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ISOLATION OF TYPE A INFLUENZA VIRUSES FROM THE MIGRATORY WATERFOWL ALONG THE MISSISSIPPI FLYWAY

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Abstract: Four type A influenza viruses were isolated from tracheal swabs taken from apparently healthy ducks (mallards, *Anas platyrhynchos*) along the Mississippi flyway in Minnesota. Initial identification of group A influenza was made possible by the use of the agar gel precipitin test.

INTRODUCTION

The importance of influenza virus infections in wild birds has not been determined and it is not known whether they are a possible source of infection for domestic avian species.[4,7,8,9,11,15,16] We have undertaken this study of isolation and characterization of influenza viruses from migratory waterfowl because of the possibility that influenza viruses in migratory waterfowl along the Mississippi flyway may be progenitors of turkey influenza epizootics in Minnesota. It has already been suggested that wild birds are involved in the dissemination of influenza virus.[5,14] Easterday, et al.[5] examined sera from nine species of wild North American birds and found antibodies against several of the avian influenza viruses in sera from two species—Canada goose (*Branta canadensis*) and snow goose (*Chen hyperborea*). Winkler, et al.[15] examined sera of wild Canada geese and found hemagglutination-inhibition (HI) antibodies against avian influenza virus strains in 4.7% of the sera examined. Laver and Webster[8] examined sera from migratory sea birds off the coast of Australia and found some sera to have antibodies against the neuraminidase of the human A/Asian/57 strain. No HI antibodies to known avian influenza virus strains were detectable. Zakestel'skaja et al.[16] showed a considerable number of sera from birds in the USSR to have HI titers against certain avian as well as equine strains. Slepuskin, et al.[12] also examined sera from a number of avian species in the far east of the USSR and found HI titers against avian and mammalian strains of type A influenza viruses. Wells[14] suggested that A/Turkey/Eng/63 (NavlNav3) highly pathogenic and closely related to fowl plague virus, was introduced into Britain by migratory birds.

The purpose of this study was to expand the information available regarding influenza viruses in waterfowl so that it might help to clarify the epizootiology of unexplained and sudden influenza infections in domestic turkey flocks in Minnesota. This paper reports the isolation and identification of four group A influenza viruses from migratory ducks.

MATERIALS AND METHODS

Collection of Material

Sera (124) and tracheal swabs (80) were collected from mallards and wood ducks (*Aix sponsa*) trapped with cannon nets at the Roseau Wildlife Refuge in the northwest corner of Minnesota along the Canadian border. This state refuge is an important resting area for migrating ducks along the Mississippi flyway. Many

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thousands of ducks inhabit this marshy refuge during the month of September each year during their southern flight.

Blood Collection

Blood samples were collected by peripheral venipuncture and the serum was stored at —20 C with one drop of 1% sodium azide as preservative.

Tracheal Swabs

Tracheal swabs were collected using calcium alginate swabs—Calgiswab® (Wilson Diagnostics, Inc., Glenwood, Illinois 60425). Swabs were placed in sterile disposable polystyrene tubes with caps (#2027 Falcon, William Drive, Oxford, California 93030) containing 2 ml veal-infusion (VI) broth, penicillin (1,000 units/ml), streptomycin (10 mg/ml) and mycostatin (10 mg/ml). The tubes were snap frozen on dry ice after tracheal swab collection; transported to the laboratory and placed in a —70 C freezer until inoculated.

Virus Isolation

The samples were thawed, let stand at room temperature for 20 min and 0.2 ml volumes were injected into the allantoic cavities of 9-11 day old chick embryos. Three eggs were used for each swab. Inoculated eggs were checked twice daily to detect embryo deaths. When death was detected or at the end of 4 days, the allantoic fluid was collected from each egg and checked for hemagglutinating activity.

Hemagglutination (HA) Test

The hemagglutination test was used to determine the HA titer of the hemagglutinating agent and confirm the presence of a virus. Chicken erythrocytes (RBC) at a concentration of 0.5% in 0.01 M phosphate buffered saline, pH 7.2 were used in the HA test.

All hemagglutinating agents were tested in the HI test against anti-Newcastle disease virus (NDV) serums. If the HI results were negative for NDV, the hemagglutinating agent was inoculated into eggs as above. Each of 10 embryonated eggs received 0.2 ml of a 1:100 dilution (VI broth diluent) of first egg passage allantoic fluid. Eggs were incubated for 48-60 h at 37 C and their chorioallantoic membranes (CAM) were processed in an attempt to detect type A influenza ribonucleoprotein (RNP) antigen by means of the agar-gel precipitin (AGP) test[2] with the following modifications: 1. prolonged incubation to 48-60 hours instead of 18-24 h, 2. CAM's after freezing and thawing thrice in acetone-dry ice mixture were sonicated for 4 min with an ultrasonic power unit (Instrumentation Associates, Inc., New York).

Antiserum

Antiserum was prepared in turkeys against all HA agents isolated. Two 5 week-old Nicholas white turkeys were given 0.25 ml of undiluted allantoic fluid from the 2nd passage intra-tracheally, followed by intra-nasal instillation of 0.25 ml fluid on day 10 and intramuscular injection of 0.25 ml fluid on day 17. All turkeys were exsanguinated 1 week after the last inoculation, and serum collected and stored at —20 C.

AGP Test

The AGP test for anti-ribonucleoprotein antibody was performed on the duck sera collected.[2]

RESULTS

Following the first egg passage, hemagglutinating agents were isolated from four of the 60 swabs collected from mallards at Roseau Wildlife Refuge, Minnesota, during September, 1973. Allantoic fluids from each of the three eggs inoculated with material from those four tracheal swabs contained high levels of HA activity.

The hemagglutinating agents isolated were bacteriologically sterile, grew rapidly at 37 C in the allantoic cavity of chicken embryos, produced high HA titers (1:640-1:1280) and killed embryos within 36-60 h. The agents absorbed to and eluted from chicken erythrocytes in the manner previously observed with other avian influenza viruses.

RNP antigens prepared with these isolates produced precipitin lines adjacent to the wells that contained specific type A influenza turkey antiserum, A/Turkey/Minn/PR/73 and A/Turkey/Minn/Kandiyohi/73/1, but no lines were formed to those that contained normal turkey serum or anti-Newcastle disease serum. A line of identity developed when the new RNP antigens were tested adjacent to type A influenza antigens.

Antiserums prepared against the duck isolates in normal turkeys reacted with lines of precipitation and identity with known anti-influenza A serum.

One hundred twenty-four sera collected from the mallards and woodducks were tested for the anti-RNP antibodies but no lines of precipitation could be demonstrated. The serum from the birds from which the viruses were isolated (#15, 19, 25 and 33) contained no detectable antibodies against the ribonucleoprotein antigen.

DISCUSSION

This paper describes the isolation of four type A influenza viruses from apparently healthy ducks. The report adds to the information of the involvement of migratory ducks with influenza A viruses under natural conditions in the Mississippi flyway. Slemons et al.[11] have made isolations of influenza A viruses from ducks in the Pacific flyway and recently Rosenberger[9] reported similar findings from migratory ducks in the Atlantic flyway.

Initial identification of these isolates as influenza was facilitated by use of the virus type specific AGP test. The isolation of type A influenza viruses from the free-flying ducks in the Mississippi flyway is potentially important for both economic and epidemiologic reasons. It has been observed in Minnesota that ducks during their fall migration utilize ponds adjacent to turkey ranges as resting points. These ponds and sloughs harbor a few non-migratory ducks and a varied collection of free-flying wild birds. This kind of a situation provides excellent conditions for interspecies transfer of influenza viruses. A multiple circulation of varied influenza viruses among ducks would provide great opportunity for genetic recombination in nature, resulting in novel viruses which may cause human pandemics once introduced into the human populations, or may cause epizootics in domestic poultry, namely turkeys. Evidence has accumulated that suggests that viruses which cause influenza pandemics in man might be derived from influenza viruses infecting lower mammals or birds.[11]

The complete absence of detectable levels of anti-RNP antibodies in the duck sera strengthens the findings of Slemons and Easterday.[10] They reported marked differences between avian species in the response to infection with avian influenza viruses. In their studies, a large proportion of experimentally inoculated ducks had low levels of HI titers against the inoculated virus, but still fewer sera were positive with the AGP test. On the other hand, in our studies the duck viruses produced high levels of easily detectable anti-RNP antibodies when susceptible turkeys were inoculated to produce anti-duck influenza virus sera.

The results of HI tests of duck sera have not been included in this study because of the difficulties encountered as mentioned by Slemons.[11] But, further efforts are being made to identify the nature of the hemagglutinin and neuraminidase and to determine whether relationships exist between the Minnesota turkey influenza isolates and the duck isolates.

Further attempts at virus isolation from migratory ducks are in progress and serologic surveys will be performed using the duck isolates as the antigen.

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