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# Little evidence of human infection with equine influenza during the 2007 epizootic, Queensland, Australia

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ABSTRACT

*Background:* Equine influenza virus (EIV) is considered enzootic in Europe (except Iceland), Asia, North Africa, and North and South America. When EIV outbreaks occur they may severely impact the equine and tourist industries. Australia faced its first EIV outbreak beginning in August of 2007. The outbreak was concentrated in New South Wales and Queensland, with more than 1400 confirmed EIV infections in horses during the first month. Rapid response from the equine industry and the federal government was successful and Australia was declared free from EIV by the end of 2007.
*Objectives:* This cross-sectional study was designed to examine associations between exposure to EIV-infected horses and evidence of EIV infection in humans.
*Study design:* Employing informed consent, between October 2007 and April 2008, 100 subjects (89 with horse exposures and 11 non-exposed) were enrolled during equine events and at the University of the Sunshine Coast. All subjects provided a blood sample and were asked to complete an online questionnaire including health history, animal exposure and demographic information. Sera samples were tested for the presence of antibodies against two H3N8 EIV strains using microneutralization, hemagglutination inhibition, and enzyme-linked lectin assays.
*Results:* Evidence for H3N8 infection was sparse, with only 9 study participants having any indication of H3N8 infection and the seroreactivity seen was low and easily explained by cross-reactions against human influenza strains or vaccines.
*Conclusions:* These data provide little evidence to support the premise that EIV infections occurred among humans exposed to EIV-infected horses during the 2007 Australian epizootic.


## 1. Background

In the 1800s, when horses were central to transportation and commerce, equine influenza virus (EIV) epizootics brought national economic calamity in North and Central America [1]. Historical reports from Australia and New Zealand during the early 1900s report thousands of horses falling ill, severely impacting mounted forces during battle [2]. Today EIV epizootics continue to sporadically occur, however, their impact is more focused upon horse racing and tourism industries. Australia's horse industry suffered a national EIV outbreak associated with imported horses in 2007. The H3N8 EIV emerged in Sydney and greatly impacted horses in New South Wales and southern Queensland [3]. This epizootic was associated with an estimated AU$24 million in economic losses to equine industries, AU$381 million in losses to households and businesses, and AU$390 million direct costs to the government [4,5]. OIE received confirmation of 1485 EIV infections in horses and 1 fatality between August 28 and September 14, 2007; fortunately, industry and government veterinary professionals quickly implemented control strategies to restrict EIV transmission, ultimately eradicating the virus from the equine population by the end of 2007 [6].

## 2. Objectives

As was observed in the 1800s [7] and experimentally proven through challenge studies in the 1960s [8–10], humans are readily infected with EIV. While some persons asymptomatically seroconvert, others develop classical influenza-like signs and symptoms. The goal of this study was to look for evidence that humans exposed to EIV infected horses in Queensland during the 2007 Australian

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epizootic were infected and if possible, to identify risk factors for those infections.

## 3. Study design

Permission for this cross-sectional study was granted by the Human Research Ethics Committees at the University of the Sunshine Coast in South East Queensland, Australia and at the University of Iowa, Iowa City, IA, USA. Potential participants were contacted between October 2007 and April 2008 throughout the enrollment target areas. Methods of contact included email messages, and attending Pony Club Association meetings and local public health information sessions.

### 3.1. Subject enrollment

Eligible participants had to be 18 years of age or older, have no recognized immunosuppressive conditions, and deny currently receiving immunosuppressive therapies. Most individuals recruited had varying degrees of direct equine contact. Direct contact was defined as: (i) touching a horse, or (ii) being less than one meter away from a horse for at least 30 min at a time. All participants signed an informed consent document. Upon enrollment, each participant was asked to provide a whole blood sample and complete an online questionnaire that captured demographic, medical history, and animal occupational and recreational exposures. This online questionnaire was developed from one used in similar studies [11–13].

### 3.2. Specimen collection

Following collection, sera aliquots were stored at −80 °C before shipping to the University of Iowa for testing.

### 3.3. Influenza viruses studied

Sera were tested for the presence of antibodies against A/equine/Pennsylvania/01/2007(H3N8), A/equine/Mongolia/01/2008(H3N8) and human influenza A/Brisbane/10/2007(H3N2). As we failed in our attempts to obtain a 2007 Australian EIV strain, the two EIV strains mentioned above were chosen because they were closely related through sequence studies to the 2007 EIV Australian epizootic strains.

### 3.4. Serological assays

A hemagglutination inhibition (HI) assay was employed to detect potentially cross-reacting antibodies against the human influenza virus or vaccine and a microneutralization (MN) assay was used against the EIVs. Additionally, an enzyme-linked lectin assay was developed using recombinantly expressed N8 from the A/equine/Pennsylvania/01/2007(H3N8).

#### 3.4.1. Microneutralization (MN) assay

A WHO-recommended MN assay adapted from that reported by Rowe [14] was used to detect human antibodies against equine viruses, with the following modification: MDCK London cells were added at $2 \times 10^4$ cells per assay well. Assays were conducted using equine influenza A virus grown in embryonated chicken eggs. Sera were first screened at a dilution of 1:10. Positive specimens were then titrated out in duplicate by examining 2-fold serial dilutions from 1:10 to 1:1280. Virus neutralization was assessed in these assays using influenza A nucleoprotein specific ELISA assay described by Rowe [14]. The ELISA endpoint titer was expressed as the reciprocal of the highest dilution of serum with optical density (OD) less than $X$, where $X$ = [(average OD of virus control wells) + (average OD of cell control wells)]/2. Test cells with an OD> 2 times the cell control OD mean were considered positive for virus growth. A back titration of the virus antigen was run in duplicate and only accepted when both replicates had matching results. Influenza viruses were obtained from acknowledged collaborators or from Biodefense and Emerging Infections (BEI) Research Resources Repository or through the Influenza Reagent Resource (IRR) program of the US CDC.

#### 3.4.2. Hemagglutination Inhibition (HI) assay

A WHO-recommended HI assay [15] was used to test for serum antibodies against influenza A (H3N2). Influenza virus strains were grown in embryonated chicken eggs. Sera were pre-treated with receptor destroying enzyme and hemabsorbed using 0.65% guinea pig erythrocyte suspension in PBS. Titer results were reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination, indicating the presence of antibodies in serum. Viral antigens and control antisera for HI assays were obtained from acknowledged collaborators or from Biodefense and Emerging Infections (BEI) Research Resources Repository or through the Influenza Reagent Resource (IRR) program of the US CDC.

#### 3.4.3. Enzyme-linked lectin assay (ELLA)

This assay as previously described by Hassantoufighi [16] was adapted to use a baculovirus expressed recombinant neuraminidase subtype 8 (rN8) (BEI Resources catalog #NR-13523) from the A/equine/Pennsylvania/1/2007(H3N8) influenza virus. The soluble rN8 used in this assay was demonstrated by the supplier to be functionally active based on its ability to cleave the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUNANA) indicating it would be appropriate for use in a functional neuraminidase inhibition assay. In addition to using rN8 protein in place of whole viral antigen, the ELLA procedure [16] was also modified by implementing a screening test at a sera dilution of 1:10 in duplicate. Sera testing positive at the 1:10 dilution were subjected to a titration assay in which serial 2-fold dilutions of the specimen were tested in duplicate. A goat polycolonal antiserum to the N8 of A/equine/Miami/63 (BEI Resources NR-3145) was included at 1:20 on screen plates and as a single serial 2-fold dilution (1:20 to 1:2560) on titration plates. An assay well was determined to be positive for N8 specific inhibiting antibodies when the measured absorbance levels were less than 50% of the average of the standardized antigen control wells (contained no serum) for the corresponding plate after background levels (average of plate control wells which contained only sample diluent) were subtracted from all wells on the plate. The standardized concentration of rN8 used for this assay was determined by performing the assay as described above with multiple replicates of serially diluted antigen in the absence of any serum specimen. The dilution of stock antigen for use in these ELLA assays was selected as the dilution which produced absorbance levels at ∼80% of saturation under the assays conditions.

### 3.5. Statistical methods

As serologic responses to zoonotic influenza infections can rapidly wane [17], as we have reported previously [11–13,18], we chose a low threshold of antibody titer (≥1:10) as evidence of previous infection with an EIV strain. Because we know cross-reactions from human influenza virus infection can confound EIV serology, we considered seroreactivity (HI titer ≥1:40) as an indicator as a potential confounder. Initially we examined risk factors for bivariate associations with assay results. When data were sparse we