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Short Communication

Infectivity of equine H3N8 influenza virus in bovine cells and calves

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Background Serological evidence for influenza A, subtype H1 and H3 virus infections of bovines, associated with respiratory disease and decreased milk production, has been reported. Equine H3N8 influenza virus circulates widely and was responsible for the introduction of H3N8 influenza into canines.

Objective To explore the possibility that equine H3N8 influenza might also infect bovines.

Methods To assess the incidence of seroconversion in the field, a retrospective survey of bovine serum samples was carried out. Also, primary cultures of bovine nasal turbinate cells, and live beef calves, were studied for their permissiveness to infection.

Results and Conclusions We found serological evidence of exposure of bovines in Kentucky to H3 influenza. We demonstrate that cultured bovine respiratory epithelium is permissive for the growth of equine H3N8 influenza virus *in vitro*, but this virus does not replicate extensively or produce disease in experimentally inoculated cattle.

Keywords Cattle, epithelium, host range, temperature-sensitive.

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Cows and steers are not commonly recognized as natural hosts for influenza viruses. However, a number of reports suggest sporadic transmission of influenza to ruminants. Significant incidence of cattle seropositive to influenza A viruses of the H1 and H3 subtypes has been reported,[1–8] and this has been associated with decreased milk production and sometimes respiratory diseases.[1,3,4] However, no influenza virus was isolated in those studies. Cattle can be experimentally infected with human and swine influenza A viruses[9,10] as well as with pandemic H5N1 avian influenza virus,[11] although generally without overt signs of disease.

Horses are a natural host to influenza A virus. Equine H3N8 influenza viruses actively circulate in horses in much of the world.[12] Equine H3N8 virus also is transmitted across species barriers into canines and since 2004 has established itself in canine populations in the USA.[13] The potential of equine influenza virus (EIV) as a source of respiratory disease in cattle and the cellular pathology of EIV infection in bovines are not clear. As cows and horses sometimes come into proximity, it was of interest to determine whether bovines are susceptible to EIV infection.

Survey of random bovine sera for HI antibodies to H3 influenza

To add the bovine sero-epidemiological picture in Kentucky to that previously published for Minnesota[5] and the United Kingdom,[1,3,4,8] we performed a retrospective serological survey of random stored bovine serum samples submitted in 1999 and 2000 for routine diagnostics at the University of Kentucky Livestock Disease Diagnostic Center. Samples were Kaolin-treated sera from Kentucky cattle of various breeds from ages 4 months to 8 years. When measured by hemagglutination-inhibition assay using chicken erythrocytes,[14] 51% of sera had detectable antibodies to either influenza A/equine/Kentucky/94 (H3N8) or influenza A/swine/Texas/98 (H3N2) (Table 1). The two antigens partially cross-react, and we observed higher incidence and significantly higher titers of positive reactions

	1999	2000	Total
Number of samples tested	241	111	352
Equine/KY/94			
Number positive	53 (22%)	7 (6%)	60 (17%)
Mean titer and (range) (pos only)	28 (20–40)	20 (20–20)	28
Swine/TX/98			
Number positive	134 (56%)	44 (40%)	178 (51%)
Mean titer and (range) (pos only)	43 (20–160)	23 (20–40)	37

Randomly selected, kaolin-treated sera from Kentucky cattle of various breeds and ages 4 months to 8 years were tested by HI assay using 0·5% chicken erythrocytes. Virus antigens were back-titrated at 4 HA units/well. Titers <20 were recorded as negative. HI titers are expressed as geometric means of the positive results (i.e. negative results are excluded from the calculation). As controls, sera from two horses with known exposure to H3N8 equine influenza virus (EIV) were tested against the same antigens, with means and ranges as follows: Equine/KY/94, mean 224 (range 80–640); Swine/TX/98, mean 40 (range 10–160).

Table 1. Survey of random bovine sera for HI antibodies to H3 influenza

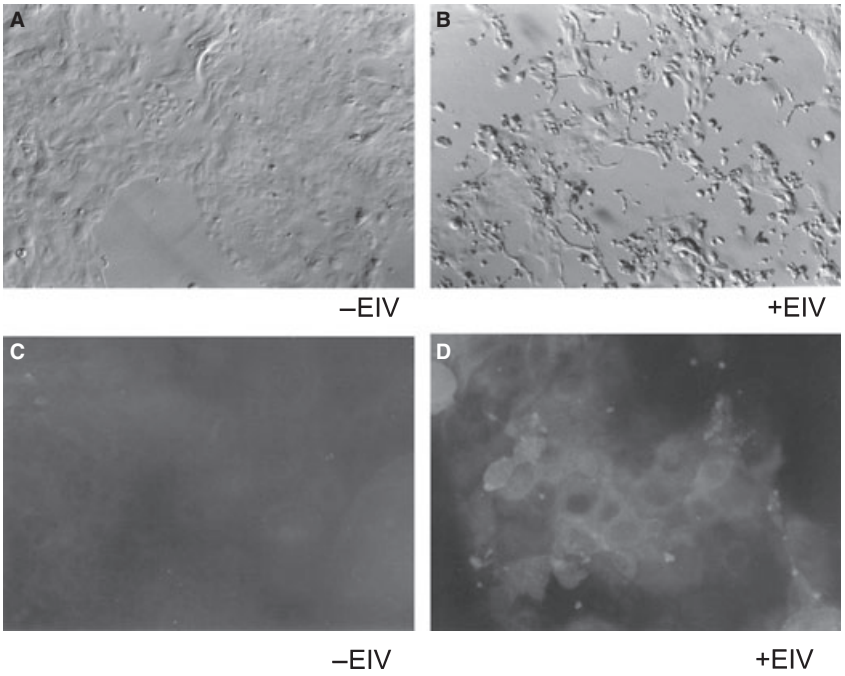

Figure 1. (A and B) Light microscopic appearance of morphological changes and (C and D) indirect IFA staining of mock (–EIV (equine influenza virus), A and C) or virus (+EIV, B and D)-infected BT cells. Cells were either mock infected with normal egg allantoic fluid alone or infected with EIV (Miami/63, H3N8) and examined at 48 hours p.i. IFA was carried out using a monoclonal antibody against the viral HA.[21]

against the swine strain ($P = 0.003$). However, there are relatively few swine in Kentucky. Therefore, the source of viruses responsible for seroconversion to H3 influenza in the bovines tested is unclear. We did not test for antibodies against subtype H1 influenza.

EIV replication in primary cultivated bovine nasal turbinate cells

Primary cultures of respiratory epithelial cells are believed to be a useful model for the study of virus–host interactions,[15,16] possibly more representative of natural infection than virus culture in embryonated chicken eggs and continuous cell lines, where adaption and passage of influenza virus can change native virus properties such as receptor binding or tissue tropism.[17,18] To establish cell growth, we isolated nasal turbinate from a freshly deceased cow, and the bovine turbinate (BT) cells were obtained by proteolysis using similar procedures as reported previously.[19] Primary cultivated BT cells were infected with influenza A/equine/Miami/63 (H3N8) virus at a multiplicity of infection (MOI) of approximately 10 EID_{50}/cell. Tissue culture

supernatants from the infected cultures were collected at 24-hour intervals post-infection (p.i.) for the analysis of virus infection kinetics by HA and EID_{50} assays as described previously.[20] EIV HA antigen present in infected cells was detected by indirect immunofluorescent antibody (IFA) staining with a mouse monoclonal antibody (F90 5H1.25, kindly provided by Dr. Judith Appleton) specific for H3N8 EIV surface HA antigen[21] and Texas-Red conjugated goat anti-mouse immunoglobulin as the detection antibody. The cytotoxic effect of EIV on BT cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell survival assay that is dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to blue formazan product. The conversion (yellow to blue) takes place only in living cells, and the amount of formazan produced is proportional to the number of cells present.[22]

Mock-infected BT cells showed almost no morphologic changes (Figure 1A), whereas EIV-infected cells at 48 hours p.i. showed cytopathic effect characterized by cell shrinkage, rounding, and detachment from the culture matrix (Figure 1B). By indirect IFA labeling, in comparison with mock-infected cultures (Figure 1C), a strong fluorescence staining was observed in EIV-infected cells (Figure 1D). Therefore, BT cells were susceptible to infection by EIV. As shown in Figure 2A, viral HA and EID_{50} titers were found to rise steadily over 72 hours p.i. from being undetectable to an HA titer of 32 and 2×10^6 EID_{50} units/ml. These data indicate that EIV not only infected but also actively replicated in BT cells. When cell survival was assessed by MTT assay (Figure 2B), the optical density (OD) in mock-infected BT cell cultures increased gradually through 72 hours p.i., indicating that the cells continued to proliferate, whereas EIV infection of BT cells caused >50% reduction in OD value at 72 hours p.i. Comparison of Figure 2A and Figure 2B shows that the OD value dropped as the virus yield increased, indicating that the decline in cell survival was associated with virus replication in BT cells and probably resulted from EIV-mediated apoptotic cell death as we have previously described in equine primary nasal turbinate cells[19] as well as MDCK cells.[23,24] Primary equine turbinate cells[19] were susceptible to the infection with the supernatant collected from EIV-infected BT cell culture (data not shown), showing that the host specificity of BT-passaged EIV retained infectivity for equine cells.

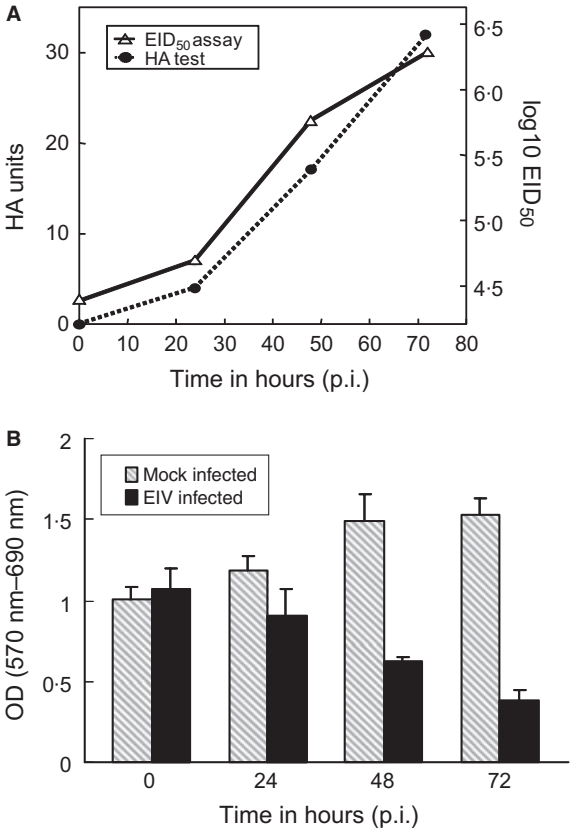

Figure 2. (A) Replication of equine influenza virus (EIV) (Miami/63, H3N8) in primary BT cells. Supernatants from infected cells were collected for virus titration at 0, 24, 48, and 72 hour p.i. Virus titers are shown as HA units (dotted line, left scale) and EID_{50} units (solid line, right scale). (B) Cytocidal effect of EIV on BT cells. Viability of mock (hatched)-infected and EIV (solid)-infected BT cells were determined by MTT assay at the same time intervals p.i. as for (A). Data are means ± SD for three cultures.

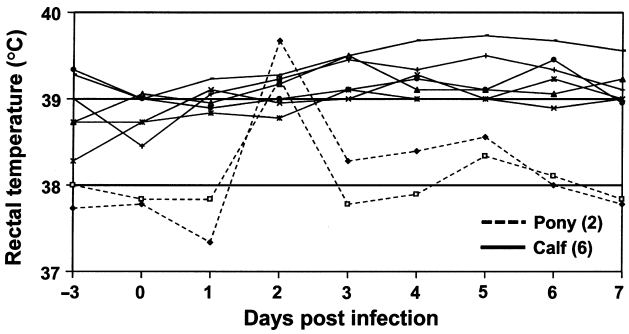

Figure 3. Daily rectal temperatures of calves (solid lines, $n = 6$) and ponies (hatched lines, $n = 2$) following experimental infection with influenza A/equine/KY/91 virus. Each line represents one animal.

Experimental infection of calves with EIV

As virus growth in primary cell culture may not correlate with the capability of EIV to infect and cause disease in the intact animal, *in vivo* experimental infection of live cattle was performed. Briefly, a group of six beef calves (6 months age) and two ponies (8 months age) that were seronegative for EIV were experimentally infected with aerosolized virus strain influenza A/equine/Kentucky/91

Table 2. Virus shedding in nasopharyngeal swabs

	Day post-infection								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Calves (ID#)									
#72	–	–	–	–	–	–	–	–	–
#74	–	–	–	–	–	–	–	–	–
#76	–	–	–	–	–	–	–	–	–
#113	–	–	–	–	–	–	–	–	–
#121	–	–	–	–	–	–	–	–	–
#139	–	–	–	–	–	–	–	–	–
Ponies (ID#)									
#9929	–	+	+	+	+	+	+	+	–
#9954	–	+	+	+	+	+	+	+	–

Nasopharyngeal swabs collected from calves and ponies were inoculated into embryonated chicken eggs and incubated for 3 days at 35°C. Virus growth +/– was determined for each swab by HA assay.

Table 3. Serological responses to experimental influenza infection

	Day 0	**Day +9**	**Day +20**
Calves (ID#)			
72	<10	<10	<10
74	<10	<10	<10
76	<10	<10	<10
113	<10	<10	<10
121	<10	<10	<10
139	<10	<10	<10
Ponies (ID#)			
9929	<10	320	320
9954	<10	160	320

Sera were collected on the indicated days post-challenge and treated with trypsin-periodate to remove non-specific inhibitors of hemagglutination, then assayed by HI assay using intact equine/KY/91 virus as antigen. <10, lower limit of detection.

(H3N8) as previously described.[25] Each animal was exposed to 10^6 EID_{50} of virus by inhalation through a mask. The ponies served as positive infection controls and were housed in a separate stall but in the same room as the calf stalls and were challenged at the same time. The animals were monitored daily for clinical manifestation of influenza, and their rectal temperatures were recorded. As shown in Figure 3, aerosol-infected calves showed no pyrexia or overt clinical signs, whereas ponies exhibited typical influenza-like symptoms including pyrexia on Day +2 post-challenge, nasal discharge, and cough on multiple days later post-challenge. Nasopharyngeal swabs were collected daily from all animals and were tested for virus shedding by inoculation into embryonated chicken eggs. EIV was detected only in the nasopharyngeal swabs from the positive control ponies, whereas no virus was detected in any of the swabs from the experimentally infected calves (Table 2). Sera were collected and tested by hemagglutination-inhibition for antibodies against equine/KY/91 virus as described.[14,25] As shown in Table 3, both ponies seroconverted following EIV infection, while calves did not. Therefore, live calves were not susceptible to infection with EIV based on standard clinical, virological, or serological measures.

We observed during the pre-challenge phase that the beef calves had a higher normal body temperature (mean 38·9°C) than the ponies (37·9°C) (Figure 3). We thank Dr. Alicia Solorzano for the suggestion that this difference in normal body temperature could be critical for *in vivo* infection if equine/KY/91 virus were temperature-sensitive. Youngner *et al.*[26] have previously described the cold-adaptation of this EIV strain and observed that the wild-type virus produced titers at 39·5°C ($1{\cdot}5 \times 10^6$ PFU/ml) that were within 1 log of those produced at 34°C (1×10^7 PFU/ml). This suggests that 38·9°C should still have been a permissive temperature. Furthermore, the temperature of the upper respiratory tract in both calves and ponies is expected to be slightly less than rectal temperature. Therefore, it seems unlikely that the 1°C difference in core body temperature alone was responsible for the suppression of detectable virus replication in beef calves, although it might have been a contributing factor.

In summary, we demonstrated productive infection of H3N8 EIV in primary cultured bovine respiratory cells. However, H3N8 EIV did not detectably replicate, produce disease, or trigger seroconversion in live beef calves.

A serological survey suggests that some bovines in the state of Kentucky were exposed to H3 influenza. It is unclear which H3 viruses were responsible for bovine seroconversion to H3 influenza in the field.

Acknowledgments

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