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## Virological and Serological Surveillance of Avian Influenza Virus in the Birds of Grenada

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**Abstract:** The zoonotic potential of the circulating influenza subtypes in avian species underscore the importance of surveillance of influenza virus in the avian population. So far there is only one published study in the Caribbean region on the presence of Avian Influenza Virus (AIV) in Barbados. A screening approach based on blood and cloacal and tracheal swabs to study the prevalence of influenza A in the avian population of Grenada was carried out in 2009-2010. We collected 230 blood samples and 230 mixed tracheal and cloacal swabs from backyard chicken (143), ducks (45), turkey (10), guinea fowl (1) and pigeon (31). Samples were screened by RT-PCR, embryo inoculation and ELISA for AIV. Neither AIV RNA was found by RTPCR nor could virus be isolated in embryonated eggs. 27 blood samples from backyard chickens were positive for AIV antibodies.

**Key words:** Influenza virus, AIV antibodies, influenza 'A' viruses

### INTRODUCTION

The Influenza virus is a member of the family Orthomyxoviridae which includes influenza A, B, C and Thogoto viruses (Da Silva *et al.*, 2005). Influenza A viruses were originally isolated in 1902 from chickens (Horimoto and Kawaoka, 2001) and are classified on the basis of the antigenic properties of the Hemagglutinin (HA) and Neuraminidase (NA) glycoproteins expressed on the surface of virus particles. Several studies investigating the prevalence of influenza A viruses in birds have revealed the presence of 16 Hemagglutinin (HA) and Nine Neuraminidase (NA) subtypes of Influenza A viruses (Fouchier *et al.*, 2005).

Wild birds in the orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds) collectively are the natural reservoir for all known subtypes of Avian Influenza Viruses (AIVs) (Webster *et al.*, 1992; Alexander, 2003). AIV in wild birds is thought to be a primarily asymptomatic infection. However in poultry species including chickens, turkeys, guinea fowl and other avian species, infection with AIV can produce a range of symptoms ranging from asymptomatic or mild clinical signs to an acute fatal disease (Alexander, 2003). Symptoms include greenish watery diarrhea, trembling, edema and cyanosis of wattles, combs and shanks (Easterday *et al.*, 1997). Once transmitted to domestic chicken, Low pathogenic Avian Influenza Virus (LPAI) subtypes H5 and H7 can evolve into Highly Pathogenic Avian Influenza Viruses

(HPAI), which can cause severe disease in infected birds (Easterday *et al.*, 1997). Viral spread in the wild birds is typically via oral infected fecal contact as waterfowl shed high titers of AIV from their intestinal tract (Fouchier *et al.*, 2007). Since 2005, H5N1 Avian Influenza (AI) has spread from South-East Asia to over 60 different countries, resulting in the direct death or slaughter of over 250,000,000 poultry (FAO, 2007). These outbreaks emphasized the importance of global surveillance of AIV infections in the natural hosts.

Grenada is an island country of 344 square kilometers in size. It is located at the southern end of Caribbean Sea. Published information regarding the detection of Avian Influenza Virus from Caribbean islands is limited. So far there is only one report in the Caribbean region on the presence of AIV in Barbados. In that study avian influenza viruses from wild waterfowl were isolated and genetically characterized (Douglas *et al.*, 2007). In the present study, we investigated the prevalence of AIV in the free range poultry and pigeons of Grenada.

### MATERIALS AND METHODS

#### Sampling

**Birds: Source and sampling:** We collected a total of two hundred and thirty mixed tracheal and cloacal swabs and sera samples from free range chicken (143), muscovy ducks (45), turkeys (10), rock pigeons (31) and guinea fowl (1) (Fig. 1). Sampling covered all parishes of Grenada and was conducted during the month of

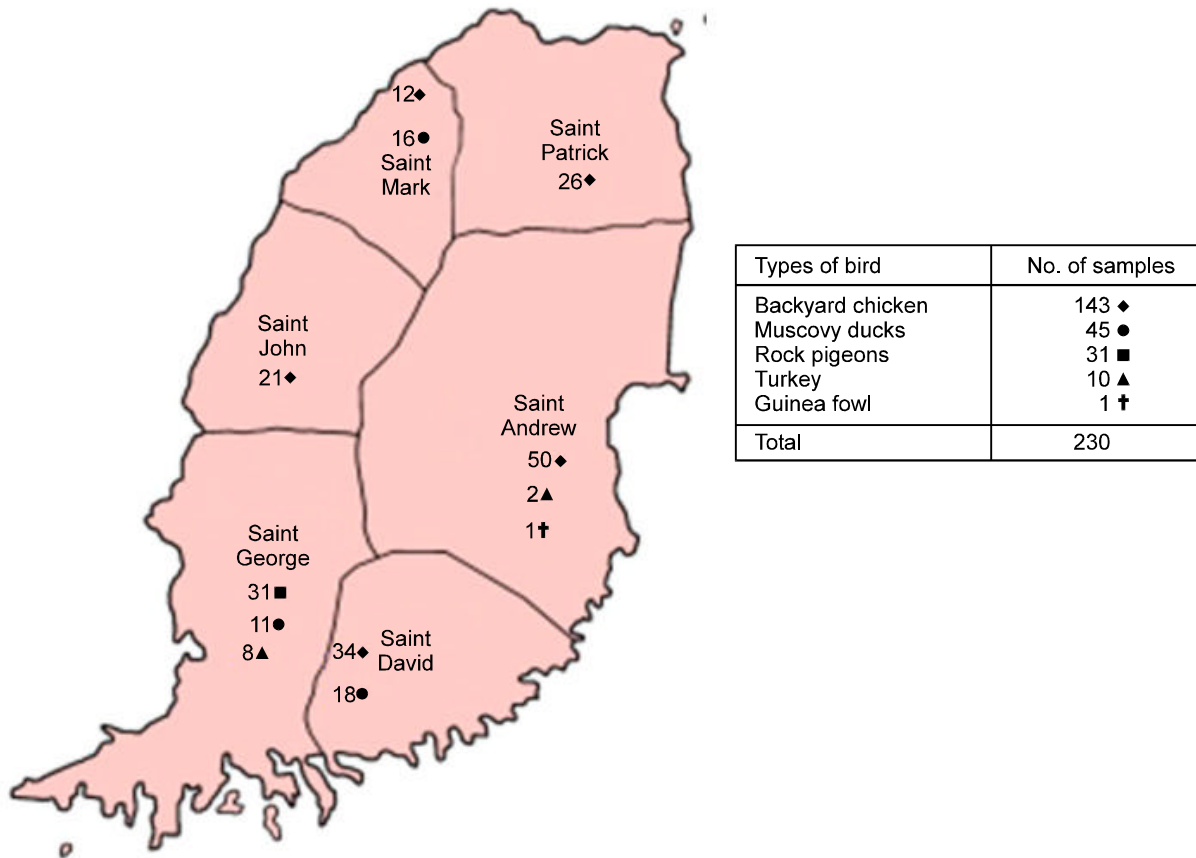


Fig. 1: Picture of Grenada showing distribution of samples collected from different parishes

September to November, 2009. Birds were apparently free from any clinical signs of disease. Approximately 2 ml blood was collected from each bird from wing vein. Sera was separated and stored at -70°C. Swab samples were collected using cotton swabs (Fisher scientific, NY, USA) which were placed in tubes containing 1 ml Transport Medium (Phosphate Buffered Saline Solution with 10% glycerol supplemented with 10 000 u/ml penicillin G, 2 mg/ml streptomycin and 1 mg/ml gentamicin). Samples were transferred to the laboratory and they were stored at -20°C until tested. From each bird, tracheal and cloacal swab samples were taken and placed in the same tube. A corresponding blood sample was available for each of the mixed swab samples.

**Serological examination:** Serum samples were tested for antibodies to influenza A with the commercial FlockChek\* AI Ab Test Kit (IDEXX Laboratories Inc., Maine, USA) as per manufacturer's instructions. The relative level of antibody in the sample was determined by calculating the sample to positive (S/P) ratio. Serum samples with S/P ratio of less than or equal to 0.2 were considered negative and those samples with S/P ratio greater than 0.20 (titer > 396) were considered positive.

**RNA extraction and RT-PCR:** After the swab samples were thawed and centrifuged, total RNA was manually extracted with Qiagen Viral RNA isolation Kit, (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. Viral sequences specific for Influenza A were amplified in a one-step RT-PCR (Invitrogen, USA) with primers M52C (5'-CTT CTA ACC GAG GTC GAA ACG-3') and M253R (5'-AGG GCA TTT TGG ACA AAG/T CGT CTA-3') targeting the matrix gene (Fouchier *et al.*, 2005).

**Virus isolation:** Transport media from swab samples were thawed, vortexed, briefly centrifuged and 50 µl Gentamicin (50 mg/ml, GIBCO®) added to the supernatant. After approximately 1 h incubation at room temperature, 100-200 µl medium from each sample was inoculated into the allantoic cavity of 9-11 days old embryonated chicken eggs. The allantoic fluids were harvested from all eggs six days post-inoculation and were tested for hemagglutinating activity. Presence of hemagglutinating agents was determined by the hemagglutination assay using 1% chicken erythrocytes (WHO, 2002). The allantoic fluids were also subjected to one step RT-PCR assay as described earlier.

Table 1: ELISA result of samples collected from different bird species in each parish

Parish	Total No. of samples	Backyard poultry	Duck	Turkey	Guinea fowl	Pigeon	ELISA positive	Positive category of birds
St. Patrick	26	26	0	0	0	0	16	Backyard poultry (16/26)
St. Mark	28	12	16	0	0	0	0	None
St. Johns	21	21	0	0	0	0	4	Backyard poultry (4/21)
St. Georges	50	0	11	8		31	0	None
St. Davids	52	34	18	0	0	0	0	None
St. Andrew	53	50	0	2	1	0	7	Backyard poultry (7/50)

**RNA isolation and RT-PCR of tissue samples from ELISA positive birds:** Ten Birds were purchased from ELISA positive flocks and were euthanized as per the Animal Ethics Committee regulations. Trachea and cecal tonsils were collected in Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated from the tissues as described by the manufacturer. One step RT-PCR targeting the matrix gene of Influenza A virus was carried out as described for the swab samples.

## RESULTS AND DISCUSSION

The present study was conducted to screen the presence of AIV in Grenada. Attempts were made to isolate the virus from resident bird species. None of the specimens were positive for AIV by the allantoic inoculation method in chicken embryonated eggs. Allantoic Fluids from inoculated eggs were negative for Hemagglutination test. All mixed swab suspensions, Allantoic fluids and tissues from ELISA positive birds were RT PCR negative for AIV RNA. Twenty seven out of 143 (18.8%) serum samples from backyard chicken were positive for AIV specific antibodies by ELISA (Table 1). AIV RNA could not be detected in birds from seropositive chicken. This indicates that though the virus is not circulating in the bird population, free range chickens in Grenada were exposed to AIV at some point. Similar findings of either negative or inconsistent isolation of AIV and or presence of AIV RNA from seropositive birds have been reported by Molia *et al.* (2010) in Mali and by De Witt *et al.* (2004) in Netherlands. A similar surveillance in Nepal reported 34 seropositive cases out of 337 samples tested with no virus isolation or antigen detection (Pant and Selleck, 2007).

Seroprevalence of AIV varied in surveys conducted in different countries. Antibody positivity (13-20%) for AIV in different periods in the small holdings has been reported by Biswas *et al.* (2009) in Bangladesh, while Al-Natour and Abo-Shehada (2005) found a very high seropositive birds in broiler breeders in Jordan. However, a similar survey conducted in Nigeria showed birds were free of AIV antibodies (Owoade *et al.*, 2006). In the present study, antibodies to Influenza A virus were not detected in ducks, pigeons or guinea fowl. Panigrahy *et al.* (1996) working on susceptibility of pigeons to avian influenza concluded that the pigeons are resistant or minimally susceptible to infection with AIV.

Gaining information on the full spectrum of influenza A viruses circulating in our environment and developing reagents for the specific detection of these viruses will remain important tasks for influenza surveillance, outbreak control and animal and public health. This is the first report of serologic evidence of an avian influenza virus infection in Grenada. Further studies including migratory birds, wild water fowls and other shore birds will be helpful in identifying the currently circulation AIV in the region.

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