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Avian influenza outbreak in poultry in the Lebanon and transmission to neighbouring farmers and swine

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Summary

Twenty-four poultry farms in four major provinces of the Lebanon were investigated to verify the first emergence of avian influenza (AI). Both the meat chicken breeders and commercial chicken layers presented a significant average drop in egg production equivalent to 46% and 47.3%, respectively. However, the average drop in egg production in the free-range layers was only 11.1%. Flocks were confirmed as positive for AI by ELISA, clinical signs and pathological lesions. The pathogenicity, including case fatality in affected chickens, was different depending of the types of chicken and farming conditions. The average fatality rates among breeders, commercial layers, free-range layers and broilers were 2%, 2%, 1% and 35%, respectively. The majority of the randomly selected ELISA-positive serum samples collected from different farms showed H9-specific haemagglutination-inhibition (HI) antibodies. The direct immunoflorescent antibody test also revealed the presence of H9 antigen in congested brains and in tracheal lesions of broilers. The virus isolated from the brains of broilers was subtype H9N2. Pigs fed with carcasses of affected chickens showed H9-specific HI antibodies at 100%. Farmers (32.3%) serving the affected chickens also revealed these antibodies in their sera.

Keywords

Avian influenza, Chickens, Enzyme-linked immunosorbent assay, H9N2, Haemagglutination inhibition, Humans, Lebanon, Pigs, Viruses.

Episodio di influenza aviaria nei polli in Libano e trasmissione dell'infezione ad allevatori e a suini

Riassunto

Al fine di studiare la prima emergenza legata all'insorgenza dell'influenza aviaria in Libano, sono stati esaminati ventiquattro allevamenti distribuiti nelle quattro principali Province. Nei riproduttori di polli da carne e nelle galline ovaiole produttrici di uova destinate al commercio è stato registrato un calo significativo della produzione, pari rispettivamente al 46% e al 47.3%. Il calo di deposizione in ovaiole allevate all'aperto è stato solamente pari all'11.1%. A seconda della categoria produttiva di appartenenza e delle condizioni di gestione degli allevamenti sono stati riscontrati differenti livelli di patogenicità e letalità. La letalità media riscontrata nei riproduttori e nelle galline ovaiole è stata del 2%, mentre per le ovaiole allevate all'aperto e per i broilers è stata rispettivamente pari a 1% e a 35%. Oltre alla sintomatologia clinica e allo studio delle lesioni patologiche, per la conferma di laboratorio della positività è stato utilizzato il test ELISA. La maggior parte dei campioni di siero, scelti a random, da diversi allevamenti e positivi in ELISA, al test di inibizione dell'emoagglutinazione

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(HI) ha dimostrato presenza di anticorpi specifici nei confronti dell'emoagglutinina virale 9 (H9). Inoltre, il test di immunofluorescenza diretta ha rilevato, nei broilers, la presenza dell'antigene H9 in campioni prelevati sia da cervelli congesti sia da lesioni tracheali. In questi il virus isolato dal cervello è stato il sottotipo H9N2. Il 100% dei suini alimentati con le carcasse di pollame infetto ha mostrato, al test di HI, la presenza di anticorpi specifici nei confronti di H9. Anche il 32.3% degli operatori addetti all'alimentazione dei suini con pollame infetto ha sviluppato anticorpi nel siero.

Parole chiave

ELISA, H9N2, Inibizione dell'emoagglutinazione, Influenza aviaria, Libano, Maiali, Polli, Virus.

Introduction

Influenza A viruses infect birds, humans and pigs. The human tracheal epithelial cells do not have receptors for the attachment of avian influenza (AI) virus, thus hindering AI transmissibility between avians and humans. However, the tracheal epithelium of pigs has two respective receptors for both the avian and human influenza viruses namely, the α2,3-N-acethylneuraminic acid-galactose and the α2,6-N-acethylneuraminic acid-galactose (13).

This dual susceptibility postulates the pig as a 'mixing vessel', a place for reassortment of influenza viruses (8), and for emergence of zoonotic transmission of influenza from avians to humans through the swine intermediate host (14).

The H9N2 viruses responsible for poultry outbreaks during the last 10 years have been reported from some parts of the world, including European countries (Germany, Italy and Ireland), Middle Eastern countries (Iran, Pakistan and Saudi Arabia), South Africa and the USA (1, 2).

The pathogenicity indexes of the H9N2 isolates in poultry are usually low, resulting in low fatalities and significant losses in egg production in chicken layers (12). However, high fatality is also reported in broilers in certain instances (3, 17).

In 1997, the H9N2 subtype influenza virus was frequently isolated from birds in the live bird markets of Hong Kong and additional isolations from pigs were reported in 1998 (15).

One year later, the H9N2 virus was isolated from two sick children in Hong Kong and from six additional human cases reported from China (11, 18). The human infections by H9N2 influenza virus alerted the community to the pandemic potential of this subtype of AI (7).

The purpose of this research is to describe the first occurrence of an outbreak of H9N2 avian influenza virus in poultry farms of the Lebanon. This is not only a problem encountered in poultry production, the virus had spread to neighbouring swine and poultry farmers since specific HI antibodies were revealed in their serum samples.

Materials and methods

Production losses on poultry farms

Twenty-four poultry farms distributed in the north, south, east and middle mountains of the Lebanon were surveyed during the avian influenza virus (AIV) outbreak in 2005. The farms investigated were meat chicken breeders, commercial layers, free-range layers and broilers. The damages to egg production during the present investigation were compared to those before the outbreak. The fatality among present cases was also included.

Clinical signs and pathological lesions

Clinical signs and pathological lesions were examined to identify the pathogenicity of AI and to differentiate between low pathogenic AI (LPAI) and highly pathogenic AI (HPAI).

Quantitating haematocrit values and avian influenza virus-specific antibodies

The percentage haematocrit value was determined in accordance with a previously described protocol (5). Fourteen birds were randomly selected from a commercial layer farm that eliminated the AIV outbreak four days earlier. Blood samples for determination of AIV-specific antibodies were collected from brachial veins of individual birds in each of the 24 farms investigated. The number of randomly collected blood samples was equivalent to about 0.4% of the total flock size of breeders, commercial layers and broilers. However, the number of collected blood in free-range layers was about 4% of the flock size. Sera separated from each blood sample were kept at –20°C for further analyses of AIV-specific antibodies.

ELISA for avian influenza virus-specific antibodies

An AIV ELISA kit was used to detect the presence of AIV-specific antibodies. The kit used demonstrates broad reactivity to all subtypes of AIV type A (9). This commercial ELISA kit categorises serum samples with positive antibodies to AIV when the serum to positive (S/P) ratio is ≥0.500, while samples are defined as suspects when the S/P ratio is between 0.300 and 0.499, and negatives when the S/P values are <0.300.

Haemagglutination-inhibition test

The haemagglutination-inhibition (HI) test (4) was performed to confirm the presence of AIV H9-specific antibodies in serum samples that proved positive by the ELISA (S/P ratio ≥0.500). Any ELISA AIV-positive sera showing a negative reaction to the H9 component of the H9N2 subtype in the HI test were sent to Intervet in Holland to determine their specificities to other AIV subtypes. It is worth noting that the H9N2 antigen, positive and negative control sera for H9N2, used in the HI test were kindly provided by Hans Vervuurt of the R&D Service Laboratory in Boxmeer, The Netherlands. The H9N2 working antigen was used in the HI test at 8 haemagglutination units/50 ml, and each serum sample was subjected to twofold serial dilution, including the positive and negative control sera. The chicken red blood cells were used in the test at 1% (V/V) in a phosphate buffered saline diluent.

Swine and human blood

In the eastern region of the Lebanon, which accounts for 50% of the total Lebanese poultry production, the poultry farmers decided to feed the dead carcasses of chicken resulting from the AIV outbreak to neighbouring pigs. One month later, we were notified of that activity and decided to include sera of three randomly chosen pigs in the same HI test described above. In addition, individual serum samples were collected from 34 farmers that spent between 2 and 5 h each day in poultry barns serving the AIV-infected flocks. The sera of these humans were also run by the same HI test described previously.

Direct H9N2 detection and identification

The direct immunofluoresence test (20) was applied on cryostat sections of brains and tracheas of four broilers during the AIV outbreak since tracheitis, cerebral congestion and nervous disorders were recognised. Specific chicken antisera against H9N2 virus used for cryostat sections were provided by H. Vervuurt. Rabbit serum against H&L chains of chicken IgG, labelled with fluorescein isothiocyanate was used as secondary antibody. The tissue sections were observed under a fluorescent microscope for AIV antigen. A homogenate of three pooled brains of these broilers was also injected through the allantoic cavity of 10-day-old embryonated chicken eggs for AI virus propagation (3). The harvested allantoic fluid virus was subjected to the haemagglutination (HA) test. One percent of chicken RBC suspension was used (4). When HA activity was positive, the agent was examined by HI against H9N2-specific

antibody. After certified HA activity, the allantoic fluid was despatched to Ruth Manvell at the Central Veterinary Laboratory in Weybridge for final identification of the AIV subtype.

Results and discussion

As illustrated by Table I, a similar average drop in egg production at the time of the current investigation was observed in both the meat breeders and commercial layers (46% and 47.3%, respectively). On the other hand, the average drop in egg production in the free-range layers was 11.1%. These differences could be mainly due to the difference of farm management systems. The free-range layers spend most of the day outdoors in the backyard where the average chicken density is 0.2 layer per m^2. In the intensive commercial layer and meat breeder systems, the birds stay indoors where the density is 3 layers per m^2 (i.e. 15 times more birds per m^2).

The indoor environment associated with a higher density of laying may result in more stress and a higher possibility of AIV infection if the virus were introduced (19). Moreover, Table II confirms that only six out of nine (66.7%) free-range layer

Table I
Production losses[a] on 24 poultry farms in four provinces of the Lebanon during an influenza A virus outbreak

Province	Farms	Flock farming type	Age (weeks)	Drop in egg production (%)
North	1	Meat breeders	24	46
(Akkar)	2	Meat breeders	41	46
	3	Meat breeders	23	46
East	1	Meat breeders	NR[b]	NR
(Bekaa Valley)	2	Commercial layers[c]	52	40
	3	Commercial layers	40	70
	4	Commercial layers	40	72
	5	Commercial layers	36	63
	6	Commercial layers	25	48
	7	Commercial layers	104	41
	8	Commercial layers	25	46
	9	Commercial layers	56	46
	10	Commercial layers	NR	NR
	11	Broilers	5	NA[d]
Middle mountains	1	Commercial layers	32	0
(Mten)				
South	1	Free-range layers	72	0
(Jezzine)	2	Free-range layers	28	10
	3	Free-range layers	36	0
	4	Free-range layers	36	10
	5	Free-range layers	72	0
	6	Free-range layers	28	45
	7	Free-range layers	28	15
	8	Free-range layers	32	15
	9	Free-range layers	36	5

a) average fatality in either commercial layers or meat breeders during 9 days of the out break is 2% while that in free range flocks was 1%; the fatality rate on the broiler farm was 35%
b) not recorded
c) intensive system
d) not applicable

Table II
Seroprevalence of antibodies specific to avian influenza (AI) on 19 of 24 poultry farms investigated in the Lebanon

Province	Farms	Flock farming type	Percent of serum samples[a] with AI antibodies[b]		
			Positive	Suspect	Negative
North	1	Meat breeders	95.8	0.0	4.2
(Akkar)	2	Meat breeders	100.0	0.0	0.0
	3	Meat breeders	10.0	15.0	75.0
East	1	Meat breeders	0.0	23.0	77.0
(Bekaa Valley)	2	Commercial layers	100.0	0.0	0.0
	3	Commercial layers	100.0	0.0	0.0
	4	Commercial layers	100.0	0.0	0.0
	5	Commercial layers	100.0	0.0	0.0
	6	Commercial layers	60.0	20.0	20.0
	7	Commercial layers	90.0	10.0	0.0
	8	Commercial layers	20.0	50.0	30.0
	9	Commercial layers	100.0	0.0	0.0
	10	Commercial layers	100.0	0.0	0.0
	11	Broilers	92.5	7.5	0.0
Middle mountains (Mten)	1	Commercial layers	0.0	0.0	100.0
South	1	Free-range layers	12.5	25.0	62.5
(Jezzine)	2	Free-range layers	12.5	0.0	87.5
	3	Free-range layers	33.3	11.1	55.5
	4	Free-range layers	33.3	0.0	66.6
	5	Free-range layers	50.0	12.5	37.5
	6	Free-range layers	100.0	0.0	0.0
	7	Free-range layers	0.0	0.0	100.0
	8	Free-range layers	0.0	0.0	100.0
	9	Free-range layers	0.0	0.0	100.0

a) the number of randomly collected and analysed serum samples was equivalent to about 0.4% of the total flock size of breeders, commercial layers and broilers, while it was approximately 4% on free-range farms
b) the commecial ELISA kit categorises positive AI samples when S/P values ≥0.5, suspects when S/P values are between 0.500-0.499 and negatives when S/P values are <0.300

farms were confirmed by ELISA to have AIV infection, while 9 out of 10 (90%) commercial layer farms and three out of four (75%) meat breeder farms were positive for AIV.

In addition, Table III shows that all of the randomly selected ELISA positive samples for AI from meat breeders and commercial layers were confirmed by the HI test to contain H9-specific antibodies. However, three out of six AIV-ELISA positive free-range layer farms, namely numbers 3, 4 and 5 had HI antibodies to AIV subtypes other than H9 (R&D Service Laboratory). The pathogenicity of different subtypes of AIV differs in layer chickens (16).

The predominant clinical signs and pathological lesions might be influenced by the different farming management methods (Table IV). Both the meat breeders and commercial layers showed typical signs of coughing, sneezing, rales, lacrimation and depression. Furthermore, lesions were typical in nature as indicated in Table IV. Both the clinical signs and lesions in meat breeders and commercial layers suggest the presence of LPAI (16, 17). In regard to the less stressed free-range layers, the

Table III
Results of haemagglutination-inhibition[a] with H9N2 antigen performed on randomly selected ELISA positive samples for avian influenza in the Lebanon

Province	Farms	Flock farming type	No. of selected ELISA (positive) samples for AI	No. positive by HI test (%)	Mean HI titre
North	1	Meat breeders	23	23 (100)	1/1228.8
(Akkar)	2	Meat breeders	24	24 (100)	1/1126.4
	3	Meat breeders	2	2 (100)	1/1024.0
East	1	Meat breeders	0	NA[b]	NA
(Bekaa Valley)	2	Commercial layers	20	20 (100)	1/563.2
	3	Commercial layers	5	5 (100)	1/563.2
	4	Commercial layers	10	10 (100)	1/819.2
	5	Commercial layers	15	15 (100)	1/780.8
	6	Commercial layers	12	12 (100)	1/115.2
	7	Commercial layers	9	9 (100)	1/1024.0
	8	Commercial layers	2	2 (100)	1/96.0
	9	Commercial layers	10	10 (100)	1/307.2
	10	Commercial layers	NES[c]	NA	NA
	11	Broilers	37	37 (100)	1/147.2
Middle mountains (Mten)	1	Commercial layers	0	NA	NA
South	1	Free-range layers	NES[c]	NA	NA
(Jezzine)	2	Free-range layers	NES[c]	NA	NA
	3	Free-range layers	3	0 (0)[d]	<1/2
	4	Free-range layers	3	0 (0)[d]	<1/2
	5	Free-range layers	4	0 (0)[d]	<1/2
	6	Free-range layers	9	9 (100)	1/486.4
	7	Free-range layers	0	NA	NA
	8	Free-range layers	0	NA	NA
	9	Free-range layers	0	NA	NA

a) HI test specific for H9 antigen of the virus H9N2 used at 8 HA units
b) not applicable
c) not enough serum volume left for HI test
d) these AI-ELISA positive sera with a negative reaction for H9N2 antigen were sent to Intervet in Holland for a specificity study for other AI subtypes

Table IV
Characteristic clinical signs and pathological lesions in all flock farming types in the Lebanon

Flock farming type	Characteristic findings	
	Clinical signs	Pathological lesions
Meat breeders and commercial layers	Coughing, sneezing, rales lacrimation, depression	Tracheal inflammation, conjunctivitis, haemorrhagic lesions associated with a complex of abnormalities*
Free range layers	Sneezing and depression	Tracheal inflammation, conjunctivitis
Broilers	Nervous disorder, coughing, sneezing, rales, depression	Conjunctivitis, tracheal inflammation, cerebral congestion

* abnormalities included blotchy red discoloration of the shanks, foci of necrosis in comb, pale combs, and pale internal organs such as in the pancreas and liver

signs of lacrimation, rales and coughing were not noticed. More important was the lack of haemorrhagic lesions including blotchy red discoloration of shanks, foci of necrosis in combs, pale combs and pale internal organs, such as the pancreas and liver. This was confirmed in free-range layer farm number 6 that showed an infection titre to the H9 component of the virus (Table III) similar to that recorded in commercial layer and breeder farms.

Broilers presented an additional sign of nervous disorder, associated with a cerebral congestion lesion; these two observations were not made in the other types of chicken. Previous research workers have indicated this difference in signs, lesions and decline in performance between layers and broilers (3, 17). The susceptibility of broilers to H9N2 virus seems to be much higher than that reported for layers.

An interesting finding not reported before in the literature is that the hemorrhagic lesions in a commercial layer flock lead to anaemia four days after the end of the AIV outbreak (Table V). Thirteen out of 14 randomly selected commercial layers in the flock had haematocrit values ≤27%, an indicator of anaemia (6). This suggests that in the future a new regimen should be introduced to increase iron levels in the feed, or soluble formulas in drinking water to help in homeostasis of red blood cells in infected flocks manifesting apparent haemorrhagic lesions.

The seroprevalence specific to H9 detected on 19 out of the 24 farms investigated (Table II) indicate the widespread nature of AI infection in different provinces of the Lebanon. Some farms had 100% seroconversion to AI-positive birds (breeder farm no. 2; commercial layer farms nos. 2, 3, 4, 5, 9 and 10; free-range layer farm no. 6); however, in other farms the infection titre was apparent, but not evenly distributed in the flock (breeder farm no. 3; commercial layer farm no. 8; and many of the free-range layer farms). In farms that have a high percentage of suspects, additional screening after 2-3 weeks is recommended to evaluate the degree of AIV infectivity in the flocks (10).

The results of the HI test, using H9N2 antigen, performed on randomly selected ELISA-positive

Table V
Haemorrhagic lesions in commercial layers lead to anaemia four days after the end of the AI outbreak[a] in one representative commercial layer flock

Randomly chosen layers	Haematocrit values[b] (%)	Anaemic status
1	22.0	Anaemic
2	22.5	Anaemic
3	25.0	Anaemic
4	23.0	Anaemic
5	27.0	Anaemic
6	26.5	Anaemic
7	23.0	Anaemic
8	22.0	Anaemic
9	27.5	Non-anaemic
10	23.0	Anaemic
11	22.0	Anaemic
12	26.5	Anaemic
13	24.0	Anaemic
14	24.5	Anaemic

a) the AI outbreak interval was 9 days in most of the commercial layers and meat breeders
b) a bird is considered anaemic when the haematocrit value is ≤27%, while the normal values range between 29-35%

Table VI
Antigen and antibody detection in different nature of samples using different tests

Tests	Sample nature (number)	No. positive (%)
Direct immunoflorescence	Brain[a] (4) Tracheal[a] (4)	4 (100) for H9-AIV 4 (100) positive for H9-AIV
HI[b]	Allantoic fluid of propagated AI for brains (1)	1 (100) positive for H9-AIV
Typing[c]	Allantoic fluid of propagated AI for brains (1)	1 (100) H9N2
HI	Sera from farmers (34) Sera from pigs (3)	11 (32.3) positive for H9-AI 3 (100.0) positive for H9-AI

a) brain and trachea of broilers affected by AI
b) haemagglutination-inhibition test
c) typing performed by the Central Veterinary Laboratory in Weybridge

samples (Table III) proved H9 AIV to be predominant. It is generally known that H9 AI viruses are easily adapted to chickens. It is worth noting that the country nearest to the Lebanon that has reported AI virus containing H9 antigen, specifically H9N2, is Saudi Arabia (12).

Three principal tests employed in this study (Table VI) confirmed the H9 antigen of the incriminating AI virus. The viruses isolated from brains and trachea were confirmed to be the H9N2 subtype by the Central Veterinary Laboratory in Weybridge.

The pigs that fed on carcasses of chickens that had died during the AIV outbreak showed H9-specific HI antibody (Table VI), one month after feeding. This indicates that the virus was also infectious in pigs. There is the possibility that genetic reassortment might have occurred in pigs. This virus was infectious for humans since one third of farmers working on the poultry farms revealed specific HI antibody. Fortunately to date none of these farmers have shown clinical signs, as reported in human cases caused by H5N1 virus in Hong Kong and China (11, 18).

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