Reverse transcriptase-polymerase chain reaction-based surveillance of type A influenza viruses in wild and domestic birds of the Lebanon

Elie K. Barbour, Houssam A. Shaib & Edward G. Rayya

Summary

A total of 1 330 oropharyngeal swabs collected from wild and domestic birds in Lebanon were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) for type A avian influenza virus (AIV) during the migratory season between the autumn of 2005 and winter of 2006. Twenty-five species of birds were included in the study. There are 14 species of migratory wild birds, 4 species of resident wild birds, 3 species of pet birds and 4 species of farm birds. The number and percentage of positive type A avian influenza viruses in collected oropharyngeal swabs was 190 positive out of 1 330 samples tested (14.3%). The 190 oropharyngeal samples positive for AIV were further tested by specific RT-PCR for H5 and H7 subtypes. The 190 AIV-positive samples were all negative for H5, while 13 of the 190 (6.8%) were positive for H7. The H7-positive samples were confined to sparrows (resident wild bird species) and to backyard chicken in the south province, located 10-20 km from the Israeli-Lebanese border.

Keywords

Avian influenza, Domestic birds, H5, H7, Migratory birds, Reverse transcriptase-polymerase chain reaction, Surveillance, Type A, Viruses, Wild birds.

Sorveglianza per influenza aviaria in uccelli selvatici e domestici in Libano basata sulla reverse transcriptase-polymerase chain reaction

Riassunto

Un totale di 1330 tamponi oro-faringei di uccelli selvatici e domestici, campionati in Libano durante la stagione migratoria compresa tra l'autunno 2005 e l'inverno 2006, sono stati esaminati mediante reverse transcriptase-polymerase chain reaction (RT-PCR) per la ricerca del virus della influenza aviaria (AIV). I campioni sono stati raccolti da 25 differenti specie aviarie. Queste includevano 14 specie selvatiche migratorie, 4 specie selvatiche residenti, 3 specie aviarie da collezione e 4 di pollame domestico. I campioni risultati positivi per influenza virus di tipo A erano 190/1330, pari al 14,3%. I 190 campioni positivi sono stati testati mediante RT-PCR specifica per il sottotipo H5 e H7. Tutti i campioni sono risultati negativi per H5, mentre 13 su 190 (6,8%) risultarono positivi per H7. La positività per H7 era limitata a specie aviarie residenti (passeri) e a polli di allevamenti rurali nella provincia meridionale, a circa 10-20 Km dal confine libanese-israeliano.

Parole chiave

Influenza aviaria, H5, H7, Reverse transcriptase-polymerase chain reaction, Sorveglianza, Tipo A, Uccelli domestici, Uccelli migratori, Uccelli selvatici, Virus.

Department of Animal Sciences, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon
eb01@aub.edu.lb

Introduction

The migration of wild birds at the end of the autumn and beginning of winter from the north, where outbreaks of H5N1 avian influenza virus (AIV) had been documented, extending from China, Russia and Europe, through Romania and Turkey (17), to the Lebanon, Israel and ending in South Africa has encouraged officials in the Lebanon to implement unprecedented surveillance of type A avian influenza viruses in migratory and domestic birds that fly over Lebanese territory.

Since 1996, epidemiological studies have shown that direct transmission of highly pathogenic avian influenza (HPAI) is transmited from birds to humans (9, 20, 29). High mortality caused by H5N1 in humans in Hong Kong in 1997 alerted many countries to a possible new pandemic that could originate from wild and/or domestic birds (27). Avian influenza specialists believe that wild birds are the principal source of the AIV in humans (1).

Most AIV isolates detected in wild birds across the world belonged to the low pathogenicity avian influenza (LPAI) viruses, and were asymptomatic in nature; however, the LPAI forms of H5 or H7 subtypes, once transmitted to intensively reared avian flocks become more pathogenic, as the virus adapt to host cells, leading to significant economic losses in the poultry industry (4).

The above facts rendered the understanding of the nature of the circulating influenza viral infections in migratory wild and domestic bird populations of paramount importance (24); the information related to these viruses needs to be shared among countries that are on the north-south migratory routes, thus contributing to the development of successful control programmes to prevent catastrophic losses caused by AIV in intensively managed avian farms and, consequently, to the prevention of transmission to human populations.

The aim of this surveillance is to use reverse transcriptase-polymerase chain reaction (RT-PCR) technology on oropharyngeal swab washings collected from 25 species of migratory and domestic birds of Lebanon to detect the presence of type A AIV, followed by the use of the same technology with inclusion of primers specific for the detection of H5 and H7 subtypes.

Materials and methods

Birds and sampling

Birds of 25 species were collected by authorities of the Ministry of Agriculture in the Lebanon. These were submitted to the Animal Science Laboratory of the American University of Beirut. The oropharyngeal region of each individual bird was sampled using a sterile cotton swab. The swab was then washed in 2 ml of sterile saline. The saline-swab washing of each bird was stored at –40°C for analysis within 2 to 3 days by RT-PCR. An accession number was attributed to each bird and the record submitted by Ministry of Agriculture authorities stored on computer, together with details concerning the species of bird, region of capture and date of sampling.

Reverse transcriptase-polymerase chain reaction

The RT-PCR diagnostic kits were supplied by Sacace Biotechnologies (Caserta, Italy). Briefly, the procedure used to detect type A AIV in oropharyngeal swab-saline wash involved the extraction of the RNA from the virus, amplification of the conserved type A AIV gene using a specific primer supplied by the kit, electrophoresis of the amplicons in 2% agarose gel, staining of the banded amplicons by ethidium bromide and saving the documented electrophorised amplicon bands by ultra violet imaging using the 'Gel-Doc' system (BioRad Laboratories, Hercules). It is worth noting that specific lanes were loaded with 100 bp-ladders of known genetic sizes, amplicons of a positive type A AIV control, and additional lanes for negative controls. The amplified conserved type A AIV gene was located by electrophoresis at 365 bp. Any type A AIV detection in the sample was followed by running another RT-PCR to detect AIV subtypes, namely: H5 and H7. The procedure is similar to that described for

type A AIV; however, the primers used for H5 and H7 detection are specific to the two subtypes. It should be noted that the electrophorised amplicons of the specific gene of H5 and H7 were located in the gel at 235 and 360 bp, respectively.

Results and discussion

Table I shows clear differences in the rate of type A AIV presence in the oropharynx of different species of migratory birds ranging between 0.0% and 66.7%. Eight migratory bird species revealed no type A AIV, namely: the *Sylvia atricapilla*, *Corvus*, *Accipiter cooperi*, *Upupa epops*, *Erithacus* spp., *Larus crassirostris*, *Ciconia abdimii* and *Dendroica striata*. This absence of infection in eight species of migratory birds could be explained by those bird species never being exposed to the virus, or due to the inability of AIV to adapt to such species. No literature exists on the infection of these birds by type A AIV.

The other six species of migratory birds that revealed positive type A AIV were as follows, namely, *Phylloscopus collybita* (66.7%), *Pelecanus* spp. (50.0%), unidentified bird species (26.1%), *Coturnix coturnix* (12.5%), *Anas creeca* (8.9%) and *Turdus* spp. (5.8%). The literature on the infection of these five species by type A AIV shows that *C. coturnix* could provide an environment for the spread of reassortant influenza viruses between avian species and humans, thus acting as a potential intermediate host (31); *Pelecanus* spp. are also found to carry the influenza virus H5N1, as documented by a follow-up report submitted by Ukraine and published by the World

Table I
Oropharyngeal swabs from different species of birds positive for type A avian influenza virus

Category	Species	Common name	No. of avian influenza-positive cases/No. tested (%)
Migratory birds	*Phylloscopus collybita*	Chiffchaff	10/15 (67)
	Pelecanus spp.	Pelican	2/4 (50)
	_	Unidentified species	6/23 (26)
	Coturnix coturnix	Quail	3/24 (13)
	Anas creeca	Duck	5/56 (9)
	Turdus spp.	Thrush	4/69 (6)
	Sylvia atricapilla	Black cap	0/2 (0)
	Corvus brachyrhynchos	Crow	0/3 (0)
	Accipiter cooperi	Hawk	0/0 (0)
	Upupa epops	Hoopoe	0/2 (0)
	Erithacus spp.	Robin	0/8 (0)
	Larus crassirostris	Seagull	0/6 (0)
	Ciconia abdimii	Stork	0/2 (0)
	Dendroica striata	Warbler	0/63 (0)
Resident wild birds	*Alectoris rufa*	Partridge	1/3 (33)
	Streptopelia senegalensis	Dove	11/78 (14)
	Arremonops rufivirgatus	Sparrow	3/24 (13)
	Luscinia megarhynchos	Nightingale	0/3 (0)
Pet birds	*Gallicolumba* spp.	Pigeon	11/78 (14)
	Serinus canaria	Canary	0/5 (0)
	Passerina cyanea	Finch	0/8 (0)
Farm birds	*Gallus domesticus*	Chicken	128/810 (16)
	Meleagris spp.	Turkey	4/26 (15)
	Struthio camelus	Ostrich	0/2 (0)
	Anser spp.	Goose	2/10 (20)

organisation for animal health (OIE: Office International des Épizooties) (15). In addition, domestic ducks have been recognised as silent carriers of H5N1 influenza virus (3, 25, 33).

Most of the resident wild bird species of Lebanon were positive for type A AIV, namely: the *Alectoris rufa* (33.3%), *Streptopelia senegalensis* (14.1%) and *Arremonops rufivirgatus* (12.5%). One species of resident wild birds, *Luscinia megarhyncho*, was negative for type A AIV. It is worth noting that the *S. senegalensis* dwell mostly in and around the large cities of the Lebanon, near the shores of the eastern Mediterranean, while *A. rufivirgatus* are found in most areas and are in direct contact with backyard poultry; *A. rufa* are mostly present in the high mountains (1 500 m above sea level). The fact that *S. senegalensis* are in direct contact with the human populations in the cities, mostly due to droppings that contaminate the environment and buildings, provides a strong motivation to create awareness programmes in the country. In addition, the spread of *A. rufivirgatus* around all the Lebanese territories, and especially the direct contact with backyard and free-range poultry, even sharing feed, is quite alarming. Literature from around the world indicates that *A. rufa* were less susceptible to AIV infection (6), while *S. senegalensis* species were listed as susceptible to avian influenza (23) and do carry AIV type A. To our knowledge, documentation on the presence of type A AIV in nightingale (*Luscinia megarhynchos)* is non-existant.

Pigeons (*Gallicolumba* spp.), one out of three pet bird species examined for AIV, show a positive rate of 14.1%, while canaries (*Serinus canaria*) and finches (*Passerina cyanea*) revealed no positive result. *Gallicolumba* spp. are alarming, especially for the many racing pigeon hobbies practised, involving direct contact with such birds. Research has documented less frequent isolations of type A AIV from pigeons than ducks; pigeons are found to be highly resistant to AIV, lacking gross and histological lesions, viral antigen expression, and failures to re-isolate the virus occur. Pigeons played a minimal epidemiological role in the maintenance of H5N1 in Hong Kong (7, 21). The presence of AIV in pigeons requires caution from poultry farmers not to include racing pigeons on their land, or to include pigeons free from AIV, that should be confined to wire-mesh fencing and roofing, to avoid contact with other domestic or migratory birds. Documentation does exist on AIV infection in canaries that were housed in the same room as an AI-infected siskin (*Carduelis pinus*), resulting in AIV transmission from the siskin to canaries, causing conjunctivitis, apathy, anorexia and a high rate mortality in canaries (8). House and zebra finches were found to be susceptible to infection with the HPAI virus with some variation in pathogenicity of the virus in different birds across the world (22). This suggests that continuous surveillance of AIV infection should be conducted in these two very common pet bird species.

In regard to farm bird species, three of four surveyed species showed positive results for type A AIV, namely: geese (*Anser spp.*) (20.0%), chicken (*Gallus domesticus*) (15.8%) and turkey (*Meleagris* spp.) (15.4%), while the fourth, ostrich (*Struthio camelus*), was negative. There is a wealth of literature on AIV in *Anser* spp. that discusses the emergence of the present strains to previous reassortment of precursor avian influenza viruses (5). A virus identified in a goose in 1996 and known as Goose/ Guangdong/1/96 (Gs/Gd/96) was the likely donor of the H5 haemagglutinin (5). This goose virus was transmissible to other geese by contact, resulting in infection of all birds (34). Most outbreaks of avian influenza in domestic poultry are caused by mildly pathogenic AIV (30). Since 1997, outbreaks of highly pathogenic H5N1 and circulation of H9N2 viruses among domestic poultry in Asia have posed a threat to public health (14) and a total of 199 flocks were diagnosed with influenza infection, with the highest frequency of infection in meat turkeys (4, 17). Over 13 million birds on 413 different premises were affected by the HPAI virus, including *Meleagris* spp., *Gallus domesticus*, guinea fowl, pheasant, *C. coturnix*, *S. camelus*, and waterfowl flocks (12). The presence of type A AIV in *S. camelus* has also been documented in the United States (10) and Europe (11).

This preliminary surveillance of type A AIV in oropharyngeal washes has shown the presence of AIV in many bird species surveyed (six of 14 migratory bird species, 3 of 4 resident wild bird species, 1 of 3 pet bird species and 3 of 4 farm bird species). This presence of circulating type A AIV in several bird species in the Lebanon, a country that is on the migratory route of birds from the north (China, Russia), towards the south (South Africa) necessitates the establishment of a centre in the Lebanon that would conduct continuous monitoring of these circulating viruses and study subtypes and molecular characteristics to monitor the emergence of mutants that could threaten the poultry industry and/or affect the health of human populations in countries and regions along this important migratory route across the globe.

The 190 type A AIV-positive samples from a total of 1 330 birds tested (Tables I and II) were subjected further to H5 and H7 subtyping by specific RT-PCR that revealed the absence of H5 subtypes and the presence of H7 positive cases in 13 of 190 type A AIV positives (6.8%) (Table II). The presence of H7 subtypes was confined to sparrows (*Arremonops rufivirgatus*) (33.3%) and to backyard chicken (*Gallus domesticus*) (9.4%), all present in the southern province of the Lebanon, near the Lebanese-Israeli border. The literature has documented the presence of AIV type A H7 subtypes in wild birds, including isolates from starlings in 1985 (A/Starling/Victoria/5156/85), and serological evidence of H7N7 viral infection in sparrows (*A. rufivirgatus*) and ducks (*Anas creeca*) (13). All avian influenza isolates reported from ostriches (*S. camelus*) were non-pathogenic to poultry, even the H5 and H7 subtypes. Some of the H7 infections have been associated with mortality in *S. camelus* chicks in localised outbreaks during periods of inclement weather and due to significant contact with wild birds, principally waterfowl (31); the isolation of AIV H7 from migratory waterfowl, including the whistling swan (*Cygnus columbianus*) and pintail (*Anas acuta*), has been documented (18). The map of the spread of H7 subtype around the world includes at the present time the United States (26, 28), Canada (16, 19), the Netherlands (15, 22) and Mexico (2).

Poultry flocks that revealed a positive H7 subtype did not demonstrate highly

Table II
Type A avian influenza-positive oropharyngeal swabs positive for H5 or H7 subtypes using the reverse transcriptase-polymerase chain reaction

Category	Species	Common name	No. of avian influenza-positive birds	No. positive/No. tested (%) for subtypes	
				H5	H7
Migratory birds	Phylloscopus collybita	Chiffchaff	10	0/10 (0)	0/10 (0)
	Pelecanus spp.	Pelican	2	0/2 (0)	0/2 (0)
	_	Unidentified species	6	0/6 (0)	0/6 (0)
	Coturnix coturnix	Quail	3	0/3 (0)	0/3 (0)
	Anas creeca	Duck	5	0/5 (0)	0/5 (0)
	Turdus spp.	Thrush	4	0/4 (0)	0/4 (0)
Resident wild birds	*Alectoris rufa*	Partridge	1	0/1 (0)	0/1 (0)
	Streptopelia senegalensis	Dove	11	0/11 (0)	0/11 (0)
	Arremonops rufivirgatus	Sparrow	3	0/3 (0)	1/3 (33)
Pet birds	*Gallicolumba* spp.	Pigeon	11	0/11 (0)	0/11 (0)
Farm birds	*Gallus domesticus*	Chicken	128	0/128 (0)	12/128 (9)
	Meleagris spp.	Turkey	4	0/4 (0)	0/4 (0)
	Anser spp.	Goose	2	0/2 (0)	0/2 (0)
Total			190	0/190 (0)	13/190 (7)

pathogenic symptoms, which probably indicates that the H7 subtype was of the LPAI category. Many countries have witnessed the spread of LPAI H7 subtypes among their poultry flocks including the United States (26, 28) and Mexico (2).

The gel documentation of control samples for confirmation of accuracy of the RT-PCR for type A AIV and for subtypes H5 and H7 is shown in Figure 1. The gel document of the type A AIV-positive oropharyngeal samples from a sparrow and from a chicken are shown in Figure 2. The amplicons for H7 positive cases are all located at the same 360 bp band (Fig. 3).

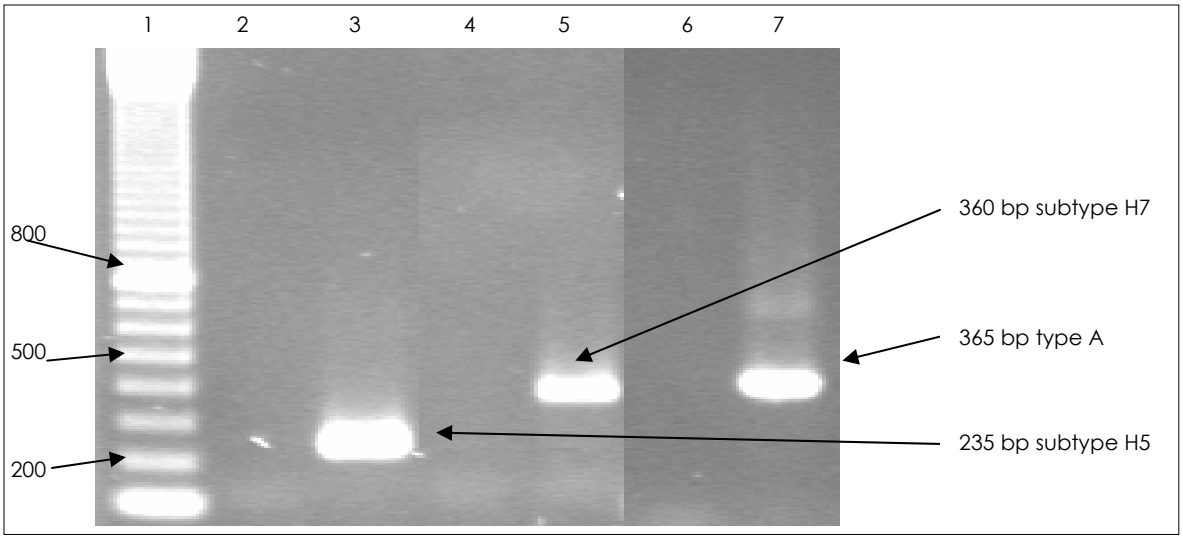

Figure 1
Gel documentation of electrophoresis reverse transcriptase-polymerase chain reaction amplicons of positive controls for type A avian influenza virus (AIV), H5, H7 and negative controls
Lane 1 =100 bp ladder, lane 2 = H5-negative control, lane 3 = H5-positive control, lane 4 = H7-negative control, lane 5 = H7-positive control, lane 6 = type A AIV-negative control, lane 7 = type A AIV-positive control

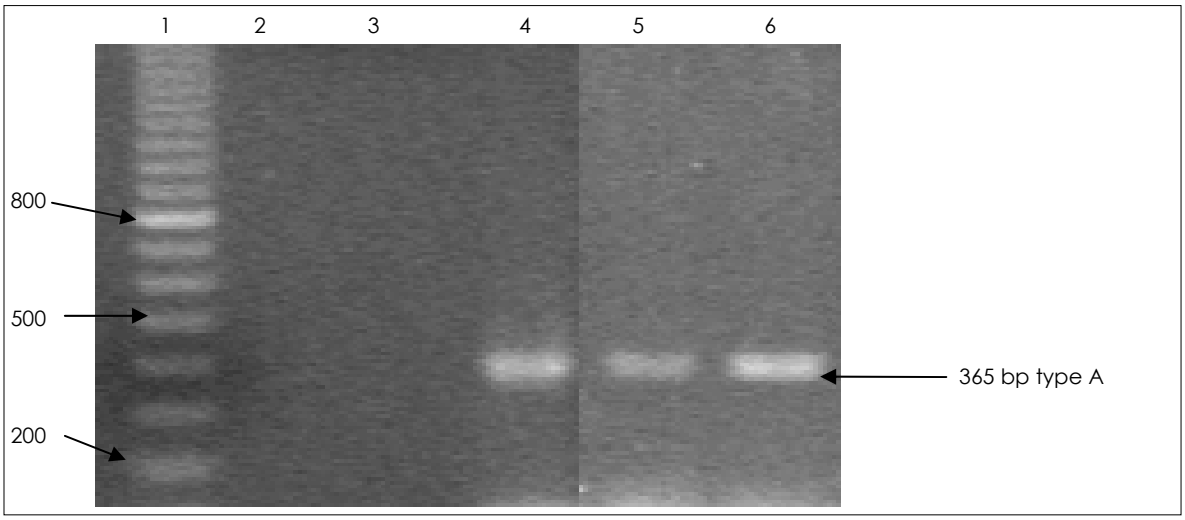

Figure 2
Gel documentation of electrophoresis reverse transcriptase-polymerase chain reaction amplicons of type A avian influenza virus (AIV) present in oropharynx of *Arremonops rufivirgatuss* and *Gallus domesticus*
Lane 1= 100 bp ladder, lane 2 = negative control of extraction for type A AIV, lane 3 = negative control of amplification for type A AIV, lane 4 = type A AIV-positive control, lane 5 = type A AIV-positive in *Gallus domesticus*, lane 6 = type A AIV-positive in *Arremonops rufivirgatus*

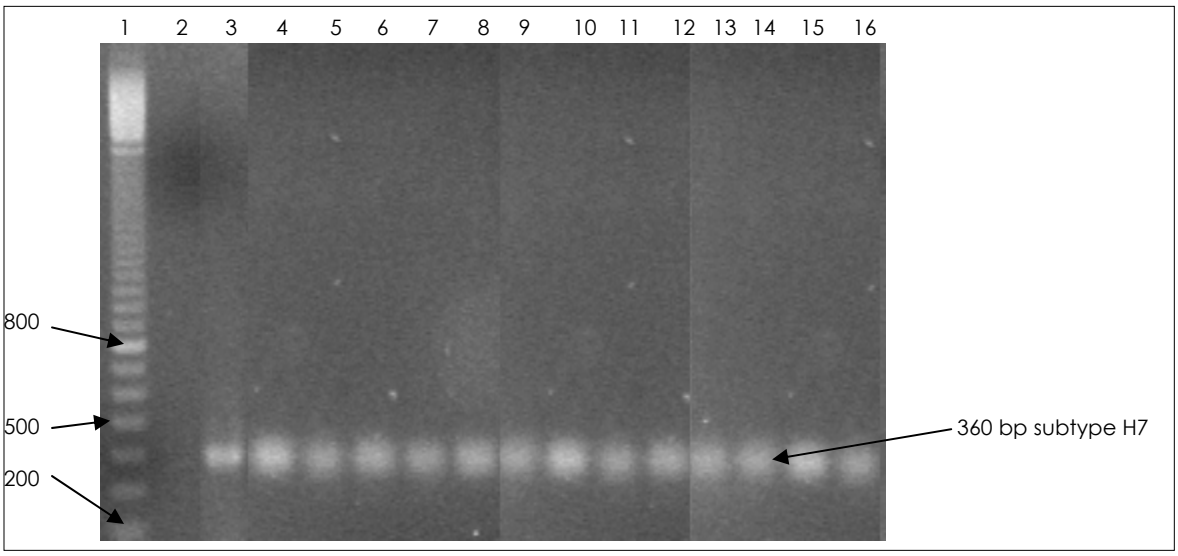

Figure 3
Gel documentation of electrophoresis reverse transcriptase-polymerase chain reaction amplicons of H7-positive birds (one *Arremonops rufivirgatus* and 12 *Gallus domesticus*)
Lane 1 = 100 bp ladder, lane 2 = H7-negative control, lane 3 = H7-positive control, lanes 4-15 = oropharyngeal samples positive for H7 in individual *Gallus domesticus*, lane 16 = an oropharyngeal sample positive for H7 in *Arremonops rufivirgatus*

Conclusion

The number and percentage of positive type A avian influenza viruses in collected oropharyngeal swabs were 190 positive out of 1 330 samples tested (14.3%). The 190 AIV-positive samples were all negative for H5, while 13 of the 190 (6.8%) were positive for H7. The H7-positive samples were confined to sparrows (resident wild bird species) and to backyard chicken in the south province, located 10-20 km from the Israeli-Lebanese border.

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