We evaluated a hypothesis that horses are susceptible to avian influenza viruses by in vitro testing, using explanted equine tracheal epithelial cultures, and in vivo testing by aerosol inoculation of ponies. Results showed that several subtypes of avian influenza viruses detectably replicated in vitro. Three viruses with high in vitro replication competence were administered to ponies. None of the three demonstrably replicated or caused disease signs in ponies. While these results do not exhaustively test our hypothesis, they do highlight that the tracheal explant culture system is a poor predictor of in vivo infectivity.

**Keywords** Avian influenza, equine influenza, host range, interspecies transmission, organ culture.

To elucidate the ability of AIV to infect equine respiratory epithelial cells in vitro, we used explanted equine tracheal cultures as previously described. The source of tracheas was horses/ponies being euthanized by other investigators for reasons unrelated to respiratory disease. Tracheas were excised within 30 minutes of euthanasia. The mucosal/submucosal layers were removed from the cartilage, cut into 1-cm² sections, and cultured in six-well plates with the epithelial surface facing up and awash in Leibovitz L15 medium (Lonza BioWhittaker) buffered with HEPES. Background infectious residue at 0 hour p.i. in this model was approximately 10⁴ EID₅₀/ml.
Using a threshold of 10^5 EID50/ml to signify virus replication, we found that avian H7 virus was replication-competent as expected (since the first-discovered EIVs are subtype H7). Additionally, the viruses of subtype H1, H2, and H6 were also replication-competent (Figure 1). This indicates that AIVs of these HA subtypes have the potential to infect horses. We did not test avian subtype H3N8 virus, as there is already a published example of avian H3N8 virus infecting horses (equine/Jilin/89). Among NA subtypes paired with H3, we found that AIVs of the N1 and N5 subtypes were replication-competent.

We next asked, are those viruses that are replication-competent in equine tracheal explants also replication-competent in horses; that is, can they infect horses resulting in shedding of progeny virus particles? This was studied using our long-established procedure for experimental infection of horses with equine influenza viruses. In brief, the test viruses were aerosolized using a DeVillbis Ultra-Neb 99 nebuliser and pumped into a tented stall (21.5 m^3) where it was inhaled by an assembled group of ponies for 45 minutes. The virus dose used, 5 × 10^7 EID50 units/m^3, was 5–10 times greater than we routinely use for equine influenza virus infection experiments. We used three influenza-seronegative weanling or yearling ponies for each of three test viruses (H1N2, H6N2, H7N2), with the aim of obtaining a qualitative yes/no answer rather than a quantitative incidence rate. Each of these viruses was studied in a separate experiment, using different animals and with the biocontainment stalls disinfected between experiments. Ponies were examined daily for clinical signs of disease. Nasopharyngeal swab samples were obtained daily through Day 7 post-infection for assessment of virus shedding by quantitative real-time RT-PCR (qRT-PCR) using M1-specific primers (InfA Forward, InfA Reverse, InfA Probe; CDC REF. #I-007-05). These primers were tested to verify reactivity with the specific AIV studied in ponies. RNA copy numbers were calculated against a standard curve generated using in vitro-transcribed RNA made from cloned M1 cDNA as described. Sera were obtained at Day 0 and again at Day 14 post-infection, and virus-specific serum antibodies were

| Table 1. Avian influenza viruses tested |
|----------------------------------------|
| Subtype     | Isolate name               |
| H1N2        | A/Mallard/SC/264/88         |
| H2N2        | A/Mallard/NY/6750/78        |
| H4N2        | A/Pheasant/PA/7128/93      |
| H5N2        | A/Turkey/MN/40550/87       |
| H6N2        | A/Mallard/NY/118/88        |
| H7N2        | A/Mallard/Alberta/223/79   |
| H9N2        | A/Turkey/CA/1/88           |
| H11N2       | A/Ruddy Turnstone/DE/2762/87 |
| H13N2       | A/Herring Gulf/DE/134/90   |
| H3N1        | A/Shorebird/DE/275/2009    |
| H3N2        | A/Shorebird/DE/485/2008    |
| H3N3        | A/Mallard/Alberta/192/2004 |
| H3N4        | A/Mallard/Alberta/205/2004 |
| H3N5        | A/Mallard/Alberta/310/2010 |
| H3N6        | A/Blue-winged Teal/Alberta/376/2007 |
| H3N7        | A/Mallard/Alberta/113/91   |
| H3N8        | A/Mallard/Alberta/582/2010 |
| H3N9        | A/Mallard/Alberta/31/2001  |

All viruses were obtained from the influenza repository at St. Jude Children’s Research Hospital, Memphis TN, USA, and grown in embryonated hen eggs.
measured using the hemagglutination-inhibition (HI) assay\textsuperscript{15} using both ether-treated and untreated virus antigens. All animal work was approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol no. 2007-0153).

Results of these pony infection experiments were almost all negative. No pony exhibited disease signs or seroconverted to the AIV used for infection. Most nasopharyngeal swabs were negative by qRT-PCR (Table 2). Two swabs (one pony in H6N2 group and one in H7N2 group) were weakly positive only on Day 1 post-infection which might be residual from the infection process rather than representative of virus replication. One swab from a pony in the H6N2 group was positive with more strength on Day 3 post-infection, but every other swab from that pony was negative.

Our intention was to isolate and sequence viruses from PCR-positive swabs, to determine whether there were genetic changes associated with virus replication in ponies, but after repeated blind passages in embryonated eggs, we failed to isolate any viruses. Genetic adaptation of avian influenza viruses to the equine host has not been rigorously examined. As several AIV subtypes tested here did exhibit virus replication in explanted equine tracheal epithelium, HA receptor binding is unlikely to be a restricting factor for those subtypes. The PB2 E627K substitution, which features prominently in the adaptation of many zoonotic AIVs to humans, is not present in the PB2 of equine influenza viruses found in GenBank, as per our own inspection. Also the AIV tested here, or similar strains in GenBank, all featured PB2 with E627.

Neither the in vitro tracheal explant experiments nor the in vivo pony infection experiments exhaustively test every AIV subtype, and our results cannot rule out the possibility that some AIVs may be infectious for horses. They do, however, demonstrate that the equine tracheal explant model is a poor predictor of AIV infectivity in the upper respiratory tract of live equines.

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Competing interests

The authors have no competing interests.

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Table 2. Detection of virus shedding by qRT-PCR following experimental infections of ponies

| Virus | Pony | qRT-PCR results |
|-------|------|-----------------|
| H1N2  | L41  | Days 0–7: All negative (C\textsubscript{t} > 40) |
| H1N2  | L46  | Days 0–7: All negative (C\textsubscript{t} > 40) |
| H1N2  | L55  | Days 0–7: All negative (C\textsubscript{t} > 40) |
| H6N2  | L52  | Days 0–7: All negative (C\textsubscript{t} > 40) |
| H6N2  | L54  | Day 3: C\textsubscript{t} = 33 (24408 RNA copies), all other days negative (C\textsubscript{t} > 40) |
| H6N2  | L72  | Day 1: C\textsubscript{t} = 40 (432 RNA copies), Day 7: C\textsubscript{t} = 39 (785 RNA copies). All other days negative (C\textsubscript{t} > 40) |
| H7N2  | M75  | Days 0–7: All negative (C\textsubscript{t} > 40) |
| H7N2  | M76  | Day 1: C\textsubscript{t} = 37 (2483 RNA copies), All other days negative (C\textsubscript{t} > 40) |
| H7N2  | M89  | Days 0–7: All negative (C\textsubscript{t} > 40) |

Ponies were infected by inhalation of nebulized virus (5 x 10^7 EID50 units per m\textsuperscript{3}). Nasopharyngeal swabs were collected prior to infection (Day 0) and daily for 7 days post-infection (Days 1–7). Results are expressed as cycle threshold (C\textsubscript{t}) values, in which lower number = stronger signal, and C\textsubscript{t} > 40 is undetectable. In parentheses are calculated RNA copies.
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