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Serological evidence of equine influenza infections among persons with horse exposure, Iowa

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ABSTRACT

Background: Equine influenza virus (EIV) is considered enzootic in North America and experimental studies have documented human EIV infections.
Study design: This cross-sectional study examined 94 horse-exposed and 34 non-exposed controls for serological evidence of EIV infection. Sera were evaluated for antibodies against three EIV and two human H3N2 viruses using microneutralization (MN), neuraminidase inhibition (NI), enzyme-linked lectin (ELLA), and hemagglutination inhibition (HI) serological assays. Risk factor analyses were conducted using logistic regression and proportional odds modeling.
Results: There was evidence of previous infection by MN assay against A/equine/Ohio/2003(H3N8) but not the other 2 EIVs. Eleven (11.7%, maximum titer 1:320) horse-exposed and 2 (5.9%, maximum titer 1:160) control subjects had MN titers ≥1:80. Among the horse-exposed, 18 (19.1%) were positive by NI assay and 8 (8.5%) had elevated ELLA titers ≥1:10. Logistic regression modeling among horse-exposed revealed that having an elevated MN or ELLA titer (≤1:10) was associated with having a positive NI titer (OR = 4.9; 95% CI = 1.3–18.7, and OR = 53.2; 95% CI = 5.9–478.5, respectively). Upon proportional odds modeling, having worked as an equine veterinarian (OR = 14.0; 95% CI = 2.6–75.9), having a history of smoking (OR = 3.1; 95% CI = 1.2–7.7), and receipt of seasonal influenza vaccine between 2000 and 2005 (OR = 2.3; 95% CI = 1.1–5.0) were important independent risk factors for elevations in MN assay.
Conclusions: While we cannot rule out confounding exposures, these data support the premise that occupational exposure to EIV may lead to human infection.

1. Background

A 2005 national survey found there were more than 9,000,000 horses in the United States with 4.6 million Americans involved in the industry, many having direct contact with horses [1]. Passive surveillance in the United States has documented sporadic equine influenza virus (EIV) infections among US horses.

Anecdotal reports from 16th to 17th century indicate that cross species (horse and man) influenza transmission was not a rare event [2,3]. Experimental research in the 1960s clearly documented that horses can be infected with human H3N2 influenza viruses [4] and that humans can be infected with equine H3N8 virus and become ill [5,6]. Serial passage experiments also document at least one EIV serially passaged among human volunteers still caused illness among horses [6]. One report from Chile documented possible EIV infection of a horse worker during an equine epizootic, but the isolate was not typed [7]. Otherwise, data are very sparse regarding the risk for humans with exposure to horses to experience EIV infections [8–10]. As horses have played a role in cross-species transmission of influenza virus to dogs [11] and camels [12], and avian influenza virus has caused clinical diseases in horses [13] it seems prudent to consider horses in the ecology of influenza viruses that infect man.

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1.1. Objectives

In this cross-sectional study we examined seroepidemiological evidence that persons with occupational or recreational exposure to horses may have experienced EIV infections.

2. Study design

2.1. Study subjects

Healthy volunteers >18 years of age were enrolled from March to May 2005 at three locations/events in the state of Iowa. Each participant provided written informed consent. Subjects were classified as horse-exposed if they had consistent (at least once per week) exposure to horses within the past six months. Participants were then asked to complete a questionnaire (demographic, medical history, and occupational and/or recreational animal exposure), available in English and Spanish, and to permit phlebotomy. Non horse-exposed controls (those with no horse exposure in the past 10 years, with sera also drawn in 2005) were taken from our serum bank of healthy adults who gave consent for their samples to be used for future studies.

2.2. Laboratory methods

Serum samples were tested against three EIV strains and two recently circulating human influenza H3N2 isolates using a microneutralization (MN) assay, a neuraminidase inhibition (NI) assay, an enzyme-linked-lectin (ELLA), and a hemagglutination inhibition (HI) assay.

Both the A/Equine/Ohio/2003(H3N8) and A/Equine/New Market/2/93(H3N8) strains were recommended for the inclusion in the 2005 equine influenza vaccine; they are representative of the Florida clade 1 and Eurasian lineages, respectively [14]. The A/equine/Ohio/2003 (H3N8) strain is representative of equine influenza viruses circulating in the USA and Canada at the time [15]. The two human influenza A isolates, A/Panama/2007/99(H3N2) and A/Nanchang/933/95(H3N2), examined evidence for cross-reactivity as confounding the EIV serological assays [obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository or through the Influenza Reagent Resource (IRR) program of the US CDC].

2.3. Microneutralization assay

A WHO-recommended MN assay adapted from Rowe [16–18] was used to detect human antibodies against the three equine viruses. The viruses were grown in fertilized eggs. Sera were first screened at a dilution of 1:10 with positive specimens titrated out in duplicate by examining 2-fold serial dilutions from 1:10 to 1:1280. Virus neutralization was then performed by adding of virus to the sera at 100 $TCID_{50}$/100 μL as determined by the Reed Muench method [19]. MDCK cells in log phase growth were adjusted to 2.0×10^5 cells/mL with virus diluent, and added to each well. The plates were then incubated at 37 °C with 5% CO_2 for 22 h. Following incubation plates were washed twice with PBS, fixed with cold 80% acetone, and allowed to dry at room temperature. Influenza on the fixed monolayers was quantified by influenza A nucleoprotein-specific indirect ELISA washing with PBS containing 0.05% Tween 20 between each antibody addition. Following the final wash, 0.1 mL of 3,3′,5,5′-tetramethylbenzidine (TMB) [Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland] was added and incubated for 10 min at room temperature, then stopped by the addition of 0.1 mL of 1N sulfuric acid. The optical density of the plates was read at 450 nm. 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. A back titration of the virus antigen was run in duplicate and only accepted when both replicates had matching results. The entire spectrum of titers was reported, but a titer endpoint ≥1:80 was considered elevated.

2.4. Neuraminidase inhibition assay

As previously reported [20,21], a qualitative NI assay was developed to examine sera for antibodies against neuraminidase (N8) subtype specificity using A/equine/Ohio/2003(H3N8) as the whole virus antigen. A virus titration was first performed to determine the optimum antigen dilution, which was the highest dilution with a "medium" pink color, or the dilution prior to a reduction in pink color. Sera were heat-inactivated at 56 °C for 30 min then diluted 1:2.25 in PBS. Using white opaque polystyrene 96-well microtiter plates [Nunc, Rochester, NY], 25 μL of test sera were added in duplicate, followed by 25 μL of standardized virus. Virus control wells with PBS only, as well as positive control wells employing equine sera were used as assay controls. Next, 25 μL of 50% sodium arsenite reagent was added to each well, causing a dark brown precipitate to form; followed by the addition of 100 μL of 0.6% thiobarbituric acid to all wells. Each plate was covered with plate sealing tape, and punctured over each well to allow for expansion while incubating in a 56 °C water bath for 30 min prior to reading [21]. A dark pink color was deemed negative, and a reduced pink or light pink color was considered positive. The NI assay was run on only the sera from equine-exposed participants.

2.5. Enzyme-linked lectin assay

This assay, as previously described by Hassantoufighi [22] was modified 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 [14,23]. In addition to using rN8 protein in place of whole viral antigen, the ELLA procedure [22] was modified by implementing a screening test at a sera dilution of 1:10 in duplicate. Sera testing positive at the 1:10 dilution were titrated in serial 2-fold dilutions of the specimen in duplicate. A goat polyclonal antiserum to the N8 of A/Equine/Ohio/2005(H3N8) was included at 1:20 on screen plates and as a single serial 2-fold dilution (1:20–1:2560) on titration plates. A positive assay well had measured absorbance levels less than 50% of the average of the standardized antigen control wells (no serum) for the corresponding plate, after background levels were subtracted from all wells on the plate. As used previously, a cut point of ≥1:10 was considered elevated [23].

2.6. Hemagglutination Inhibition (HI) assay

A WHO-recommended HI assay [24] was used to test for serum antibodies against human influenza strains A/Panama/2007/99(H3N2) and A/Nanchang/933/95(H3N2), which had been in recent circulation. Influenza virus strains were grown in fertilized chicken eggs. Sera were pre-treated with receptor destroying enzyme, heat inactivated at 56 °C for 30 min, and hemadsorbed using 0.65% guinea pig erythrocyte suspension in phosphate buffered saline (PBS). Similarly, HI assays were also run using 0.5% rooster erythrocytes and then 0.5% turkey erythrocytes to test serum antibodies to equine influenza A/Equine/Ohio/2003(H3N8) for cross-reactivity [25]. A titer ≥1:40 was considered elevated.