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Ocular infectivity and replication of a clade 2.3.4.4b A(H5N1) influenza virus associated with human conjunctivitis in a dairy farm worker in the USA: an in-vitro and ferret study

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Contributors

JAB designed the study, performed experiments, processed samples, and analysed data. JAB, JAP-P, NB, XS, and CP performed experiments with live viruses. TJK and XS performed the sequence analysis. JAB and TJK performed the statistical analyses. HZ assisted with plaque assays. MINV and SSL provided the pre-immune ferrets used in this work. JAB wrote the initial draft of the manuscript with input from TMT and TRM who also supervised the work and verified the underlying data. All authors had access to the data in the study and reviewed, modified, and approved the manuscript. All authors accept responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

See Online for appendix

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Summary

Background: The human eye represents a potential site of influenza A virus (IAV) replication, and an entry point for the virus to reach the respiratory tract. The frequent detection of conjunctivitis among farm workers with confirmed infection with clade 2.3.4.4b A(H5N1) IAV from this ongoing outbreak represents an atypical disease presentation for this virus subtype. We aimed to investigate whether the occurrence of ocular complications reported following clade 2.3.4.4b A(H5N1) virus infection was associated with an enhanced capacity of this virus to replicate in mammalian ocular tissue and cause infection following ocular exposure.

Methods: Primary human nasal and corneal tissue constructs were infected with A(H5N1) A/Texas/37/2024 (Texas/37), A(H1N1)pdm09 A/Nebraska/14/2019 (Neb/14), and A(H7N7) A/Netherlands/219/2003 (NL/219) viruses (multiplicity of infection [MOI] of 0.01–0.02, 33°C). Corneal tissue constructs were also infected with an expanded panel of IAVs (Texas/37, A[H5N1] A/Michigan/90/2024 [MI/90], A[H5N1] A/Chile/25945/2023 [Chile/25945], NL/219, A/Netherlands/230/2003 [NL/230], and Neb/14; MOI of 0.01, 37°C). In-vitro infections of tissue constructs were used to assess replication kinetics by infectious virus titration. Induction of innate host antiviral responses in infected corneal tissue constructs was assessed by PCR array (MOI of 2.00, 37°C). Ferrets (serologically naive or pre-immune to A[H1N1)pdm09 virus) were inoculated by the ocular route with Texas/37 A(H5N1) virus—using a liquid inoculum (10^6 plaque forming units [PFU]), aerosol inhalation (15–16 PFU), or ocular-only aerosol exposure (18–132 PFU)—to assess pathogenicity and tropism of the virus following different exposure routes. Transmissibility was assessed by placing serologically naive or pre-immune ferrets inoculated by ocular-only aerosol exposure in direct contact with serologically naive ferrets, monitoring pathogenicity in contact animals, and measuring viral titres in nasal washes of both inoculated and contact ferrets.

Findings: Nasal and corneal tissue constructs supported replication of all IAVs tested. In corneal tissue constructs, A(H7N7) and A(H1N1)pdm09 viruses reached 10-fold higher overall titres than A(H5N1) isolates. Relatively few genes ($n=13$) related to antiviral responses were significantly differentially expressed in corneal tissue constructs infected with IAV, with no consistent differential expression among clade 2.3.4.4b A(H5N1) viruses associated with either conjunctivitis or severe respiratory disease, although strain-specific differences were observed. Serologically naive ferrets inoculated by liquid ocular, aerosol inhalation, or aerosol-only ocular routes with Texas/37 virus exhibited a systemic and fatal infection in all animals, transmitting the virus to naive cagemates. By contrast, reduced disease severity following ocular-only aerosol inoculation was observed in animals with pre-existing heterosubtypic immunity. No serologically naive ferrets placed in direct contact with pre-immune ferrets inoculated with Texas/37 virus by the ocular-only aerosol route became infected.

Interpretation: A clade 2.3.4.4b A(H5N1) virus from the dairy cattle outbreak in the USA that was first detected in March, 2024, does not appear to possess features indicative of an ocular tropism. However, this virus can maintain a virulent and transmissible phenotype in ferrets following ocular exposure, highlighting the importance of ocular protection.

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Introduction

The abundant expression of sialic acid receptors on human respiratory tract epithelia makes this tissue an attractive site of influenza A virus (IAV) replication, with respiratory disease being a frequent clinical presentation of infection. However, human ocular tissue (notably corneal and conjunctival epithelial cells) also bear sialic acid receptors and can support the replication of multiple IAV subtypes,¹ highlighting the need to study the capacity of IAVs to replicate in non-respiratory tissues and infect humans via non-respiratory routes. Ocular complications (primarily conjunctivitis) have been reported following human infection with seasonal, avian-origin, and swine-origin IAVs,² with or without known ocular exposure to the virus. Because ocular and respiratory systems are connected by the nasolacrimal duct, fluidics exchange between these sites occurs, and renders ocular tissues susceptible to both primary IAV infection following ocular exposure, and secondary infection following the establishment of a respiratory tract infection.¹ Furthermore, expired air from individuals with IAV infection could expose their ocular surface to infectious virus via convective flow.³ Although the properties governing IAV ocular tropism are not well understood, in-vitro studies support that ocular-tropic IAVs—notably those within the A(H7) subtype—elicit differential induction of cytokines and signalling pathways in human ocular cells compared with respiratory-tropic IAVs.^{4,5}

Outbreaks of clade 2.3.4.4b highly pathogenic avian influenza (HPAI) A(H5N1) virus in cattle, poultry, and other species in the USA since March, 2024 have resulted in multiple cases of human infection.⁶ Notably, many of these infections have been associated with conjunctivitis, with or without concurrent respiratory illness.^{7,8} It is unclear whether virus isolates linked to outbreaks on dairy farms in 2024 possess an enhanced ability to use ocular tissue for virus entry or replication, or both, compared with other clade 2.3.4.4b isolates or other IAV subtypes, or whether the unique conditions associated with these outbreaks are facilitating conjunctival presentation in the absence of an ocular tropism.

Detection of higher viral RNA levels in a conjunctival specimen than in nasal specimens from the first confirmed human case from this outbreak⁷ could suggest that ocular tissues were initially exposed to the virus, that the virus was capable of enhanced replication in ocular tissues, or both. Reports of ocular disease and detection of viral RNA from ocular specimens of A(H5N1) virus-infected cats and cows on affected farms⁹ further supports the capacity of these viruses to replicate in mammalian ocular tissue.

In this study, we assessed the ability of A/Texas/37/2024 (Texas/37) and A/Michigan/90/2024 (MI/90) A(H5N1) viruses, isolated from conjunctival specimens from the first two confirmed infections in dairy farm workers in Texas and Michigan,^{7,8} to replicate and elicit antiviral host responses in human corneal tissue constructs, compared with contemporary human and avian IAVs. Ferrets were used to assess pathogenicity, transmissibility, and tropism of Texas/37 virus (previously shown to cause fatal infection in serologically naive ferrets inoculated by the intranasal route with a high dose of the virus^{10,11}) following exposure of the respiratory tract or ocular surface.

Methods

Study design

This study included in-vitro experiments investigating the cellular tropism of clade 2.3.4.4b A(H5N1) viruses in primary human nasal and corneal tissue constructs, and inflammatory or antiviral responses elicited in corneal constructs, as compared with a panel of IAVs. The panel of IAVs included Texas/37 and MI/90 viruses, an additional 2.3.4.4b A(H5N1) virus isolated from a human with severe respiratory infection (A/Chile/25945/2023 [Chile/25945]),¹⁰ a contemporary A(H1N1)pdm09 respiratory virus (A/Nebraska/14/2019 [Neb/14]), and two HPAI A(H7N7) virus isolates that caused either a fatal outcome in a human (A/Netherlands/219/2003 [NL/219]), or conjunctivitis in a human (A/Netherlands/230/2003 [NL/230]) from an outbreak in the Netherlands in 2003 that caused ocular complications in 83 (93%) of 89 individuals with confirmed infection.¹² This study also included in-vivo experiments that assessed the capacity of Texas/37 virus to cause severe disease following ocular exposure in a ferret model and transmit to naïve animals in close contact.

All in-vivo studies were conducted under the guidance and approval of the US Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee (IACUC; protocol 3347MAIFERC) in an AAALAC International-accredited animal facility, in compliance with all policies and regulations of the Office of Laboratory Animal Welfare, as part of the US National Institutes of Health.

Procedures

Texas/37 (for in-vivo studies¹⁰), MI/90, and Neb/14 viruses were propagated for 2 days in MDCK cells (ATCC, Manassas, VA, USA), whereas Texas/37 (for in-vitro studies), Chile/25945, NL/219, and NL/230 viruses were propagated for 24–26 h in the allantoic cavity of 10-day-old embryonated hens' eggs (Hy-Line, Mansfield, GA, USA).¹³ All viruses were propagated at CDC and sequenced before use (appendix p 2). Quantification of infectious viral titres for all in-vitro and in-vivo experiments was performed by standard plaque assay titration in MDCK cells (limit of detection of 10 plaque forming units [PFU]). All experiments were performed in CDC Biosafety Level 3 containment laboratories, including enhancements to meet all biosafety and biosecurity requirements of the US Department of Agriculture and the Federal Select Agent Program for avian influenza A viruses.

To investigate the cellular tropism of clade 2.3.4.4b A(H5N1) viruses, we assessed IAV replication kinetics in primary human nasal tissue constructs (EpiNasal, MatTek, Ashland, MA, USA), emulating the nasal passages, and corneal tissue constructs (EpiCorneal, MatTek), emulating the ocular surface. EpiCorneal and EpiNasal tissue constructs, derived from normal human corneal and nasal epithelial cells, respectively, were maintained at air–liquid interface with serum-free basolateral media as per the manufacturer's instructions. Estimate cell counts on the apical surface for multiplicity of infection (MOI) determination were 100 000 (EpiCorneal) and 200 000 (EpiNasal). We used a sample size of three constructs per experiment, in which the viral strain was the variable for comparison. Constructs were infected (within 24 h of receipt from manufacturer) with all viruses to assess replication kinetics at the temperature and MOI indicated (0.01 and 0.02 for EpiNasal

and EpiCorneal constructs cultured at 33°C with Neb/14, NL/219, and Texas/37 viruses, and 0-01 for EpiCorneal constructs cultured at 37°C with the entire panel),⁵ with timepoints generated by adding 200 µL construct-specific assay media to the apical surface for 15 min before collection (2, 24, 48, and 72 h post-inoculation) and frozen at -80°C.

We then examined whether the frequency of ocular complications in human cases associated with 2024 A(H5N1) 2.3.4.4b outbreaks could be due to differences in these viruses eliciting inflammatory or antiviral responses in corneal constructs compared with viruses associated primarily with respiratory disease in humans, by isolating total RNA from EpiCorneal constructs infected with the entire panel of viruses and testing them with a PCR array. Total RNA was extracted (RNeasy Mini Kit, Qiagen, Venlo, the Netherlands) from triplicate mock-infected (assay media only) or virus-infected EpiCorneal constructs (MOI of 2-00 for all viruses) 24 h post-inoculation and tested by a PCR array including 84 genes to assess regulation of antiviral response-related genes using an RT² Profiler PCR Array Human Antiviral Response (PAHS-122Z, Qiagen). Arrays were run and analysed as per manufacturer's instructions (appendix pp 2, 5-17).

For in-vivo studies of infection with Texas/37 virus, we used male Fitch ferrets (Triple F Farms, Sayre, PA, USA; n=3 for all experiments) that were either serologically naive to IAV before inoculation or pre-immune to A(H1N1) pdm09 IAV. Pre-immune ferrets were serologically negative, then exposed to A/California/7/2009 A(H1N1)pdm09 virus as contacts in an unrelated transmission experiment (all ferrets showing peak nasal wash titres greater than 10⁴ 50% tissue culture infective dose [TCID₅₀] during infection) and allowed to convalesce for 12 weeks (with serological titres to homologous virus of 32-64) before inoculation with Texas/37 virus.

Serologically naive ferrets were inoculated with Texas/37 virus by the liquid ocular route (to assess the capacity of Texas/37 virus to cause severe disease following ocular exposure), the respiratory inhalation route (to assess whether Texas/37 maintained a virulent phenotype following more physiologically relevant exposure conditions), or an ocular-only aerosol inoculation route, bypassing respiratory inhalation (to assess infectivity and disease severity following this secondary exposure route that can lead to a productive respiratory infection in mammals).² Pre-immune ferrets, included to assess whether previous immunological exposure to IAV could modulate Texas/37 disease severity, were exclusively inoculated by the ocular-only aerosol inoculation route. Liquid ocular inoculation was performed by depositing 100 µL of diluted virus (10⁶ PFU) onto the right eye of the ferret and massaging it into the eye with the eyelid, as previously described.¹⁴ For aerosol exposures, aerosolisation efficiency of Texas/37 virus was established by calculation of a stock spray factor as described.¹⁵ Respiratory inhalation of aerosolised Texas/37 virus was performed as previously described,¹⁶ with all animals exposed concurrently for 15 min, leading to a presented dose of 15-16 PFU per ferret; variability in the presented dose following respiratory inhalation is due to per-animal variability in minute volume respiration. Ocular exposures to aerosolised Texas/37 virus (both eyes exposed) were performed for 15 min as previously described,¹⁷ with animals exposed sequentially with the same preparation of virus in the nebuliser, leading to a presented dose of 18-132 PFU per ferret; variability in the presented dose is due to different goggles fitted to each ferret. Virus aerosolisation

procedures were performed at ambient room temperature (approximately 21°C) and at approximately 65% relative humidity.

All ferrets (both serologically naive and pre-immune) inoculated with Texas/37 virus using aerosol inoculation methods served as donors (1:1 ratio) to assess transmission, with a serologically naive contact animal placed in an adjacent cage with perforated side-walls to an inoculated animal to assess respiratory droplet transmission (RDT), or in the same cage with an inoculated animal to assess transmission in the presence of direct contact (DCT), with contact established 24 h post-inoculation. All inoculated and contact ferrets were observed daily for 14 days for clinical signs of infection (including weight loss, temperature rise, and lethargy assessments). Nasal washes, conjunctival washes, and rectal swabs were collected every other day from all inoculated and contact animals to assess changes in viral load over time.¹⁴ Transmission was assessed by detecting infectious virus in specimens collected from contact ferrets and by seroconversion of contact ferrets to the homologous virus at the end of the study. Extrapulmonary spread of virus was assessed by collecting and titrating systemic tissues from inoculated ferrets that reached humane endpoints during the experiment. Complete methodological details of in-vivo experiments are in the appendix (p 2). To assess the emergence of genetic changes to Texas/37 virus during in-vivo infection, viral RNA from ferret specimens was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) and processed for influenza genome amplification and next generation sequencing as described previously (appendix p 2).¹⁰

Statistical analysis

For assessments of viral replication kinetics, statistically significant differences between all viruses were assessed by calculation of area under the curve using GraphPad Prism (version 7.05) followed by two-way ANOVA with a Tukey post-hoc test with viral strain as the variable. For PCR arrays, analyses focused on the genes included in the array that were significantly ($p < 0.05$ by Student's t test) upregulated or downregulated by more than two-fold compared with the control specified (either mock-infected or Texas/37 virus-infected constructs). Specifically, raw expression values were normalised using the formula $2^{-\text{mean}(\Delta\text{Ct})}$, where ΔCt is the difference between the cycle threshold (Ct) of the gene of interest and the Ct of housekeeping genes, then fold change was calculated by dividing the experimental normalised raw expression by the control normalised raw expression, and the results were reported as fold regulation (down [or negative] fold regulation is reported as the negative reciprocal fold change) for all virus strains versus mock infection and for MI/90 versus Texas/37; no logarithmic transformations were used (appendix pp 5–17). For in-vivo experiments, no formal sample size calculation was performed, and no statistics were conducted. For the in vivo experiments, a group size of three ferrets was chosen to match standard conventions in the field.¹⁸

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We infected primary human nasal (EpiNasal) and corneal (EpiCorneal) tissue constructs with Texas/37, Neb/14, or NL/219 viruses. Although all viruses exhibited productive replication, no significant differences in total area under the curve were observed across all three viruses in either EpiNasal (figure 1A) or EpiCorneal (figure 1B) constructs (appendix p 4). EpiCorneal constructs infected with the full panel of viruses showed that Neb/14, NL/219, and NL/230 viruses all replicated faster (24 h titre) and to higher mean peak titres (4.2–4.7 log₁₀ PFU/mL) than a panel of 2.3.4.4b HPAI A(H5N1) viruses comprising Texas/37, MI/90, and Chile/25945 (3.2–3.8 log₁₀ PFU/mL; figure 1C). NL/219 and NL/230 viruses, and Texas/37 and MI/90 viruses, each differ between each other by 13 amino acids, including at amino acid position 627 in PB2. NL/219 and Texas/37 both have a lysine at position 627, an adaptive mutation associated with overcoming host range of avian influenza viruses.^{7,8,12} Despite different disease profiles in humans, replication of the 2.3.4.4b A(H5N1) viruses Texas/37 and Chile/25945 was indistinguishable in EpiCorneal constructs (figure 1C).

All viruses exhibited productive replication (mean peak titre per virus 3.7–5.7 log₁₀ PFU/mL) in EpiCorneal constructs before collection of total RNA for inflammatory or antiviral response assessment, with no significant difference in mean titres among viruses of the same subtype. Only 13 genes were significantly upregulated or down-regulated by more than two-fold in virus-infected constructs compared with mock-infected constructs among all IAVs tested (all with fold regulation <10; figure 2A). All but one of these genes (in Chile/25945-infected constructs) were detected in constructs infected with either NL/230 or Neb/14 viruses; none of these were detected in constructs infected with Texas/37 virus (figure 2A). However, infection with MI/90 virus induced significantly higher levels (more than two-fold upregulation) of numerous Toll-like receptor (TLR) signalling, type 1 interferon (IFN) signalling, and IFN responsive genes than following infection with the Texas/37 virus (figure 2B).

In in-vivo experiments, ferrets that were serologically naive to IAV prior to inoculation exhibited clinical disease following inoculation with a high viral dose (10⁶ PFU) by the liquid ocular route, with elevated temperatures, weight loss, and presentation of clinical signs such as diarrhoea and nasal discharge detected in all animals (table). All inoculated animals reached humane endpoints 3 days after inoculation. Furthermore, similar to intranasally inoculated animals receiving the same viral dose,¹⁰ infectious virus was recovered from all tissues collected, including high-titre detection (>10⁴ PFU/ml) in the eyes and conjunctiva (figure 3A).

Ferrets inoculated by the aerosol inhalation route with a low dose of virus (15–16 PFU) displayed a delay in viral shedding in the upper respiratory tract (compared with ferrets receiving a high viral dose by the liquid ocular route; figure 4A, B), but they developed severe disease indistinguishable from that following the high-dose liquid ocular challenge described above, warranting humane euthanasia 3 days after inoculation (table). Post-mortem necropsy revealed elevated viral loads throughout the respiratory tract and numerous extrapulmonary sites (notably the liver, spleen, and intestine; figure 3B). Infectious virus

was only infrequently detected in ocular (eyes and conjunctiva) and brain (cerebrum and olfactory bulb) tissues at low titre ($<10^2$ PFU/mL or PFU/g). Animals inoculated by the low-dose aerosol inhalation route did not transmit Texas/37 virus to contact animals in an RDT setting (table), probably due to a delay in productive viral replication in the upper respiratory tract resulting in a shortened interval of virus shedding into the environment before infected animals reached humane endpoints (figure 4B), compared with previous ferret experiments using a high challenge dose of virus.¹⁰ For this reason, subsequent transmission assessments were performed using a more permissive direct contact transmission model.

Ferrets exposed to aerosolised Texas/37 virus by the ocular-only route (52–132 PFU) became productively infected, resulting in clinical disease (table) and viral shedding in the upper respiratory tract (figure 4C), which was indistinguishable from animals inoculated by the respiratory inhalation route. All animals infected by this inoculation route reached euthanasia criteria 4 days after inoculation. The magnitude and distribution of viral detection in systemic tissues from these animals was similar to ferrets inoculated by the aerosol inhalation route (figure 3C), with ocular and brain tissues again exhibiting markedly reduced viral titres compared with respiratory tract specimens and other extrapulmonary sites. Using the DCT setting, contact animals who were cohoused with ferrets inoculated by the ocular-only aerosol route (n=3) all became infected, reaching humane endpoints 4–6 days post-contact (table, figure 4C).

Pre-immune ferrets rechallenged by the low-dose ocular-only route with Texas/37 virus (18–44 PFU) had fewer clinical signs of illness and showed reduced frequency and magnitude of infectious virus detection in nasal wash, conjunctival wash, and rectal swab specimens, compared with similarly exposed serologically naive animals (table, figure 4D). All pre-immune animals survived the challenge and seroconverted to Texas/37 virus. Serologically naive ferrets placed in direct contact with these animals did not become productively infected (table, figure 4D).

Discussion

We found that A(H5N1) viruses isolated from the first two confirmed infections in dairy farm workers with conjunctivitis did not exhibit an enhanced capacity to replicate in human ocular tissue compared with an A(H5N1) isolate from a severe respiratory human infection or a A(H1N1) pdm09 virus. Ocular-only aerosol challenge with Texas/37 virus resulted in infection of ferrets with or without preexisting IAV immunity but caused severe and fatal illness only in serologically naive animals. This work suggests that 2.3.4.4b A(H5N1) viruses currently causing mammalian and poultry outbreaks in the USA do not possess a marked ability relative to other IAVs to infect ocular epithelia, but are nonetheless capable of mounting a productive, transmissible, and fatal infection in this mammalian model following low-dose ocular exposure.

Human corneal and conjunctival epithelial cells primarily express α 2-3 linked sialic acid (ie, sialic acid linked to galactose via α 2,3 glycosidic bonds), although α 2-6 linked sialic (ie, sialic acid linked to galactose via α 2,6 glycosidic bonds) can also be detected and are present in other tissues (including the nasolacrimal duct) in the ocular milieu.

^{1,19} Despite strong binding of Texas/37 virus to α 2,3-linked sialic acid,¹⁰ all A(H5N1) viruses replicated to lower overall peak titre than either A(H7N7) or A(H1N1)pdm09 viruses in EpiCorneal constructs. In agreement with previous work,⁵ relatively few genes deviated significantly from mock-infected EpiCorneal constructs when tested by PCR array. Furthermore, generally similar induction of innate antiviral responses among all A(H5N1) viruses examined 24 h after infection suggests that Texas/37 or MI/90 viruses do not possess a particular affinity to replicate or elicit robust host responses within ocular tissue compared with other IAVs, although additional investigation of later timepoints (eg, 48–72 h) is warranted. IAV infection of human ocular cells can lead to differential production of pro-inflammatory cytokines and chemokines.^{4,5} Although changes in RNA transcript levels following IAV infection were generally within five-fold of mock-infected cells (figure 2A), further examination of the capacity of A(H5N1) viruses that have continued to emerge throughout 2024 to produce pro-inflammatory mediators in human corneal and conjunctival cells is still needed, as is a greater understanding of the host immune responses that govern IAV-associated conjunctivitis. The high titres observed with Neb/14 virus in EpiCorneal cells are in agreement with previous work supporting the capacity of some A(H1N1)pdm09 viruses to use ocular tissue to mount a productive infection in-vitro.^{19,20} The eye represents an immune-privileged site, and caution should be applied in extrapolating results from this tissue to other anatomical locations. Ongoing assessments of host responses to clade 2.3.4.4b viruses in both human and bovine respiratory tract epithelial cells could reveal further insight into unique properties of these viruses compared with historical A(H5N1) strains.^{21,22} Further investigation of the capacity of clade 2.3.4.4b A(H5N1) viruses to maintain infectivity during passage through human tear film is also warranted.¹⁹ Studies using different in-vitro and ex-vivo systems are needed to better emulate the complexity of both human corneal and conjunctival tissues.^{4,11,20} Furthermore, including additional readouts (eg, immunohistochemistry) could help explore possible differences in viral infectivity during the first round of virus replication in these cells.¹⁹

Clade 2.3.4.4b HPAI A(H5N1) viruses isolated from both humans and animals since 2022 have shown a remarkable capacity to cause severe disease in mammals.^{10,23–25} However, studies of these strains have rarely used inoculation doses of less than 10^6 infectious units.²³ Although a high-dose liquid challenge can reveal the full capacity of an IAV to cause severe, systemic infection, low-dose aerosol-based challenge methods can better emulate infection progression without compromising virulence readouts.¹⁶ For these reasons, we conducted ferret ocular challenge studies using both liquid and aerosol inoculation procedures, and a range of inoculum doses. Although the sample size per group (n=3) in our study was small, the strongest conclusions drawn from pathotyping and transmission are informed by results that span multiple independent experiments.¹⁸ Of note, ferrets do not consistently present with macroscopic conjunctivitis following ocular exposure or infection with IAV, restricting our assessments to detection of infectious virus in ocular specimens. Unlike a high-dose ocular challenge, ferrets inoculated via inhalation or ocular-only aerosol-based challenges did not have high viral titres in the brain or olfactory bulb, suggesting that the virulent phenotype observed was not due to neurological invasion of the virus. In agreement with previous work showing that ocular inoculation does not select for mutations compared with intranasal routes,²⁶ sequencing of specimens from ferrets inoculated by the high-dose liquid

ocular route revealed no consistent amino acid variants compared with ferrets inoculated by the respiratory inhalation route. Furthermore, despite detection of Texas/37 virus in the eyes, conjunctiva, and conjunctival wash specimens of ferrets inoculated with a high dose of virus,¹⁰ low-dose aerosol inoculation supports that this virus might not be detected at high titres in ocular tissue in ferrets once productive infection is established. Although additional research is warranted to better understand how Texas/37 virus so rapidly spreads systemically in serologically naive animals after inoculation, these data support the utility of aerosol inoculation approaches for these investigations. Abrogation of Texas/37 virus transmissibility by the airborne route in ferrets inoculated by low-dose aerosol inhalation (compared with those who received the high-dose challenge)¹⁰ further supports the utility of these methodologies to risk assessment activities.

The capacity of seasonal and zoonotic IAVs to cause productive infection in ferrets following ocular inoculation has been shown previously,² including fatal disease reported in ferrets following ocular challenge with the 2.3.4.4b A(H5N1) virus Chile/25945.²⁷ We extend these findings to show that, compared with serologically naive animals, ferrets with pre-existing heterosubtypic immunity to A(H1N1)pdm09 exhibit less severe disease following ocular virus exposure. Ferrets inoculated by the ocular route can release viral-laden aerosols into the environment after inoculation.²⁶ However, further study is needed to assess the effect that pre-existing immunity to IAV has on subsequent shedding of infectious virus into the environment, compared with serologically naive animals. Although our data, which are consistent with other work showing reduced virulence of a 2.3.4.4b A(H5N1) bovine isolate following intranasal inoculation of similarly pre-immune ferrets,^{28,29} provide a potential explanation for why human cases have been generally mild, the complexity of innate and adaptive immune responses governing this phenotypic difference requires dedicated study.³⁰

This study has several limitations. For the in-vitro work, host responses were limited to PCR-based detection methods and did not include protein quantification of secreted cytokines. Aerosol exposure-based methods can result in variability in presented doses between animals that is not observed when using a liquid inoculum.¹⁶ For the in-vivo work, although multiple inoculation routes were assessed, each individual experiment had a small sample size. The reduced viral shedding by virus-challenged pre-immune ferrets further limited interpretation of the ways in which pre-existing immunity might modulate viral transmission to susceptible contact animals.

Despite the general absence of an ocular tropism observed for Texas/37 virus in ferrets in this study, there has been a high incidence of ocular complications among humans with confirmed infection. Therefore, additional study is needed to ascertain whether this high incidence is due to specific virological features of currently circulating 2.3.4.4b A(H5N1) viruses, or whether the unique conditions associated with human exposure activities during this outbreak (including milk splashing on the ocular surface,⁸ or self-inoculation of the ocular surface with hands exposed to contaminated milking equipment, or both²⁹) represent a contributing factor. Of note, aerosol-based experiments were conducted at elevated (approximately 65%) relative humidity to better emulate environmental conditions in dairy farms,³¹ but further experimentation (eg, assessing IAV infectivity in aerosols in milk) could

be informative. Collectively, this work supports that the human ocular system represents a secondary mucosal surface susceptible to IAV exposure and infection, and highlights the importance of following recommendations for both ocular and respiratory protection when working with farm animals in locations with potential A(H5N1) detection.⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data sharing

PCR array data are provided in this Article and sequence data have been deposited to the NCBI Sequence Read Archive (BioProject identification number PRJNA891284). All other data included in this manuscript are shown in the figures and the table, and raw data are available upon request to the corresponding author.

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Research in context

Evidence before this study

We searched PubMed using the terms (“H5N1” OR “A(H5N1)”) AND (“ocular” OR “cornea” OR “conjunctivitis”) AND (“human”) for articles without language restrictions published between Jan 1, 2000, and Dec 9, 2024. We identified fewer than 20 primary laboratory-based research studies, conducted either in vitro or in vivo, showing that diverse influenza A viruses (IAVs) can replicate in human ocular cell types and cause mammalian infection following ocular exposure. Although A(H5N1) influenza viruses have sporadically been associated with ocular complications in humans, the outbreaks among dairy farm workers in the USA since March, 2024 represent the first examples of this virus subtype causing conjunctivitis as a frequently reported clinical observation.

Added value of this study

To our knowledge, this is the first study to perform a comparative analysis of the capability of two human conjunctivitis case clade 2.3.4.4b A(H5N1) virus isolates from the 2024 outbreak in the USA to replicate in human ocular tissue constructs alongside other IAVs associated with respiratory or ocular disease in humans. This study is among the first to show that viruses from this outbreak can lead to a productive and transmissible infection in mammals following ocular exposure, and that prior immunity to IAV could modulate disease severity in a mammalian model following ocular exposure to IAV.

Implications of all the available evidence

An A(H5N1) virus associated with human conjunctivitis in a dairy farm worker could cause a severe and transmissible infection in mammals following low-dose ocular exposure, despite generally mild disease presentation observed in humans to date. Further research is warranted to investigate why clade 2.3.4.4b A(H5N1) viruses from US outbreaks are so frequently associated with ocular complications in humans. This study contributes to a body of work that highlights the importance of following recommendations for both ocular and respiratory protection during potential high-risk exposure to all IAVs, including A(H5N1) viruses.

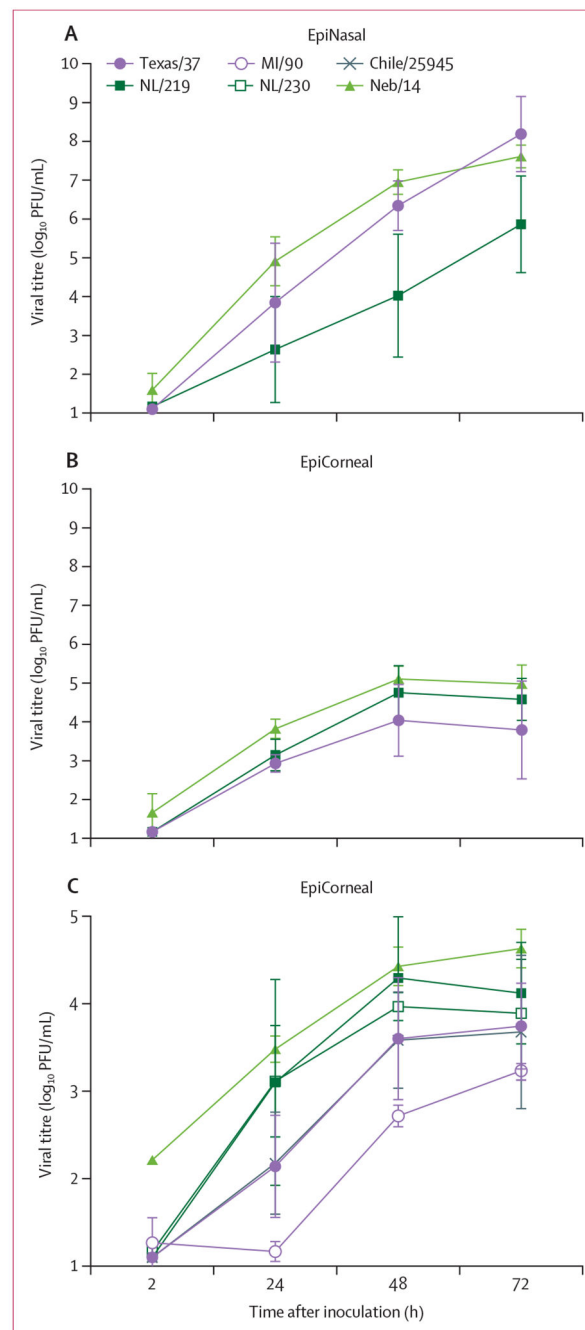


Figure 1: Replication of IAV in respiratory and ocular tissue constructs

Replication of Texas/37, NL/219, and Neb/14 viruses in EpiNasal (A) and EpiCorneal (B) tissue constructs incubated at 33°C. (C) Replication of Texas/37, MI/90, Chile/25945, NL/219, NL/230, and Neb/14 viruses in EpiCorneal tissue constructs incubated at 37°C. Infection experiments were done in triplicate and data points represent mean and SD. Statistics associated with these data are shown in the appendix (p 4). Chile/25945=A/Chile/25945/2023. IAV=influenza A virus. MI/90=A/

Michigan/90/2024. Neb/14=A/Nebraska/14/2019. NL/219=A/Netherlands/219/2003. NL/230=A/Netherlands/230/2003. PFU=plaque forming units. Texas/37=A/Texas/37/2024.

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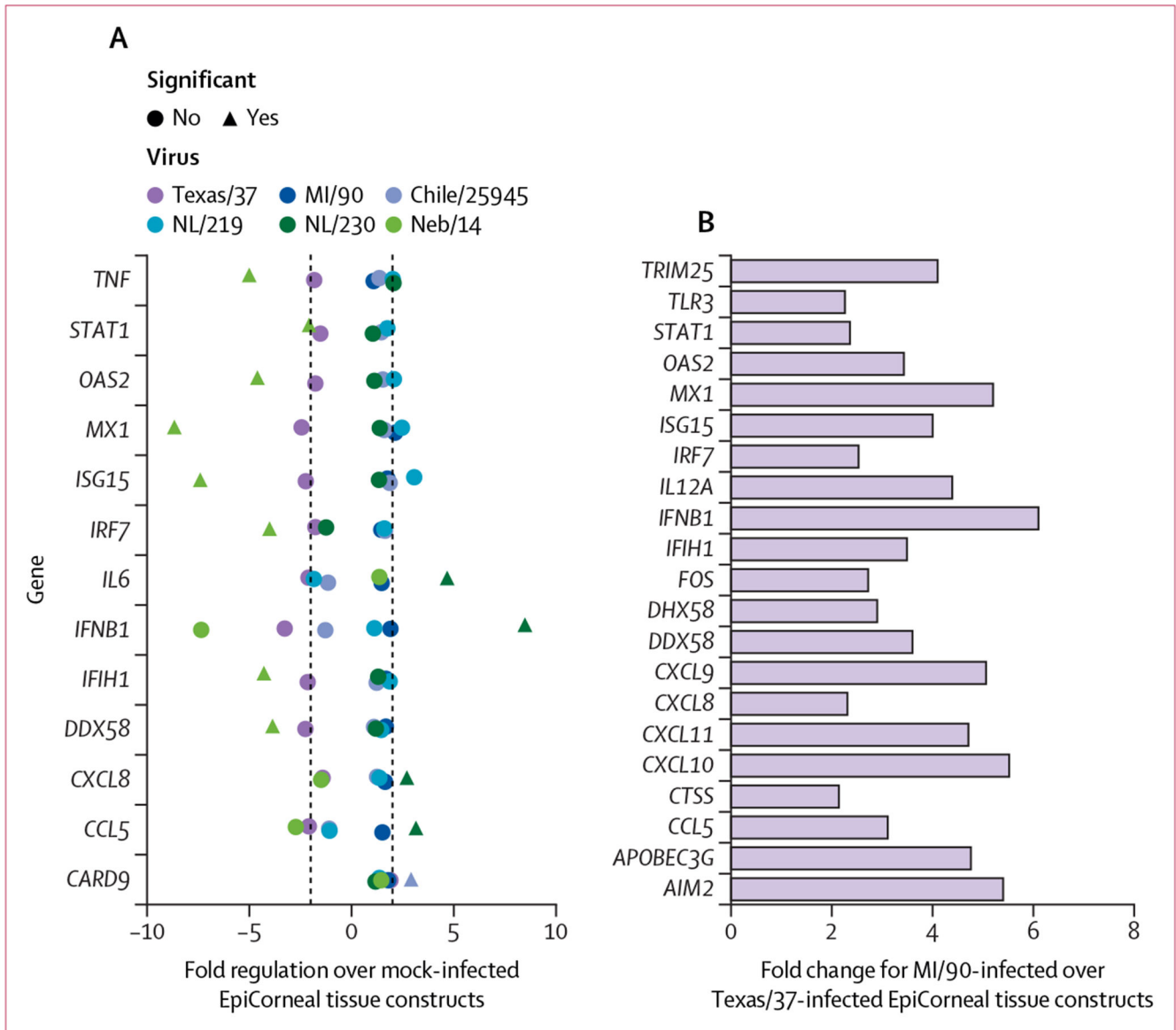


Figure 2: Regulation of specific genes related to human antiviral responses in EpiCorneal tissue constructs following infection with IAV

(A) Genes that exhibited a significant ($p < 0.05$) fold regulation in expression compared with mock-infected constructs and for which at least one of the viruses elicited a fold regulation of > 2 (as indicated with dashed lines) are shown. (B) Genes from tissue constructs infected with MI/90 virus that exhibited a significant ($p < 0.05$) change in expression (more than two-fold) compared with constructs infected with Texas/37 virus are shown. All information associated with shown data and p values are available in the appendix (pp 5–17). Chile/25945=A/Chile/25945/2023. IAV=influenza A virus. MI/90=A/Michigan/90/2024. Neb/14=A/Nebraska/14/2019. NL/219=A/Netherlands/219/2003. NL/230=A/Netherlands/230/2003. Texas/37=A/Texas/37/2024.

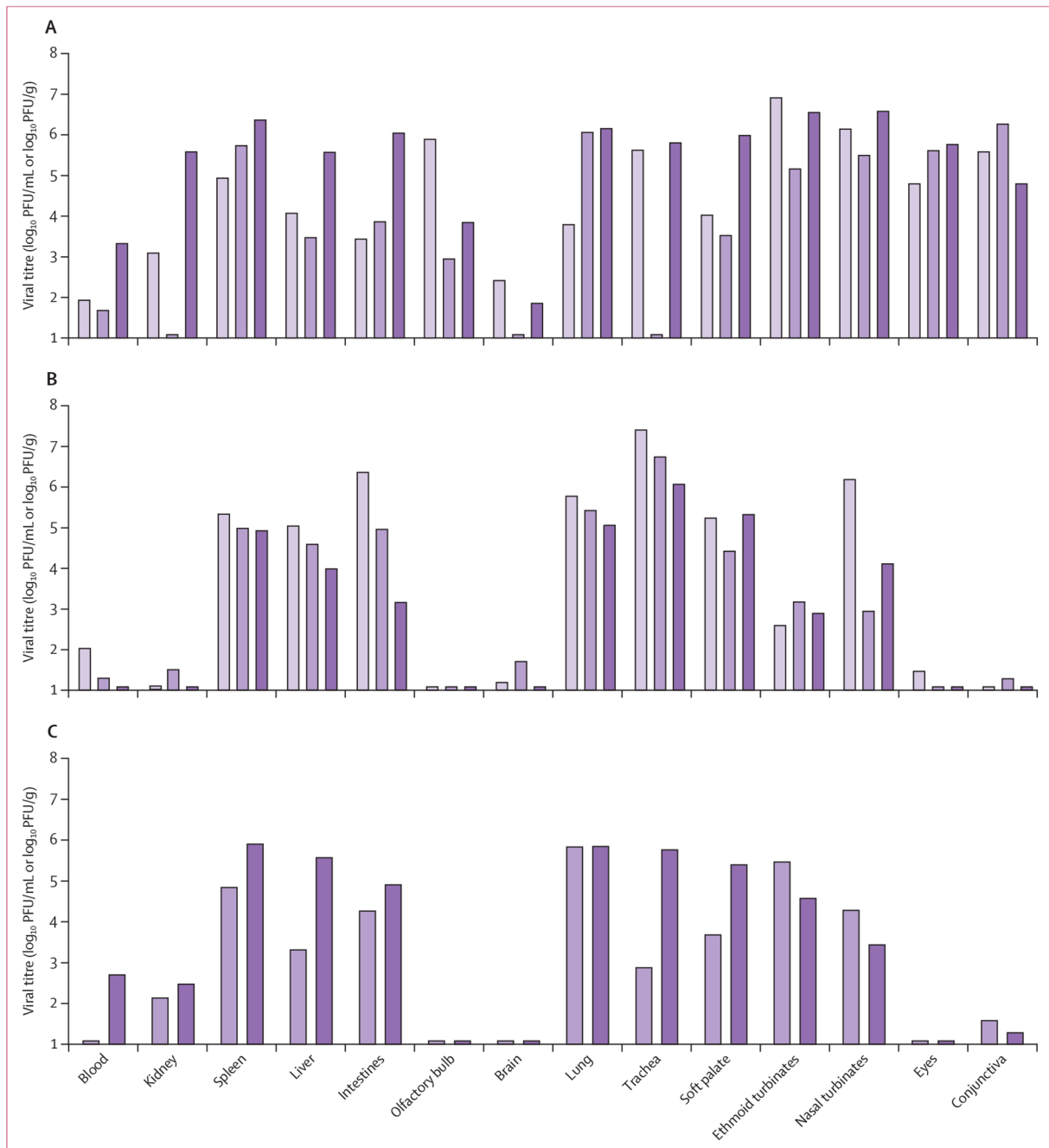


Figure 3: Systemic spread of Texas/37 virus in ferrets following multiple inoculation routes

Viral titres measured in systemic tissues collected from serologically naive ferrets that were inoculated by the ocular route using a high-dose liquid inoculum (A), by low-dose aerosol respiratory inhalation (B), or by low-dose ocular-only aerosol exposure (C) and that reached humane endpoints (3 days after inoculation for all ferrets in panels A and B and 4 days after inoculation for ferrets in panel C). Titres are expressed as log₁₀ PFU/mL (blood, soft palate, ethmoid turbinates, nasal turbinates, eyes, and conjunctiva) or PFU/g (all other tissues). Bars

represent individual ferrets with a different colour coding for each individual ferret. The LOD was 10 PFU. LOD=limit of detection. PFU=plaque forming units.

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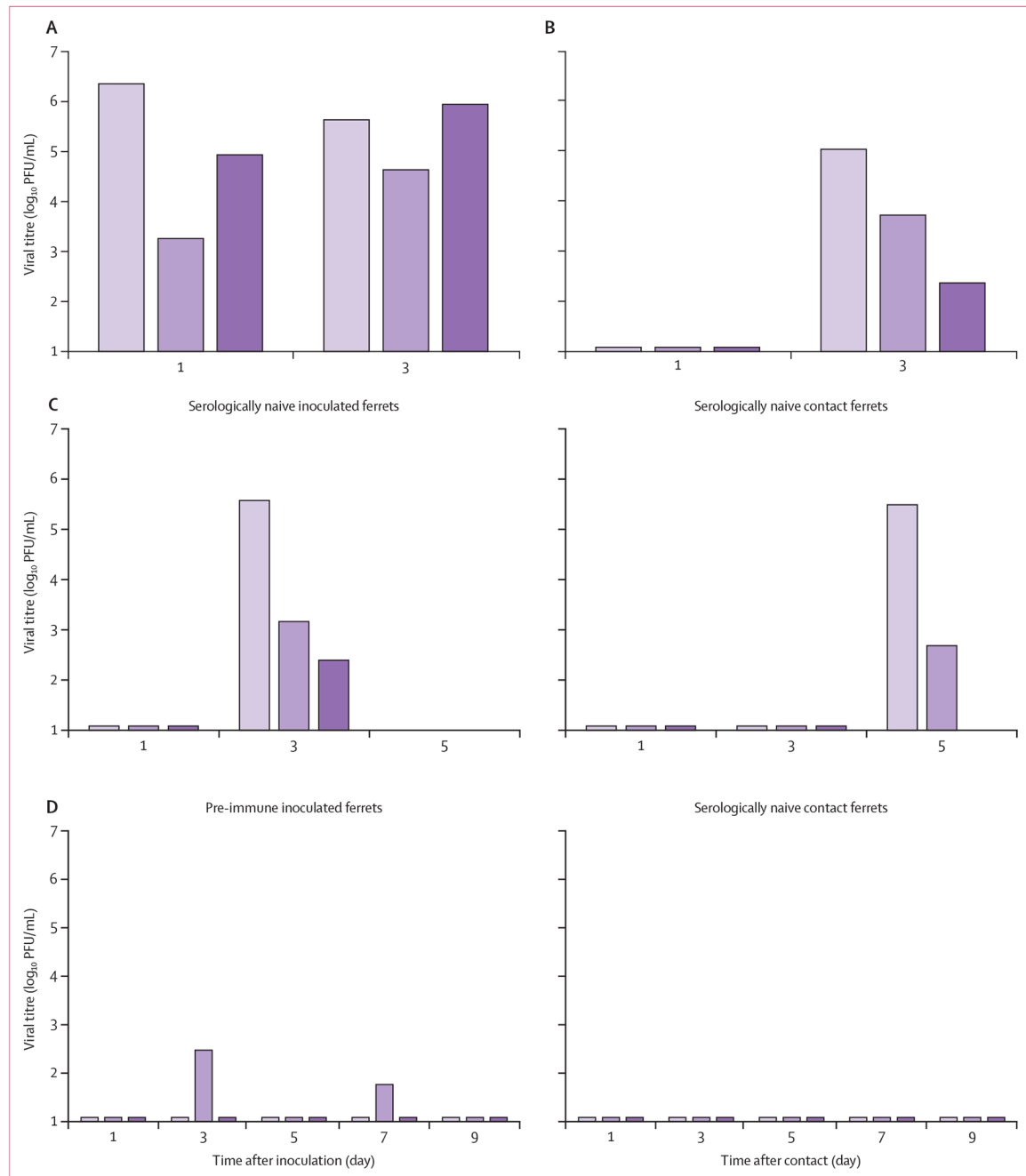


Figure 4: Replication and transmission of Texas/37 virus in ferrets following multiple inoculation routes

Viral titres in nasal wash specimens collected over time in serologically naive ferrets inoculated by the ocular route using a high-dose liquid inoculum (A) or low-dose aerosol respiratory inhalation (B). (C) Viral titres in nasal wash specimens collected over time in serologically naive ferrets inoculated by low-dose ocular-only aerosol exposure (left graph) and in serologically naive ferrets placed in direct contact with the inoculated ferrets (right graph). (D) Viral titres in nasal wash specimens collected over time in pre-immune ferrets inoculated by low-dose ocular-only aerosol exposure (left graph) and in serologically naive

ferrets placed in direct contact with the inoculated ferrets (right graph). Bars represent individual ferrets with a different colour coding for each individual ferret; no bar indicates the animal did not survive the infection and no specimen was collected. The LOD was 10 PFU. LOD=limit of detection. PFU=plaque forming units. Texas/37=A/Texas/37/2024.

Table: Pathogenicity and transmissibility of Texas/37 A(H5N1) virus in ferrets following different exposure routes and immune backgrounds

Immune status	Mean maximum weight loss (SD), % [*]	Maximum temperature rise (SD), °C [†]	Lethal outcome n/N (days after inoculation) [‡]	Mean maximum viral titre (log ₁₀ PFU/mL) in nasal wash (SD), n/N [§]	Mean maximum viral titre (log ₁₀ PFU/mL) in conjunctival wash (SD), n/N [§]	Mean maximum viral titre (log ₁₀ PFU/mL) in rectal swab (SD), n/N [§]	Seroconversion, n/N [¶]
Liquid ocular exposure							
10 ⁶ PFU Naive	8·8 (1·2)	2·2 (0·6)	3/3 (3)	5·7 (0·9), 3/3	5·0 (0·4), 3/3	4·2 (1·0), 3/3	NT
Respiratory inhalation route							
15–16 PFU Naive	10·3 (0·5)	2·3 (0·8)	3/3 (3)	3·7 (1·3), 3/3	0/3	2·1 (0·6), 3/3	NT
RDT Naive	NA	NA	0/3	NA	NA	NA	0/3
Ocular-only aerosol inoculation route							
52–132 PFU Naive	6·0 (1·2)	2·0 (0·6)	3/3 (4)	3·7 (1·7), 3/3	4·2, 1/3	3·1 (0·7), 2/3	NT
DCT Naive	5·7 (2·2)	2·3 (0·3)	3/3 (4–6)	3·3 (1·9), 3/3	1·7, 1/3	2·1, 1/3	NT
18–44 PFU Pre-immune	4·2 (2·4)	1·0 (0·4)	0/3	2·5, 1/3	0/3	1·7, 1/3	3/3
DCT Naive	NA	NA	0/3	NA	NA	NA	0/3

Data are shown for both virus-inoculated (with PFU dose specified) and contact animals (following RDT or DCT). All groups shown reflect a group size of three ferrets. DCT=direct contact transmission. LOD=limit of detection. NA=not applicable because no contact animals became infected. NT=not tested. PFU=plaque forming units. RDT=respiratory droplet transmission. Texas/37=A/Texas/37/2024.

^{*} Among all ferrets inoculated with or seroconverted to Texas/37 virus (expressed as a percentage of gram weight loss from baseline).

[†] Among all ferrets inoculated with or seroconverted to Texas/37 virus.

[‡] Number of ferrets out of total per group that did not survive the infection.

[§] Mean maximum viral titre among all ferrets with positive virus detected between 1–5 days after infection (number of positive ferrets over total is specified in parentheses when not 3/3); LOD was 10 PFU.

[¶] Number of inoculated or contact ferrets with antibodies to Texas/37 virus in serum (seroconversion titre range 10–80, LOD <10) over the total number of ferrets from which sera was collected; serology was not conducted on animals that became infected with Texas/37 virus and did not survive the 14-day experimental period.