-2 surface glycoprotein. SARS-CoV-2 surface glycoprotein (S) sequences from natural (mink) and experimental (ferret) infections were compared, and three mutations were identified. (A) A schematic diagram (not to scale) of the S protein with Subunit 1, which is involved in host receptor protein attachment, and Subunit 2, which is involved in host cell fusion. Mutation N501T is located in the RBD and receptor binding motif (RBM), shown in red. Mutation D614G is located in Subunit 1 downstream of the RBD, and mutation S686G is located directly adjacent to the novel S1/S2 cleavage motif (PPAR↓S) processed by furin. A second S1/S2 cleavage site (IAY↓TMS) seen in SARS-CoV is conserved. The S2′ cleavage site (KPSKR↓S) processed by TMPRSS2 is also conserved. Viral amino acid sequences from regions of interest are shown below the schematic, and dots represent conserved residues, using the top sequence as a reference (hCoV-19/Netherlands/NA_296/2020). Viruses from mink are separated into two clades from distinct farms (NB01 and NB02-4, respectively), and are preceded by the closest observed human sequence (hCoV-19/Netherlands) for reference. Experimentally infected ferrets are in the bottom half (F1102 to F1113). The sequence from the human inoculum (hCoV-19/Germany) is included for reference. Ferrets are separated into three groups: donors, which received direct inoculum; direct contact, which were housed with donors; and indirect contact, which were housed adjacent to donors without physical contact. Identical sequences were found from samples taken at 3 and 7 d post inoculation (dpi) in three of four donors. Donor F1105 exhibited two equivalent single-nucleotide variants (A1502C and A2056G) resulting in N501/N501T and S686/S686G, respectively, and not consensus called (“X”) in those locations. (B) The 9,253 human-derived SARS-CoV-2 S protein sequences and 57 animal-derived SARS-CoV-2 or SARS-CoV−like virus S protein sequences were aligned to calculate percent amino acid representation at three positions: N501 (top), D614 (middle), and S686 (bottom).
also the only distinct residue between ferret (G354R) and American mink (G354H) (7). Mink have been naturally infected by virus without the N501T mutation, and there have now been dozens of independent human-to-mink spillover events; therefore, we do not expect that the ACE2 G354H mutation significantly limits infection. However, the appearance of N501T in all infected ferrets suggests ACE2 G354R may provide a host barrier to SARS-CoV-2 entry in ferrets. N501T has appeared as a spontaneous mutation in four mink from four independent farm outbreaks (38). Additional work is needed to determine whether N501T is a required adaptation for ferret transmission and, if so, how it affects transmission dynamics. The increasing prevalence of N501Y among human populations has further raised concerns of increased transmissibility, virulence, and immune evasion related to mutations at this position (39, 40).

SARS-CoV-2 S protein S686G is another intriguing mutation, as it lies directly adjacent to a motif that is likely to enhance virulence (25). As of sequence accession on July 15, 2020, S686 is perfectly conserved in 9,189/9,189 human sequences, indicating strong purifying selection. S686G changes a neutral polar residue to a nonpolar one, which we estimated to decrease furin efficiency. Furthermore, S686 completes a novel glycosaminoglycan (GAG)-binding motif (XBVBXB/PRRARS) that enhances binding, and the two flanking serines in the S1/S2 site (SPRRARSXV) have been shown to be permissive to host phosphorylation and consequent down-regulation of furin activity (26, 41). For these reasons, we were surprised to see evidence of positive selection over time for this potentially unfavorable mutation in ferrets as described by Richard et al. (19). If there is further evidence of S686G selection in experimentally or naturally infected ferrets, it is essential to fully investigate changes in viral fusion activity, kinetics, and pathobiology to determine whether ferrets are an appropriate model for human disease.

Beginning with our initial community science-based surveillance efforts, we observed an unexpected result and went on to use publicly available data to investigate biologically relevant mutations correlated to species-specific infection in an important laboratory model. In addition to providing data to better evaluate risk to pet ferrets, this study unexpectedly led to broader hypotheses about mustelid susceptibility and resistance to SARS-CoV-2 with implications for experimental research and wildlife disease ecology. We propose that the mustelid-specific viral mutations we have identified have biological relevance to infection efficiency and transmission. In laboratory models, positive selection for rare variants away from human wild-type virus may affect outcomes and should be further investigated. Recent campaigns to vaccinate the endangered black-footed ferret and farmed mink are also important for continued, targeted investigation, as our results may mean that inoculation with a nonmustelid adapted variant may not provide sufficient protection.

Our results suggest that virus and host genetic barriers significantly limit natural infection in ferrets, and these are only likely to be overcome by a concentrated and/or diverse inoculum of human-derived virus. To date, successful experimental ferret infections have used ~3 TCID50 virus, and at least one inoculum contained a minority of virus with the N501T and S686G variants (18, 19). These limitations and putative host adaptations may negatively affect ferrets as a disease or transmission model and should be further investigated. The data presented here remind us that synthesis of data from surveillance work, natural experiments, and controlled, laboratory-led studies can lead us to novel hypotheses and investigations and allow us to better respond to this pandemic and prepare for the next. This household provides evidence that human-to-ferret SARS-CoV-2 transmission in domestic settings may be lower risk than would be expected from laboratory experiments.
Mammalian Gene Collection, Assembly, and Phylogenetic Analysis. PSC1-7 and CTS1 sequences were collected from NCBI Orthologs from Homo sapiens (human), Pan troglodytes (chimpanzee), Sus scrofa (pig), Ovis aries (sheep), Bos Taurus (cow), Canis lupus familiaris (dog), Canis lupis dingo (dingo), Vulpes vulpes (fox), Felis catus (cat), Panthera tigris altaica (Siberian tiger), Lontra canadensis (river otter), Enhydra lutris (sea otter), Phoca vitulina (harbor seal), Mustela erminea (ermine), Myotis lucifugus (little brown bat), Eptesicus fuscus (big brown bat), Rousettus aegyptiacus (Egyptian fruit bat), Rhinolophus ferrumequinum (greater horseshoe bat), and Pteropus vampyrus (large flying fox). M. p. furo (ferret) orthologs were inconsistent with related species by preliminary RAxML orthology analysis (50). Seven available RNAseq run from M. p. furo (SRR11517721-SRR11517724, SRR391982, SRR391968, SRR391966) were downloaded, and putative PSC1-7 CTS1 reads were extracted using Basic Local Alignment Search Tool (BLAST) (51). Reads were assembled using Pommoxi mini assembly with ermine references. Reads were then mapped back to the proposed ferret assembly with BWA, and well-supported consensus sequences were called using SAMT (51). Ortholog collections were analyzed using maximum likelihood phylogenetics via RAxML (77) using empirical base frequencies, 5,000 bootstraps) (50). RAxML output are available on a GitHub repository (49).

Data Availability. Alignment and phylogenetic data from genetic sequences have been deposited in a GitHub repository, https://github.com/kssawatzki/Mustelid_COVID_PNAS (49). All other data are included in the manuscript and/or supporting information.

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