Detection of Airborne Influenza A and SARS-CoV-2 Virus Shedding following Ocular Inoculation of Ferrets

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ABSTRACT Despite reports of confirmed human infection following ocular exposure with both influenza A virus (IAV) and SARS-CoV-2, the dynamics of virus spread throughout oculonasal tissues and the relative capacity of virus transmission following ocular inoculation remain poorly understood. Furthermore, the impact of exposure route on subsequent release of airborne viral particles into the air has not been examined previously. To assess this, ferrets were inoculated by the ocular route with A(H1N1)pdm09 and A(H7N9) IAVs and two SARS-CoV-2 (early pandemic Washington/1 and Delta variant) viruses. Virus replication was assessed in both respiratory and ocular specimens, and transmission was evaluated in direct contact or respiratory droplet settings. Viral RNA in aerosols shed by inoculated ferrets was quantified with a two-stage cyclone aerosol sampler (National Institute for Occupational Safety and Health [NIOSH]). All IAV and SARS-CoV-2 viruses mounted a productive and transmissible infection in ferrets following ocular inoculation, with peak viral titers and release of virus-laden aerosols from ferrets indistinguishable from those from ferrets inoculated by previously characterized intranasal inoculation methods. Viral RNA was detected in ferret conjunctival washes from all viruses examined, though infectious virus in this specimen was recovered only following IAV inoculation. Low-dose ocular-only aerosol exposure or inhalation aerosol exposure of ferrets to IAV similarly led to productive infection of ferrets and shedding of aerosolized virus. Viral evolution during infection was comparable between all inoculation routes examined. These data support that both IAV and SARS-CoV-2 can establish a high-titer mammalian infection following ocular exposure that is associated with rapid detection of virus-laden aerosols shed by inoculated animals.

IMPORTANCE Documented human infection with influenza viruses and SARS-CoV-2 has been reported among individuals wearing respiratory protection in the absence of eye protection, highlighting the capacity of these respiratory tract-tropic viruses to exploit nonrespiratory routes of exposure to initiate productive infection. However, comprehensive evaluations of how ocular exposure may modulate virus pathogenicity and transmissibility in mammals relative to respiratory exposure are limited and have not investigated multiple virus families side by side. Using the ferret model, we show that ocular exposure with multiple strains of either coronaviruses or influenza A viruses leads to an infection that results in shedding of detectable aerosolized virus from inoculated animals, contributing toward onward transmission of both viruses to susceptible contacts. Collectively, these studies support that the ocular surface represents a susceptible mucosal surface that, if exposed to a sufficient quantity of either virus, permits establishment of an infection which is similarly transmissible as that following respiratory exposure.

KEYWORDS ocular, SARS-CoV-2, aerosols, influenza
SCOV2 have caused human illness and ocular complications following ocular exposure (2–4), highlighting the potential of these viruses to exploit nonrespiratory routes of entry and replicate in ocular tissues. Like the respiratory tract, the human eye represents a susceptible mucosal surface exposed to viral pathogens (5, 6). Fluidics exchange can occur between ocular and nasopharyngeal tissues, which are bridged by the lacrimal duct, and distribution of viral receptors permissive to both IAV and SCOV2 on the eye, surrounding conjunctiva, lacrimal tissues, and associated sites further facilitates virus exchange between tissue systems (7–10). Transocular entry of IAV-containing aerosols in humans wearing respiratory protection has been demonstrated previously (11); use of eye protection has been associated with lower COVID-19 infection risk (12), and reports of modest reductions of COVID-19 disease in people wearing eyeglasses (relative to populations wearing contact lenses or to those with infrequent use of glasses only) further support a role for ocular exposure or contact in SCOV2 virus entry (13–16). However, the relative risk that IAV and SCOV2 ocular exposure contributes to mammalian infection and transmission remains poorly understood.

Mammalian models can be invaluable for assessing the capacity of respiratory viruses to cause disease by nonrespiratory routes. Ocular inoculation models (encompassing both liquid and aerosol-based exposures) have been established for most principal respiratory pathogens, most frequently employing the mouse and ferret (17–20). These studies have shown that IAV (both human seasonal and zoonotic) have a capacity to replicate within mammalian ocular tissues and use the eye as a portal of entry to establish a productive, and transmissible, respiratory infection (2). However, ocular exposure to coronaviruses has been rarely evaluated in laboratory settings, with a paucity of studies in small mammalian models (21–23). Furthermore, ocular specimens have been only rarely collected from intranasally (i.n.) inoculated animals (24–26), limiting our understanding of how SCOV2 disseminates to ocular tissues and the degree to which IAV and SCOV2 may differ in this ability.

Prior laboratory studies have suggested that while ocular exposure can lead to a robust viral infection in vivo, disease progression, host responses, and viral replication metrics may be delayed relative to those for respiratory exposure (17, 27, 28), and there remain areas where comparative evaluations between exposure routes are still lacking. Transmission of both IAV and SCOV2 strains has been shown to be mediated by aerosols in small mammalian models following respiratory inoculation (29, 30). However, viral aerosol shedding profiles in animals exposed by the ocular route have not been investigated previously in vivo with any respiratory virus, reducing our ability to judge the relative risk ocular exposure may contribute to onward transmissibility to susceptible contacts. Additionally, while intra-host and interhost virus adaptation is frequently assessed following respiratory inoculation routes in vivo (31, 32), it is unknown if the delayed kinetics of virus replication associated with ocular exposure has implications for virus evolution during the acute phase of infection.

Here, we inoculated ferrets by the liquid ocular route (o.c.) with two IAV and two SCOV2 strains previously shown to replicate efficiently following intranasal (i.n.) inoculation (26, 33, 34). Following inoculation, we examined virus pathogenicity, transmissibility, aerosolized virus shedding profiles, and emergence of variant virus populations during the course of infection. All viruses examined caused a productive respiratory infection following ocular inoculation, with the duration and magnitude of viral shedding in the upper respiratory tract and in aerosols comparable to those following respiratory inoculation. Delivery of a low-dose aerosolized IAV inoculum exclusively to the ferretocular surface (OA) was also capable of establishing a respiratory infection of a magnitude comparable to that of aerosol inhalation (AR), characterized by shedding detectable virus particles in the air. These studies support the risk posed by ocular exposure to respiratory pathogens, even when respiratory exposure is not present.

RESULTS

Transmission of IAV in ferrets following liquid ocular inoculation. Previous studies have shown that inoculation of influenza A virus (IAV) in a liquid suspension, deposited onto the ocular surface (o.c.), can lead to a productive and transmissible infection
Airborne Influenza and SARS-CoV-2 Shedding in Ferrets

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TABLE 1 Pathogenicity and viral titer following liquid ocular inoculation of ferrets with IAV and SCoV2 strains

| Virus   | Group  | Wt lossb | Tempb | Peak NW (PFU)c | Peak NW (RNA)c | Peak CW (PFU)d | Peak CW (RNA)d | Peak RS (RNA)d | Serologyd |
|---------|--------|----------|-------|----------------|----------------|----------------|----------------|----------------|-----------|
| Neb/14  | inoc   | 6.0 1.7  | 6.6 ± 0.6 | 9.6 ± 0.1 | 4.3 ± 0.1 | 7.6 ± 0.4 | 2.7 ± 0.2* | 3/3            |
|         | RDT    | 5.1 1.1  | 6.7 ± 0.5* | 9.2 ± 0.4 | 4.0 ± 0.7* | 7.3 ± 0.6 | 5.9*          | 2/3            |
| Anhui/1 | inoc   | 2.0 1.2  | 6.1 ± 0.3 | 8.7 ± 0.2 | 1.6 ± 0.6 | 5.2 ± 0.2 | <1            | 3/3            |
|         | DCT    | 5.7 1.6  | 6.2 ± 0.3 | 8.7 ± 0.1 | 1.9 ± 0.1* | 5.2 ± 0.3 | 3.8 ± 0.6* | 3/3            |
| WA/1    | inoc   | 2.1 0.9  | 4.5 ± 0.8 | 8.1 ± 0.7 | <1            | 4.1 ± 0.5 | 5.7 ± 0.5 | 3/3            |
|         | DCT    | 4.4* 0.8 | 4.1 ± 0.3 | 7.8 ± 0.2 | <1            | 4.2 ± 0.3 | 6.3 ± 0.5* | 3/3            |
| Delta   | inoc   | 4.3* 0.6 | 5.0 ± 0.2 | 9.0 ± 0.3 | <1            | 4.5 ± 0.2 | 5.8 ± 0.5 | 3/3            |
|         | DCT    | 2.9 0.6  | 5.0 ± 0.0 | 7.3 ± 2.0 | <1            | 4.0 ± 0.6 | 5.6 ± 1.0* | 2/3            |

*Ferrets were inoculated (inoc) ocularly with 10^7 PFU of virus in a 100-μL volume (n = 3). At 24 h p.i., a contact ferret was introduced in an adjacent cage with perforated side walls (respiratory droplet transmission [RDT], 3 ferret pairs) or in the same cage (direct contact transmission [DCT], 3 ferret pairs).

Mean maximum weight loss (percent loss from baseline) or mean peak temperature rise (degrees Celsius) above baseline (37.6 to 38.9) among all ferrets in the group between days 1 and 14 p.i./p.c. (values are inclusive of all ferrets per group unless indicated by *, in which case values are n = 2 due to no weight loss or elevated temperature detected in one ferret).

Peak viral titers as measured by log10 PFU per milliliter or log10 copies RNA per milliliter in nasal wash (NW), conjunctival wash (CW), or rectal swab (RS) specimens ± standard deviation, between days 1 and 15 p.i./p.c. Values are inclusive of all ferrets per group unless indicated by *, in which case values are reflective of all but one ferret in the group due to lack of detectable virus above limit of detection (10 PFU/mL or 1 copy RNA/mL).

Serum was tested by hemagglutination inhibition assay using homologous virus and 0.5% red blood cells (IAV) or by ELISA using homologous virus recombinant S proteins (SCoV2); number of ferrets seroconverted among all inoculated animals is shown.

One contact ferret in this group had no detectable virus in any specimen examined and did not seroconvert to homologous virus; data from this ferret have been excluded from these analyses, and values in this row reflect n = 2 infected contact animals unless otherwise specified.

in ferrets (28, 35). To extend this work and further study viral infection dynamics following ocular exposure by examining virus release into the air, we inoculated ferrets by the o.c. route with two different IAV: a contemporary A(H1N1)pdm09 virus derived from the 2009 pandemic well adapted for human infection (A/Nebraska/14/2019 [Neb/14]) and a first-wave low-pathogenic avian influenza (LPAI) A(H7N9) virus (A/Anhui/1/2013 [Anhui/1]) (33, 34). Virus transmissibility was assessed by establishing contact 1 day later with an uninfected ferret; contact ferrets were either placed in an adjacent cage with perforated side walls to permit air exchange in the absence of direct or indirect contact (respiratory droplet transmission [RDT] model) or cohoused in the same solid-walled cage (direct contact transmission [DCT] model). Nasal wash (NW), conjunctival wash/swab (CW), and rectal swab (RS) specimens were collected from all animals on alternate days postinoculation (p.i.) or postcontact (p.c.) for 11 to 13 days for quantification of infectious virus by standard plaque assay or viral RNA by quantitative real-time reverse transcription-PCR (qRT-PCR).

All ferrets inoculated by the ocular route became productively infected, shedding infectious virus to high titers in respiratory tract specimens (>6 log10 PFU/mL) and seroconverting to homologous virus (Table 1). Viral RNA persisted in NW following clearance of infectious virus for both viruses (Fig. 1A). Beyond the respiratory tract, all IAV-inoculated ferrets had detectable viral RNA in CW through day 7 p.i., and all but one ferret had detectable infectious virus in this specimen (Fig. 1B); peak mean infectious viral titers in CW were >100-fold higher in Neb/14 than in Anhui/1 virus-inoculated ferrets (Table 1). In contrast, viral RNA was only infrequently detected in RS specimens from these animals (Fig. 1C). Transient weight loss and fever were observed in all animals, and intermittent sneezing was detected in 3/3 and 2/3 Neb/14 and Anhui/1 virus-inoculated animals, respectively, but clinical signs were overall mild and in agreement with previous assessments of these viruses in ferrets (33, 34).

Neb/14 and Anhui/1 IAV have been shown previously to transmit efficiently after intranasal inoculation in RDT or DCT models, respectively (33, 34). Following ocular inoculation, 2/3 (Neb/14, RDT) and 3/3 (Anhui/1, DCT) contact ferrets were productively infected in these settings and seroconverted to homologous virus by the end of the experimental period (Table 1). In both experiments, transmission was delayed 1 to 2 days relative to that for ferrets inoculated by the i.n. route, as is typical when donor ferrets are inoculated by the ocular route (35). Generally comparable clinical signs and similar
FIG 1 Pathogenesis, transmission, and release of airborne viral RNA collected from ferrets inoculated by the ocular route with IAV. Ferrets (n = 3) were inoculated with 6.0 log₁₀ PFU of Neb/14 or Anhui/1 virus by the ocular route (100 μL). The next day, a naive ferret was placed in (Continued on next page)
peak titers in NW, CW, and RS specimens between o.c.-inoculated ferrets and contact ferrets indicate that ocular inoculation can result in a transmissible infection that is similarly robust as that in ferrets that contract IAV infection via direct, indirect, or airborne exposure (Table 1; Fig. 1).

Ferrets infected with IAV have been shown to exhale virus-laden aerosols (36), but quantification of exhaled aerosols from ferrets following ocular inoculation has not been performed previously. Air was sampled for 1 h from conscious and alert ferrets inoculated by the o.c. route with either Neb/14 or Anhui/1 IAV every other day 1 to 11 p.i., employing National Institute for Occupational Safety and Health (NIOSH) cyclone samplers that size fractionate airborne particles into >4-μm, 1- to 4-μm, and <1-μm fractions (37). For both IAV examined, ferrets inoculated by the o.c. route shed viral RNA in aerosols at comparable levels, predominantly in the >4-μm fraction; Neb/14 and Anhui/1 virus-infected ferrets shed peak mean titers of 4.5 and 4.9 log_{10} viral RNA copies/L of air or 6.8 and 7.3 log_{10} viral RNA copies over the course of an hour, respectively (Fig. 1D). Persistence of viral RNA in collected air through day 11 p.i. was in agreement with prolonged detection of viral RNA in NW specimens (Fig. 1A). Taken together, we confirmed that high-dose, liquid ocular inoculation of IAV leads to a productive and transmissible infection in ferrets and showed that this is associated with increased shedding of aerosolized viral RNA from inoculated ferrets during the acute phase of infection.

Transmission of SCOV2 in ferrets following liquid ocular inoculation. Similarly to IAV, SARS-CoV-2 (SCOV2) is a predominantly respiratory virus that has occasionally been associated with ocular exposure or ocular complications in humans. We recently found that SCOV2 RNA could be detected sporadically in ferret CW specimens following i.n. inoculation (26), but the capacity of SCOV2 to cause infection following ocular exposure in ferrets was not known. Employing the same experimental design as described above, ferrets were inoculated by the o.c. route with two different SCOV2 strains: Washington/1/2020 (WA/1) and Delta variant (B.1.617.2).

Previous studies have identified that ferrets do not recapitulate most clinical signs of COVID-19 following i.n. inoculation (38); unsurprisingly, ferrets inoculated by the o.c. route with either SCOV2 strain exhibited only mild signs of disease, with peak mean weight loss of <5% and transient rises in temperature of <1°C above baseline (Table 1). Nonetheless, o.c. inoculation with SCOV2 strains led to productive infection of all animals, with ferrets shedding detectable virus in NW specimens through days 5 to 7 p.i. and viral RNA persisting in this sample until days 11 to 13 p.i. (Fig. 2A). While infectious virus was not recovered from CW or RS specimens, viral RNA was detected from all animals in both specimen types, during periods of active virus replication in the respiratory tract (Fig. 2B and C).

WA/1 and Delta SCOV2 strains were previously shown to transmit efficiently in a ferret DCT model following i.n. inoculation (26); comparable transmissibility with the two viruses was detected following o.c. inoculation, despite a 1- to 2-day delay in infectious virus detection in contact animals relative to donor animals inoculated intranasally (Fig. 2A). WA/1 virus transmitted efficiently to 3/3 contact ferrets; Delta viral RNA was detected in NW, CW, and RS specimens from all contact ferrets, but only 2/3 contact ferrets shed infectious virus to detectable levels and seroconverted to homologous virus by the end of the study (Table 1). Overall, contact ferrets exhibited mild signs of illness and shed virus in NW specimens with peak titers similar to those of inoculated ferrets.

FIG 1 Legend (Continued)
FIG 2 Pathogenesis, transmission, and release of airborne viral RNA collected from ferrets inoculated by the ocular route with SCOV2. Ferrets \( (n = 3) \) were inoculated with 6.0 log10 PFU of WA/1 or Delta virus by the ocular route (100 μL). The next day, a naive ferret was cohoused (DCT) (Continued on next page)
Anhui/1 Ocular 0.9
study, but viral RNA was sporadically detected postexposure in NW (4.7 and 4.1 log10 with aerosolized IAV did not shed infectious virus or seroconvert at the end of the study (Table 2; Fig. 3A). The remaining two ferrets that were exposed by the OA route NW and CW specimens and seroconversion to homologous virus at the end of the ferrets became productively infected, as evidenced by detection of infectious virus in viruses by the AR route at similar presented doses (Table 2).

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ties of aerosolized IAV are delivered exclusively to the ocular surface of sedated ferrets (17). To assess the potential of viral RNA shed into the air following a physiologically relevant ocular-only challenge, ferrets were inoculated by the OA route with Neb/14 and Anhui/1 IAV. For comparison, an additional set of ferrets was inoculated with both viruses by the AR route at similar presented doses (Table 2).

Following low-dose (presented dose of <5 PFU) OA exposure to Neb/14 virus, 1/3 ferrets became productively infected, as evidenced by detection of infectious virus in NW and CW specimens and seroconversion to homologous virus at the end of the study (Table 2; Fig. 3A). The remaining two ferrets that were exposed by the OA route with aerosolized IAV did not shed infectious virus or seroconvert at the end of the study, but viral RNA was sporadically detected postexposure in NW (4.7 and 4.1 log10 viral RNA copies at day 1 and day 3 p.i., respectively; these quantities were indistinguishable from those from ferrets inoculated by the i.n. route (26). Collectively, like IAV, SCOV2 strains led to a productive and transmissible infection in ferrets following o.c. inoculation, with shedding of virus-laden aerosols during the acute phase of infection.

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trast, exposure of ferrets by the AR route to Neb/14 IAV led to a robust infection in all exposed animals (Fig. 3B), reaching peak mean NW titers comparable to those of donor animals that can subsequently establish a robust infec-
tion in contact animals following virus transmission.

Exhaled aerosols from donor animals were collected with NIOSH devices to investigate if o.c. inoculation with SCOV2 strains leads to release of virus-laden particles from infected animals. As with IAV, all ferrets inoculated by the o.c. route shed viral RNA in aerosols of >4 μm (Fig. 2D). For WA/1 and Delta viruses, ferrets exposed by the o.c. route shed peak mean titers of 3.7 and 4.6 log10 viral RNA copies/L of air, or 6.1 and 6.6 log10 viral RNA copies over the course of an hour, respectively; these quantities were indistinguishable from those from ferrets inoculated by the i.n. route (26). Collectively, like IAV, SCOV2 strains led to a productive and transmissible infection in ferrets following o.c. inoculation, with shedding of virus-laden aerosols during the acute phase of infection.

FIG 2 Legend (Continued)
model) with each inoculated ferret (3 ferret pairs/virus) to assess virus transmissibility. NW, CW, and RS were collected on alternate days p.i./p.c. (A to C, respectively). All specimens had titers determined by standard plaque assay to determine infectious virus load (left y axis, solid bars) or were subjected to real-time qRT-PCR to determine viral RNA load (right y axis, gray shading); limit of detection was 1.0 log10 PFU/mL or 2.9 log10 RNA copies/mL, respectively. Aerosol samples were collected from all inoculated animals on alternate days p.i. for 1 h using a cyclone sampler which size fractionated aerosol particles: >4 μm (solid bars), 1 to 4 μm (horizontally striped bars), or <1 μm (diagonally striped bars) (D). Viral RNA in each sample was quantified as described above; stacked bars depict total viral load in all fractions of >100 RNA copies/L of air.

### TABLE 2 Ferret pathogenicity and viral titer data following aerosol-based inoculation

| Virus Group | PD | Wt loss | Temp | Peak NW (PFU) | Peak NW (RNA) | Peak CW (PFU) | Peak CW (RNA) | Peak RS (RNA) | Serology |
|-------------|----|---------|------|--------------|--------------|--------------|--------------|--------------|----------|
| Neb/14 Ocular | 0.4–2.1 (1/3) | 14.3 | 1.3 | 5.6 | 9.5 | 5.0 | 1.8 ± 0.3 | 5.6 ± 0.1 | 5.9 ± 0.1 | 1/3 |
| Neb/14 Inhalation | 5.8–6.9 (3/3) | 7.5 | 1.3 | 6.6 ± 0.4 | 9.6 ± 0.1 | 1.3 | 1.7 ± 0.3 | 5.7 ± 0.1 | 5.5 ± 0.1 | 3/3 |
| Anhui/1 Ocular | 0.9–5.2 (2/3) | 1.4 | 1.1 | 5.6 ± 1.3 | 9.2 ± 2.7 | <1 | 5.4* | 5.1* | 2/3 |
| Anhui/1 Inhalation | 40.8–48 (2/3) | 1.4 | 1.5 | 6.1 ± 0.4 | 11.1 ± 0.9 | <1 | 6.1* | 5.9* | 2/3 |

*aFerrets were inoculated with aerosolized IAV by o.c. or inhalation routes with presented dose (PD, reported in PFU per milliliter) for 15-min exposures (n = 3). Number of ferrets with detectable infectious virus following inoculation is specified in parentheses.

*bMean maximum weight loss (percent loss from baseline) or mean peak temperature rise (degrees Celsius) above baseline (37.8 to 38.8) among all productively infected ferrets in the group during days 1 and 14 p.i./p.c.

*cPeak viral titers as measured by log10 PFU per milliliter or log10 copies RNA per milliliter in nasal wash (NW), conjunctival wash (CW), or rectal swab (RS) specimens ± standard deviation, among productively infected ferrets, between days 1 and 15 p.i./p.c. Values are inclusive of all ferrets per group unless indicated by *, in which case values are reflective of all but one ferret in the group due to lack of detectable virus above limit of detection (10 PFU/mL or 1 copy RNA/mL).

*dSera were collected from ferrets and tested by hemagglutination inhibition assay; number of ferrets that seroconverted to homologous virus (day 21 p.i.) out of total number of inoculated animals in each group is shown.

animals (Table 1), further supporting that o.c. inoculation with SCOV2 strains leads to a productive infection in donor animals that can subsequently establish a robust infection in contact animals following virus transmission.

Extent of aerosolized virus particles delivered to the ocular surface of ferrets is unknown. Aerosolized IAV was delivered to the ocular surface of sedated ferrets using an OA exposure method (Fig. 2). This OA exposure method delivered virus as a size fractionated aerosol with the majority of particles (95%) falling in the size range of 3.7 to 4.6 μm (Fig. 2D). Viral RNA loads in aerosols (peak NW and CW) were highest during the acute phase of infection (Table 2). A small fraction of aerosolized virus particles was deposited in the nasal wash (NW) specimens of ferrets inoculated with high doses of virus by standard intranasal or liquid ocular inoc-
Two of three ferrets became productively infected following either OA or AR exposure to Anhui/1 virus (Table 2; Fig. 4A and B). As with Neb/14 AR exposures, ferrets that remained seronegative to Anhui/1 virus at the end of the study nonetheless had sporadic detectable viral RNA in specimens collected through day 11 p.i.: for the OA challenge, day 9 RS and day 11 NW specimens had 4.1 and 3.9 log\(_{10}\) viral RNA copies, respectively, and for the AR challenge, day 7 NW and CW specimens had 4.2 and 4.8 log\(_{10}\) viral RNA copies, respectively, and day 9 RS had 5.6 log\(_{10}\) viral RNA copies.

Viral RNA was found in air sampled from all ferrets exposed to aerosolized IAV, regardless of the exposure route or subsequent infectivity postexposure. For Neb/14 virus, ferrets exposed by the OA or AR route shed peak mean titers of 4.2 and 5.8 log\(_{10}\) viral RNA copies/L of air, or 6.5 and 8.1 log\(_{10}\) viral RNA copies over the course of an hour, respectively (Fig. 3C and D). Overall, total counts of virus-containing aerosols at days 1 to 11 p.i. were higher for ferrets inoculated by the AR route (4.8 to 6.4 log\(_{10}\) viral RNA copies/L of air) than for ferrets inoculated by the OA route (3.3 to 4.7 log\(_{10}\) viral RNA copies/L of air). For Anhui/1 virus, ferrets exposed by the OA or AR route shed...
peak mean titers of 5.8 and 5.0 log_{10} viral RNA copies/L of air, or 8.1 and 7.3 log_{10} viral RNA copies over the course of an hour, respectively (Fig. 4C and D). Independent of inoculation route, higher overall total counts of virus-containing aerosols at days 1 to 11 p.i. were found among ferrets with detectable Anhui/1 virus in NW specimens (4.0 to 6.3 log_{10} viral RNA copies/L of air) than among ferrets that remained seronegative at the end of the study (3.6 log_{10} viral RNA copies/L of air). Collectively, these data support that low-dose ocular-only aerosol IAV exposure can lead to a productive infection, with virus-laden aerosols released by infected ferrets at levels similar to those observed following inhalation challenge.

**Contribution of inoculation route to interhost and intrahost virus adaptation.** While o.c. inoculation with IAV and SCOV2 led to productive and transmissible infections in ferrets comparable to those observed following i.n. inoculation, it was unknown if delayed kinetics of virus replication and transmission following an ocular exposure route altered the selection of mutants conferring host adaptation relative to traditional i.n. methods. NW specimens from all ferrets were sequenced and analyzed for the emergence of minor variant populations. With regard to SCOV2, nucleotide
polymorphisms (notably resulting in spike Q498H and N501T variants) which emerged in ferrets following i.n. inoculation of WA/1 were also detected with similar dynamics and frequency in ferrets inoculated by the o.c. route and infected contact animals; no major polymorphisms (>10% frequency) were detected in ferrets inoculated o.c. with Delta virus. Similarly, Neb/14 virus variants at HA positions 156, 187, and 222 were detected in most inoculated ferrets (at ≥30% frequency) regardless of inoculation route, with the same variants often detected at increased frequencies (≥45%) in virus-positive contact ferrets.

The Anhui/1 virus inoculum harbored a mixture of variants at HA positions 123D/N (59.3%/38.7%), 125A/T (83.7%/15.2%), and 149D/N (61.1%/38.4%) and NA position 10T/I (63.1%/36.9%). Variant species and frequencies observed from ferrets inoculated either o.c. or AR with Anhui/1 virus were largely reflective of virus inoculum, with the variants HA-123N, -125A, and -149N becoming predominant with ≥98% frequency in at least 2 out of 3 contact animals cohoused with ferrets inoculated by the ocular liquid route (o.c) (Fig. 5). Interestingly, both ferrets inoculated by OA exposure harbored only single polymorphisms at HA positions 123, 125, and 149 and NA position 10 with disparate mutations (either D or N) at HA positions 123 and 149, suggesting that productive infection during ocular-only aerosol exposure (OA) was most likely initiated by a very low number of infectious viral particles.

To further determine if within-host genetic diversity was dependent on inoculation route, viral diversity in NW specimens was assessed using the Shannon diversity index. Comparable Shannon diversity indexes were found between all IAV-infected ferrets (including directly inoculated animals and contact animals independent of inoculation method), with mean Shannon index (H) per group ranging from 1.19 to 1.97 and 1.23 to 1.91 for

**FIG 5** Frequency of HA and NA variants following Anhui/1 IAV infection in ferrets. Ferrets were inoculated or exposed to Anhui/1 virus by the routes specified (Ino, stock used for inoculation; o.c., liquid ocular; o.c. direct contact, contact animals for o.c.-inoculated ferrets; OA, ocular-only aerosol; AR, inhalation aerosol). Bars represent individual inoculated (F) or contact (C) ferrets; day (D) of NW collection is specified.
Neb/14 and Anhui/1 viruses, respectively. Taken together, these results suggest that both IAV and SCOV2 adapt to the ferret host by acquiring similar mutations regardless of the route of virus inoculation, with population bottlenecks possible during either respiratory or ocular low-dose exposures.

**DISCUSSION**

Influenza virus and SCOV2 are respiratory tract-tropic viruses in humans, but the presence of viral receptors on the human ocular surface and detection of virus in ocular fluids in infected individuals support the capacity for these viruses to nonetheless replicate within ocular tissue and use the eye as a portal of entry to establish a productive respiratory infection (5, 9, 39). Ocular complications, typically conjunctivitis, have been reported in humans for both circulating influenza viruses and coronaviruses (2, 40, 41). For example, conjunctivitis has been reported in 0.45 to 0.7% of confirmed seasonal influenza A and B cases and approximately 0.8% of confirmed SARS-CoV-2 cases (2, 42, 43). Collectively, epidemiological studies support that these ocular complications represent an uncommon but important clinical sign of influenza and COVID-19. While infectious virus and/or viral RNA has been detected in ocular specimens collected from humans infected with both IAV and SCOV2 (2, 44–46), a lack of routine collection of ocular samples limits our understanding of the role ocular tissues may play in infection or disease progression. Understanding the relative risk that ocular exposure contributes to mammalian infection and disease is needed to capture the full range of disease possible with respiratory viruses. Ferrets are naturally susceptible to both human and zoonotic influenza viruses and coronaviruses and have ocular anatomy and vision systems generally comparable to those of humans, making them a desirable small mammalian model to emulate ocular exposure of both viruses (47–49). In this study, we performed parallel studies with two respiratory viruses of public health relevance, IAV and SCOV2, to examine the capacity of each virus to cause infection following ocular exposure. We found that both viruses were able to mount a productive and transmissible mammalian infection following inoculation. Detection of respiratory viruses in ocular tissues following i.n. inoculation of ferrets has been reported previously. A(H1N1)pdm09 IAV derived from the 2009 pandemic exhibits an enhanced capacity for ocular involvement following i.n. inoculation compared to other circulating seasonal virus subtypes in humans (28, 50); detection of Neb/14 virus in ocular specimens from ferrets inoculated by aerosol inhalation in this study further supports this finding (Table 2). While LPAI A(H7N9) viruses do not exhibit the ocular tropism present among many H7 subtype viruses, they have been detected in ferret ocular samples following i.n. inoculation (33). Multiple isolates of SCOV2 have been detected in ocular tissues and specimens following i.n. inoculation in ferrets, including both the WA/1 and the Delta strain used in this study; these viruses were previously detected in ocular wash and tissues (eye and conjunctiva) collected during necropsy at higher titers and frequency than those of Alpha or Beta variants in ferrets (24, 26). Collectively, these studies support the need to more frequently examine ocular tissues during intranasal inoculation with a range of respiratory viruses, as strain-specific differences in the capacity to spread to ocular tissues have been noted. Reports of inflammation of ocular tissues and alteration of cellular processes in ocular cell types following i.n. inoculation of ferrets with IAV further demonstrate the need to examine ocular tissues in the context of respiratory virus infection (28, 51). Macroscopic ocular disease was not observed in ferrets following ocular inoculation in this study (either o.c. or OA routes), which is not surprising considering the generally mild infection and limited clinical symptoms presented by ferrets following i.n. inoculation with seasonal IAV and SCOV2 (38, 47).

Ocular inoculation with a respiratory pathogen can take place via numerous modes, including exposure of the ocular surface to virus-laden aerosols or respiratory droplets or direct contact with a contaminated surface (such as a fingertip) or via splashing of a virus-containing liquid (5). Previous studies have shown that inoculation of respiratory viruses (including both IAV and SCOV2) with a liquid inoculum deposited on the ocular
surface represents a reliable technique to infect mammals in the laboratory (18, 19, 21, 35, 52); however, results can vary based on the ocular inoculation method and dose employed. For example, inoculation of a murine coronavirus into the anterior chamber of the mouse eye resulted in ocular pathology and detection of infectious virus in this tissue; topical administration of virus to the corneal surface resulted in ocular pathology only following concurrent scarification (22). It should be noted that in the intraocular (i.o.) model employed here, use of a liquid inoculum permits both virus exposure to the ocular surface (leading to virus replication in ocular tissues) and replication-independent drainage via the lacrimal duct to nasopharyngeal sites (28, 35), emulating a different mode of ocular exposure than OA inoculation, which is mediated by an aerosol and not liquid exposure (17). Generally comparable peak mean titers in nasal wash (NW) and conjunctival wash (CW) specimens, and shedding of viral RNA in aerosols, between ferrets inoculated with IAV following either i.o. or OA inoculation support the capacity of IAV to mount a productive mammalian infection via multiple ocular transmission modes. Both IAV strains employed in this study have been shown to replicate in tissues within and beyond the respiratory tract following ocular inoculation (28, 53); examination of the capacity of SCOV2 to replicate in discrete tissues following ocular inoculation would be warranted. As CW specimens capture virus present on the ocular surface but cannot show if the source of this virus is due to replication specifically within ocular tissue or due to the presence of virus from fluidics exchange with nasopharyngeal tissues, evaluation of ocular tissues from SCOV2-inoculated animals would be of high interest. Further examination of additional inoculation routes, notably those which employ an aerosolized inoculum, will provide greater information regarding the capacity of SCOV2 specifically, and respiratory viruses in general, to establish infection via nonrespiratory exposures.

We have shown previously the capacity of ferrets inoculated by numerous ocular routes with IAV to establish a transmissible infection (17, 35). Our finding that o.c. inoculation with SCOV2 can also result in productive virus transmission to susceptible contacts at frequency comparable to that of i.n. inoculation (Fig. 2) underscores the capacity of multiple respiratory viruses to transmit between mammals following ocular exposure. Here, we extend this work to further demonstrate that a viral infection initiated by an ocular exposure can result in release of virus-containing aerosols at sizes, quantities, and kinetics generally comparable to those of respiratory exposure (26, 29; Pulit-Penaloza, unpublished). Detection of aerosolized viral RNA for several days after infectious virus clearance in NW specimens in ferrets inoculated by the o.c. route is in agreement with similar detection patterns following i.n. challenge (26; Pulit-Penaloza, unpublished). The NIOSH two-stage cyclone sampler employed in these studies permits size fractionation of aerosol particles and recovery of viral RNA but does not retain virus viability; future studies to quantify infectious virus in aerosols are warranted. Furthermore, collection of aerosols directly from exhaled breath (36), and not from just the air surrounding inoculated animals, would provide additional contextual information about virus-containing aerosols released from inoculated ferrets.

Within-host and between-host viral diversity is typically examined following respiratory exposure (31), but examination of variant emergence and global viral diversity following ocular exposure has not been performed previously. Generally comparable emergence of variants between ferrets inoculated with either IAV or SCOV2 by different routes supports similarities in disease progression once a productive infection has been established. Furthermore, infection bottlenecks were detected in ferrets following exposure by both low-dose aerosol ocular and inhalation routes, suggesting that, like respiratory inhalation, ocular aerosol exposure might provide a unique opportunity for a minor variant to quickly become a dominant species in the host. Considering the diversity of modes by which mammals can be infected with RNA viruses, further study of virus evolution as a function of exposure route and dose is warranted. Of note, the Anhui/1 A(H7N9) virus used in these studies harbored a mixture of variants at HA positions 123D/N (133, H3 numbering), 125A/T (135, H3 numbering), and 149D/N and NA position 107/I. The presence of HA-123N and -125T can lead to a glycosylation site,
previously observed in immune escape mutants (54). Gaining a glycan at HA position N123 in the H7N9 virus has been shown to reduce receptor binding affinity for both human and avian-like receptors (55). Viruses recovered from contact ferrets cohoused with animals inoculated with H7N9 virus by the o.c. route predominantly harbored variants without the glycan at 123 with the residues HA-123N and -125A, confirming that the virus with better affinity for receptors had a transmission advantage.

Ferrets represent just one of multiple mammalian species shown to support influenza virus or coronavirus replication in ocular tissues. Zoonotic coronaviruses have been shown to cause ocular complications in different mammalian species, including mice, felines, and ferrets (22, 56–58). Both coronavirus and IAV have been detected in conjunctiva tissue from common marmosets or macaques following joint inoculation at respiratory tract and ocular sites (59–61). Recovery of SCOV2 from eyelid tissue following high-dose i.n. inoculation in Golden Syrian hamsters supports the need to more closely examine ocular involvement in other mammalian models that better recapitulate the severity of COVID-19 (25, 38), especially as studies in this model support differences in disease severity based on the route of inoculation (23). Routine collection of ocular specimens and tissues during standard pathotyping assessments for both viruses would improve our understanding regarding the frequency with which these strains exhibit extrapulmonary spread to proximal tissues.

While both IAV and SCOV2 productively infected ferrets following ocular inoculation, infectious virus in ocular specimens was recovered only from ferrets challenged with IAV. This is supported by higher levels of viral RNA in CW specimens collected from ferrets challenged with IAV than in those collected from ferrets challenged with SCOV2, despite comparable high levels of viral RNA detected in NW specimens from all ferrets. Further investigation regarding the relative density and distribution of permissive receptors for both viruses throughout periocular tissues will permit greater insight regarding their relative capacity to support viral replication. However, while differences in the ability of mammalian ocular tissues to support viral replication are present at both strain-specific and virus-specific levels, it is clear that heterogenous RNA viruses are collectively able to exploit the ocular surface to various degrees to cause a productive infection in mammals. As confirmed infection of humans wearing respiratory protection in the absence of eye protection has been documented for both IAV and SCOV2 (2, 3, 62), collectively this work supports existing recommendations to include eye protection in addition to respiratory protection when IAV or SCOV2 infection risks are present (63, 64).

MATERIALS AND METHODS

Viruses. The A(H1N1)pdm09 influenza A viruses (IAV) A/Nebraska/14/2019 (Neb/14) and LPAI A(H7N9) IAV A/Anhui/1/2013 (Anhui/1) were propagated in MDCK cells and 10-day-old embryonated chicken eggs at 37°C, respectively, as described previously (33, 34). SARS-CoV-2 (SCOV2) strains hCoV-19/USA/TN-CDC3956481-001/2021 (WA/1) and hCoV-19/USA/KY-CDC-2-4242084/2021 (Delta) were propagated in Vero E6 cells expressing serine protease TMPRSS2 (Vero E6/TMPRSS2) at 37°C as described previously (26, 65).

Ferret experiments. All animal procedures were approved by the CDC IACUC and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institutional facility. Male Fitch ferrets (Triple F Farms, Sayre, PA), 9 to 12 months of age, were housed in Duo-Flow Bioclean mobile units (Lab Products, Inc., Seaford, DE) for the duration of each experiment. Ferrets were sedated with a ketamine-xylazine cocktail intramuscularly (i.m.) for all inoculation and sampling procedures. For ocular inoculations with a liquid inoculum, 5 log10 PFU of each virus diluted to a final volume of 100 μL in phosphate-buffered saline (PBS) was deposited onto the right eye of the ferret and massaged into the eye with the eyelid as previously described (35). For ocular aerosol inoculations, ferrets were fitted with goggles within a secondary exposure chamber, and the ocular surface of both eyes was exposed to aerosolized IAV for 15 min, as previously described (17). For respiratory inhalation aerosol inoculations, ferrets were fitted with goggles within a secondary exposure chamber, and the ocular surface of both eyes was exposed to aerosolized IAV for 15 min, as previously described (35). All aerosol inoculation procedures were conducted at 20°C and 50% relative humidity. Presented doses are shown in Table 2. The aerosol and inhalation presented doses were calculated by multiplying the concentration of virus in the aerosol by the exposure time (ocular) and respiratory minute volume (inhalation). In both settings, the presented dose represents the quantity of virus to which the ocular surface was exposed or the quantity of virus inhaled by the ferret, not the amount deposited onto the ocular or respiratory surface. Respiratory droplet transmission (RDT) or direct contact transmission (DCT) models were established 24 h p.i. by housing a serologically naive ferret in a cage with perforated side walls adjacent to
the cage of an inoculated ferret or within the same solid-walled cage as an inoculated ferret, respectively; all transmission experiments were conducted at a 1:1 inoculated/contact ratio.

Ferrets were observed daily for clinical signs of infection. Temperature readings were obtained using a subcutaneous temperature transponder (IPTT-300; BMDS, Seafood, DE) inserted into the dorsal space between the scapulae. Nasal wash (NW), conjunctival swab/wash (CW), and rectal swab (RS) specimens were collected from both donor and contact ferrets (as previously described [35]) on alternate days p.i./p.c. as indicated; all specimens were immediately frozen at −80°C until viral titer determination. Convalescent-phase serum was collected from ferrets on days 21/20 p.i./p.c. (IAV) or days 27/26 p.i./p.c. (SCOV2), respectively. Seroconversion to homologous virus was determined by hemagglutination assay with 0.5% red blood cells (IAV; naive ferrets, <10; positive seroconversion, 80 to 5,120) or by enzyme-linked immunosorbent assay (ELISA) using recombinant SARS-CoV-2 S1 + S2 proteins (SCOV2; naive ferrets, 0 to 0.12; positive seroconversion, 1.28 to 2.17) as previously described (26, 66).

**Aerosol collection experiments.** Aerosol samples were collected on alternate days 1 to 11 p.i. from all inoculated ferrets with a NIOSH BC 251 two-stage cyclone aerosol sampler as described elsewhere (Pulit-Penalosa et al., unpublished). Briefly, prior to all other scheduled aerosol sample collections and at the same time each day, awake and conscious ferrets were removed from their standard housing environment (68.58 cm long [L] by 28 cm wide [W] by 40.6 cm high [H]) and individually held in enclosed and ventilated smaller transport containers (23.9 L in size) for 1 h, during which time air was sampled from each container at 68.58 cm/s by two-stage aerosol samplers. Airborne viruses were collected on alternate days 1 to 11 p.i. from all stages of the experiment. Viral RNA was isolated from all stages for IAV or for SCOV2. Viral RNA copy numbers were normalized and expressed as RNA copy number per milliliter (ferret specimens) or RNA copy number per liter of air (aerosol samples); specimens with gene copy numbers of >10^3 copies/L of air in the first (>4-μm) stage only for influenza A virus when present, and were all negative for the remaining stages for influenza virus and for all stages for SARS-CoV-2.

**Viral quantification.** Infectious virus was quantified by plaque assay using confluent MDCK (IAV) or Vero E6/TMPRSS2 (SCOV2) cells seeded in 12-well or 6-well plates, respectively, as previously described (26, 66). Samples were incubated on cell monolayers for 1 h before removal of inoculum, and all plates were incubated at 37°C for 2 days prior to fixation with 70% ethanol and staining with crystal violet to observe plaques. Limit of detection for both viruses was 10 PFU.

For quantification of viral RNA, 140 μL of each sample (out of the total sample volume) was inactivated in 560 μL of AVL buffer (Qiagen) and stored at −80°C until extraction using the QiAamp 96 viral RNA mini-extraction kit and QIAcube HT automated high-throughput nucleic acid purification platform with a 100-μL elution volume (Qiagen). All samples were tested in duplicate by real-time RT-PCR using the CDC influenza virus/SARS-CoV-2 multiplex assay as previously described (26). Viral copy numbers in each specimen were quantified against a 10-fold serial dilution of IAV or SCOV2 viral RNA included on each plate. Mean viral RNA copy numbers were normalized and expressed as RNA copy number per milliliter (ferret specimens) or RNA copy number per liter of air (aerosol specimens); specimens with gene copy numbers of <1/μL of extracted RNA were declared negative.

**Sequencing analysis.** Next-generation sequencing (NGS) analysis was performed as previously described (26). Briefly, RNA samples of >10^3 copies/μL were reverse transcribed and amplified using SuperScript III Platinum One-Step (Invitrogen) with universal IAV primers (67) or the SCOV2 Midnight primer panel from IDT, respectively. Amplified product was purified using a 1:1 ratio of SeraMag beads (Fisher Scientific) and made into NGS libraries with the Nextera DNA Flex library prep kit following manufacturer’s instructions at half-volume. Subsequent libraries were sequenced with paired-end 150-bp sequences on an Illumina iSeq 100. FASTQ files were processed in Geneious Prime with 18-bp N spacers between genes. Variants were called from the alignments using a threshold above 5% frequency and a minimum sequencing depth of 200× coverage. Alignment files were exported from Geneious as SAM files and used to determine haplotypes greater than 5% in abundance using ClqueSNV v2.0.3 (69) with default settings and frequency value set to four decimal places. From the haplotype frequencies, Shannon diversity was calculated in R v4.0.3 (R Core Team, 2020) using the package QSutils v1.8.0 (70). Figure 5 was constructed in R using ggplot2 v3.3.5 (71) and patchwork v1.1.1 (72) packages.

**Data availability.** Raw sequencing data used in this study have been deposited in the NCBI Sequence Read Archive under BioProject identifier (ID) PRJNA891284.

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The findings and conclusions are those of the authors and do not necessarily reflect the official position of ASTDR/the Centers for Disease Control and Prevention (CDC).

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