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Serological evidence for avian H9N2 influenza virus infections among Romanian agriculture workers

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Summary In recent years, wild birds have introduced multiple highly pathogenic avian influenza (HPAI) H5N1 virus infections in Romanian poultry. In 2005 HPAI infections were widespread among domestic poultry and anecdotal reports suggested domestic pigs may also have been exposed. We sought to examine evidence for zoonotic influenza infections among Romanian agriculture workers. Between 2009 and 2010, 363 adult participants were enrolled in a cross-sectional, seroepidemiological study. Confined animal feeding operation (CAFO) swine workers in Tulcea and small, traditional backyard farmers in Cluj-Napoca were enrolled, as well as a non-animal exposed control group from Cluj-Napoca. Enrollment sera were examined for serological evidence of previous infection with 9 avian and 3 human influenza virus strains. Serologic assays showed no evidence of previous infection with 7 low pathogenic avian influenza viruses or with HPAI H5N1. However, 33 participants (9.1%) had elevated microneutralization antibody titers against avian-like A/Hong Kong/1073/1999(H9N2), 5 with titers $\geq$1:80 whom all reported exposure to poultry. Moderate poultry exposure was significantly associated with elevated titers after controlling for the subjects' age (adjusted OR = 3.6; 95% CI, 1.1—12.1). There was

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no evidence that previous infection with human H3N2 or H2N2 viruses were confounding the H9N2 seroreactivity. These data suggest that H9N2 virus may have circulated in Romanian poultry and occasionally infected man.

Introduction

Human infections with novel influenza viruses, such as highly pathogenic avian influenza (HPAI) H5N1 virus and the swine-like 2009 H1N1 pandemic influenza virus, are often only identified through clinical encounters. Little is known about subclinical human infections and risk factors for zoonotic transmission to man.

One of the newest members of the European Union (EU), Romania is economically behind many other EU nations. Approximately 45% of Romania's population of 22 million live in rural areas where small farms are a common form of subsistence [1]. HPAI H5N1 virus has been periodically detected through domestic bird die-offs in the southeastern part of Romania in 2005, 2006, 2007, and 2010 [2—4]. In 2005 and 2006 the infections in domestic poultry were widespread and caused much economic distress. Anecdotal reports suggested pigs may also have been exposed. Field studies documented that these viruses are being introduced through migrating birds that frequent the Danube Delta's flyways [5,6].

We sought to study agricultural workers in Romania for evidence of previous infection with avian influenza (AI) viruses.

Materials and methods

Study design

Cohort enrollment focused on two areas in Romania: lightly populated, Tulcea (population approximately 100,000), in the Danube Delta region, and densely populated Cluj-Napoca (population approximately 340,000) (Fig. 1). In Tulcea, we enrolled chiefly workers associated with large commercial swine CAFOs, while in Cluj, we enrolled farmers exposed to animals in their small traditional backyard farms. A total of five institutional review boards reviewed and approved the study.

Study personnel engaged village or business leaders who at an appointed time invited study subjects to areas where the study team explained the study and invited them to participate via an informed consent process. As we were targeting agricultural workers with intense and prolonged exposure to animals we chose to enroll persons $\geq$18 years of age. Consenting adult participants were interviewed by staff field workers who completed enrollment forms and collected sera. Animal exposure was classified as exposure to domestic poultry, wild birds, or pigs as part of daily activities for $\geq$5 cumulative h/wk. Age-group matched controls who did not meet the inclusion criteria for animal exposure were recruited from Babes-Bolyai University in Cluj-Napoca. Along with demographic information and medical history, community, household, and occupational animal exposures were assessed with the study's enrollment questionnaire. The questionnaire captured flock/herd size for various types of domestic poultry, wild birds, and other animals, well as years of exposure, such that animal exposure could be classified in an ordinal or continuous fashion (e.g. 1000 chicken-years or 1000 duck-years).

Laboratory methods

Whole blood specimens (10 mL red top tube) were transported at 10—15 °C to the field laboratory in Tulcea or to Babes-Bolyai University in Cluj-Napoca within 24 h after collection. Upon arrival, specimens were accessioned and blood tubes spun at 3000 $\times$ *g* for 15 min to separate serum. All collected serum was aliquoted and frozen at −80 °C. Frozen sera were transported on dry ice to the University of Florida for testing.

Influenza virus strains were selected by hemagglutinin (H) type for their best geographic and temporal proximity to the population (Table 1). Influenza virus strains were grown in fertilized eggs. The hemagglutination inhibition (HI) assay was employed as previously reported [7—12] to study human sera for antibodies against human and swine influenza viruses (SIVs). Sera were pre-treated with receptor destroying enzyme and hemabsorbed with either guinea pig or turkey erythrocytes. Titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.65% (guinea pig) or 0.50%