A NEW INFLUENZA VIRUS INFECTION IN TURKEYS
I. ISOLATION AND CHARACTERIZATION OF VIRUS 6213

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Introduction

THE ROLE played by influenza A viruses in avian pathology, especially in North America, has usually been considered negligible. *Diseases of Poultry* by Biester and Schwarte (1965 Edition) mentions the word "influenza" only once, in connection with "Goose influenza", and even this reference is inappropriate because the disease is caused by a bacterial agent, not by an influenza virus. The book does not mention that fowl plague is caused by a true influenza A virus, as demonstrated by Schaefer in 1955 (19). This may not appear too serious, because classical fowl plague is deemed a somewhat exotic disease and was last seen in North America in 1929. Since 1950 other influenza A virus isolations from birds have been reported, but they have been made mainly in the Eastern hemisphere from sporadic or enzootic disease events.

Recent developments, however, indicate that influenza A viruses are responsible for major disease problems in the North American turkey breeding industry. Wilmot virus, for example, was first recognized in several turkey flocks in Canada in 1963 (11) and since that time the infection has persisted, as demonstrated by virus isolations from diseased turkeys in Ontario during 1964, 1965, 1966 and 1967. Identical or closely related viruses were also isolated from turkeys in California (*Myxovirus meleagrium*), Massachusetts, Wisconsin and England (2, 13, 15, 22).

The first attempt to classify serologically the known avian influenza A isolates was made in 1965 by Pereira *et al.* (14). Four serological families were proposed at this time. Further studies at the World Influenza Centre in London, England, on more recent isolates brought about an amendment of this classification scheme into six serological groups (15):

(1) Classical fowl plague viruses,
(2) Dinter's virus N and related viruses,
(3) Duck viruses of Czechoslovakia 1956 and England 1962,
(4) Duck virus of England 1956,
(5) Scottish fowl virus of 1959 and South African tern virus of 1961,
(6) Turkey viruses related to Wilmot virus.

This report deals with the recognition of a natural infection in turkeys by an influenza A virus related to the tern-Scottish fowl serotype. This influenza serotype has not been recorded previously on this continent.

Case History

In December 1965, several laying flocks belonging to a large turkey breeding enterprise were affected simultaneously or successively, by an apparently contagious, but clinically ill-defined disease. Housing, feeding and lighting programs for all flocks were identical, and all flocks reacted in a similar fashion. The birds were approximately the same age, and had been laying for about two or three months.

Early signs of disease were decreased activity and feed consumption, increase in broodiness of the hens, and inconsistent mild respiratory signs. Daily death rates rose markedly with the appearance of these signs. Hens often died on the nests. Total mortality attributed to the infection varied from 2% to 13% for individual flocks. The increasing mortality was accompanied by decreasing egg production (Table I). Eight to ten days elapsed from the time disease manifestations were first noticed until production was at its low point. Many eggs laid during this period were misshapen and fertility declined markedly.

Birds found dead during the epizootic had generalized inflammation of the air

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TABLE I

EGG PROUDCTION, MORTALITY AND FERTILITY OF EGGS OF FOUR FLOCKS INFECTED BY VIRUS 6213

	Eggs laid				Weekly mortality				Fertility of eggs set at indicated dates in (%)			
Flock No.	S-55	S-56	S-57	L-51	S-55	S-56	S-57	L-51	S-55	S-56	S-57	L-51
No. birds	2500	2498	2487	2476								
Dec. 21	963	871	1016	1300								
22	892	886	1036	1339								
23	861	915	983	1429								
24	733	844	965	1340					70.0	79.4	69.3	77.1
25	532	793	817	1209	34	2	0	1				
26	650	1164	1096	1399								
27	442	890	1004	1345					69.4	74.5	65.1	
28	424	1002	975	1290								
29	305	929	1050	1405								
30	208	880	901	1320								74.8
31	247	704	886	1037					62.0	69.5	62.6	
Jan. 1	242	709	948	1116	43	22	4	0				
2	235	774	875	959								
3	264	618	767	818								
4	292	442	593	601								76.6
5	286	362	456	448								
6	320	344	351	362								
7	356	304	261	307								67.7
8	320	314	157	260	2	32	90	12	65.5	65.3	52.3	

sac and serous membranes. Spleens were normal, but lungs, liver, kidneys, ovary and oviduct were slightly to severely congested. In a few cases the entire carcass was congested.

The disease picture was suggestive of Newcastle disease, Wilmot virus infection, or fowl cholera. The first two virus infections could be ruled out by consistent negative results in the hemagglutination-inhibition (HI) test with these two agents, using sera obtained from turkeys during and after the disease outbreaks. At first confusion existed regarding fowl cholera, because at the beginning of the disease a few birds yielded *Pasteurella multocida* on bacteriological examination. Sulfaquinoxaline (19.2%) given in the drinking water resulted in slight improvement in two flocks only. Oxytetracycline and chloramphenicol were administered in the drinking water without response.

After the virus was isolated the flocks were slaughtered; all birds examined at the time of slaughter had HI antibodies to the virus in their serum.

MATERIALS AND METHODS

On January 6, 1966, two tracheas from turkeys that died two days after the disease appeared in the flock, were submitted to the Virus Research Institute, Ontario Veterinary College.

The experimental methods employed for isolation and study of the virus were the same as those outlined previously (11, 12).

RESULTS

(A) Isolation of the Agent and Growth Characteristics in the Chicken Embryo

A filtered (0.45μ Millipore membrane) suspension of mucosa from the two tracheas was inoculated into the allantoic cavity of six nine-day-old embryos, and into the yolk sac of six five-day-old embryos. All embryos inoculated into the allantoic cavity died within three days; those given the inoculum into the yolk sac died within 48 hours.

Allantoic fluids from the first group of embryos (allantoic inoculation) agglutinated chicken red blood cells (RBC) at titers from 1/80 to 1/1280, whereas these fluids from the second group (yolk sac inoculation) did so at titers not exceeding 1/20, or failed to hemagglutinate at all.

The hemagglutinating embryo-lethal agent was passed easily in the allantoic cavity of the chicken embryo. The mean death time of the minimal lethal dose (16) was 52.5 hours at the third passage level. Titration endpoints based on embryo mor-

tality were identical to infectivity endpoints by allantoic inoculation since hemagglutinins could not be detected in allantoic fluids of surviving embryos until the eighth day after inoculation. Hemagglutinin titers were highest in chorio-allantoic membrane (CAM) extracts and allantoic fluids, and negative or very low in yolk and extracts from yolk sac or embryos.

The virus also killed chicken embryos when inoculated by the dropped membrane method. Hemagglutinin titers of allantoic fluids from embryos infected by this route were slightly lower than after allantoic inoculation, and sometimes they were negative. Lower HA titers were also found in CAM extracts, whereas yolk, yolk sac extracts and embryo extracts were of the same magnitude as found in embryos infected by the allantoic route. Infection of the CAM did not result in pock lesions.

Inoculation of the virus into the yolk sac of six-day-old embryos was lethal in two to four days. Highest amounts of hemagglutinin were detectable in yolk sac extracts, in contrast to other anatomical elements of the embryo which yielded only low titers (Table II).

Embryos were usually congested, regardless of the route of infection. Petechial or ecchymotic hemorrhage was present in some, but not all embryos; the most common form was cranial ecchymosis. Specific virus lesions were not found in internal organs.

(B) Effect of Physical and Chemical Agents on Virus Infectivity and Hemagglutinin

Data on thermo-inactivation of virus 6213 are given in Table III, demonstrating that infectivity and hemagglutinin are destroyed rapidly by heating at 56° C. At lower temperatures, the hemagglutinating activity is more stable than infectivity; the virus is relatively thermo-stable under laboratory conditions. These values, however, were established with undiluted allantoic fluids. When fluids are diluted to eight HA units in saline, the hemagglutinating activity is not maintained for more than one week in the refrigerator.

The addition of 0.1% commercial formalin to undiluted allantoic fluid destroys infectivity completely within 24 hours, but the hemagglutinating activity remains stable to the same extent as virus in untreated allantoic fluid kept at 4° C.

Ether sensitivity tests using the method of Andrewes and Horstmann (1), and chloroform sensitivity tests using the method of Feldman and Wang (6) demonstrated that the virus was sensitive to these lipo-solvents. Ether treatment usually increased the HA titer of the virus preparations two to four-fold.

Tests for stability of the virus in suspensions of different pH values (9) demonstrated that its infectivity and hemagglutinin were labile at low pH but were unaffected in the alkaline range up to pH 8.

TABLE II

HEMAGGLUTINATION AND INFECTIVITY TITERS IN FLUIDS AND TISSUE EXTRACTS FROM CHICKEN EMBRYOS FOLLOWING INFECTION WITH VIRUS 6213 BY DIFFERENT ROUTES

Test fluid or Extract		Route of inoculation		
		Allantoic cavity	Dropped membrane	Yolk sac
Allantoic Fluid	EID_{50}	8.7 (8.2–9.4)	8.7 (8.2–9.0)	6.7 (3.2–7.0)
	HA	1280 (160–2560)	640 (0–1280)	20
CAM Extract	EID_{50}	8.7 (8.0–9.2)	8.2 (7.0–8.7)	6.7 (6.5–7.3)
	HA	2560 (640–10240)	640 (10–5120)	80
Yolk sac Extract	EID_{50}	8.0 (8.0–8.2)	6.7 (6.5–6.7)	8.0 (6.5–8.2)
	HA	40	40	1280
Yolk	EID_{50}	7.2 (7.0–8.2)	7.7 (7.5–7.7)	8.0 (7.0–8.2)
	HA	<5	<5	40
Embryo Extract	EID_{50}	6.5 (6.2–7.2)	7.7 (6.7–8.2)	7.7 (7.0–8.2)
	HA	<5 (0–5)	40 (0–320)	10 (5–80)

TABLE III

THERMO-INACTIVATION OF VIRUS 6213 (UNDILUTED ALLANTOIC FLUIDS)

Time of exposure	Temperature of exposure									
	56°C		37°C		22°C		4°C		−20°C	
	$CEID_{50}$	HA	$CEID_{50}$	HA	$CEID_{50}$	HA	$CEID_{50}$	HA	$CEID_{50}$	HA
To	8.4	256	9.2	512	9.2	512	9.2	512	9.2	512
5 min.	6.9	256								
10 min.	6.0	256								
15 min.	4.2	128								
30 min.	2.6	32								
60 min.	neg.	neg.								
Day 2			6.2	1024	9.0	1024	9.0	512		
Day 3			4.0	1024	8.7	1024	9.0	512		
Day 6			2.0	1024	8.9	1024	9.0	512		
Day 10			neg.	1024	5.2	1024	9.0	512		
Day 14				1024	4.0	1024	8.4	1024		
Day 25				1024	2.2	1024	8.2	1024		
Day 33				512	neg.	512	7.2	512		
Day 44				256		512		1024		
Day 75				256		512	512	1024		
Day 120									9.2	1024

(C) Morphology of Virus 6213

Examination of virus preparations (allantoic fluid) concentrated by high-speed centrifugation, using an electron microscope, showed rounded particles about 80 to 100 mμ in diameter, or elongated forms measuring about 240 by 80 mμ (Figure 1). These particles were composed of a striated surface structure (envelope) and an inner core in which details could not be recognized. These features are similar to those described for human influenza viruses (8), and for Wilmot virus (12).

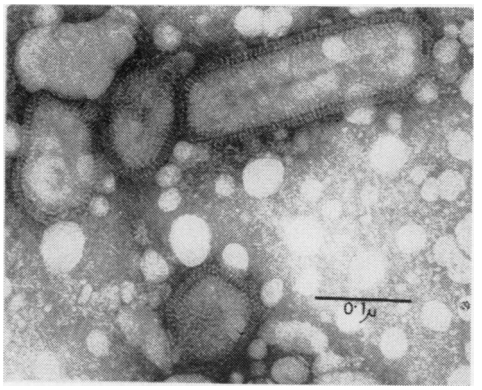

FIGURE 1. Electron-micrograph of Virus 6213 sedimented from allantoic fluid by centrifugation. Negative staining method.

(D) Serologic Properties and Identification of Virus 6213

Allantoic fluids from infected chicken embryos agglutinated not only RBC from chickens, but also those of the turkey, guinea pig, man, horse, calf, and sheep. Hemagglutination titers were highest using chicken, turkey and guinea pig RBC. Tests with human RBC sometimes reached the titers obtainable with chicken RBC, or were lower by one doubling dilution. Horse, calf and sheep RBC gave titer differences of two dilution steps or more, as compared to titers obtained with chicken RBC.

Saline extracts of CAM from chicken embryos infected by virus 6213 reacted specifically in the CF test with guinea pig antisera to the group antigen of influenza A viruses, but not with antisera to influenza type B, ND virus or to Myxovirus yucaipa. Since virus 6213 could not be identified in the HI test with the authors' various reference antisera, it was sent to the World Influenza Centre, London, England. The presence of the influenza A group antigen in virus 6213 was confirmed by CF tests. Furthermore, by means of HI and strain-specific CF tests a serological relationship of the influenza A strain Chicken/Scotland/59 and virus 6213 was detected. These findings were verified in HI tests with antisera to Chicken/Scotland/59 and Tern/South Africa/61. A

TABLE IV

SEROLOGICAL IDENTIFICATION OF VIRUS 6213 BY HEMAGGLUTINATION-INHIBITION TESTS

Reference virus strains		Hemagglutination-Inhibition Titers (in reciprocals)	
Serotype	Strain identification	6213 Hemagglutinin (8 units) vs. Reference antisera	6213 Rooster antiserum vs. Reference Hemagglutinins
Virus 6213	Turkey/Ontario/65	160	160
Infl. AHO	Puerto Rico 8/34	neg.*	neg.
Infl. AH1	New Jersey/FM 1/47	neg.	neg.
Infl. AH2	Lyon/57	neg.	neg.
Infl. AS	1976/31	N.T.	neg.
Infl. AS	Illinois 1/63	N.T.	neg.
Infl. AS	Manitoba 674/67	neg.	neg.
Infl. AE1	Toronto/61	neg.	neg.
Infl. AE2	Toronto/63	neg.	neg.
Infl. AA1	Chicken/Rostock/FP	neg.	N.T.
Infl. AA2	Chicken/Bavaria N/59	neg.	N.T.
Infl. AA3	Duck/England/56	neg.	N.T.
Infl. AA4	Duck/Czechoslovakia	neg.	N.T.
Infl. AA5	Chicken/Scotland/59	40	160
Infl. AA5	Tern/South Africa/61	40	160
Infl. AA6	Turkey/Ontario 3724/63	neg.	neg.
Infl. AA6	Turkey/Massachusetts 3740/64	neg.	neg.
Infl. AA6	Turkey/California/64	neg.	N.T.
Infl. BH	New York/Lee/40	neg.	neg.
Parainflu. Newcastle		neg.	neg.
Parainflu. Yucaipa		neg.	neg.

summary of the authors' HI results with virus 6213 is given in Table IV.

(E) Pathogenicity spectrum of virus 6213

(a) Chickens. Ten infection experiments were carried out with two adult roosters, 46 one-day-old, 48 two-week-old, and 30 four-week-old chickens. Allantoic fluid from the third passage of the virus was given intranasally, intramuscularly, intravenously or intraperitoneally with varying doses of virus. Clinical signs attributable to the infection were not observed but serological responses were elicited regularly, provided approximately 10^5CEID_{50} were given; with lower doses the serological response was erratic. Chickens older than two weeks had HI titers of 1/80 to 1/320; titers were slightly lower in younger birds.

(b) Turkeys. The same virus preparation employed in the chicken infection trials, was used in twelve transmission experiments in turkeys. Sixty-six poults less than one week old, 47 poults between two and six weeks of age, and seven adult turkeys were infected by an administration scheme similar to that used for chickens. Clinical signs were not seen in turkeys six weeks of age, or older, following infection by any route, even with high (10^9CEID_{50}) infecting doses.

In turkey poults less than one week old, 10^2CEID_{50} of the virus was not infectious by nasal instillation; 10^4CEID_{50} or more usually resulted in disease and sometimes in death. Clinical signs were marked depression during the early stages (from day 2 to day 5), and chronic sinusitis appearing during the first week and persisting for about four weeks. The fatality rate at this age often reached 50%. Death occurred mainly during the first week, but individual birds were found dead as late as 22 days after infection. Virus could always be isolated from some tissues (liver, lung, brain, spleen, or sinuses) of birds dying from the infection, but the recovery of virus was less regular from birds killed at random during the chronic stage of the disease. Virus administered intraperitoneally or intramuscularly seldom caused nasal sinusitis. Mortality by intraperitoneal infection was higher than by intranasal administration.

Poults two weeks of age were less susceptible to the infection. Clinical disease could be induced only by nasal instillation of at least 10^6CEID_{50} of the virus, and mortality was less than 5%.

Serological responses in turkeys following single administration of virus were lower (HI titers from 1/20 to 1/80) than were found in chickens under similar circumstances.

(c) Laboratory animals. Two adult rabbits injected intravenously, and four young guinea pigs injected intraperitoneally, did not show signs of disease. Three injections at weekly intervals were necessary to obtain antisera with HI titers of 1/160 to 1/320.

Discussion

In the disease outbreak under study, the fall in egg production first attracted the breeder's attention and motivated him to seek assistance from a poultry diagnostic service. The other signs of disease, such as reduced activity and feed consumption, or even the mortality rate, were considered less important, and came to light only upon questioning.

The fall in egg production of a flock is a frequent event, yet it is surprising how little information on this pathological aspect of avian medicine can be found in the literature. A diminishing laying performance may result from any type of stress put upon the birds (17). The stressing factor may be a disease, in which case the drop in egg yield assumes the significance of a clinical sign (7). If the disease is infectious, the economical consequences may assume disastrous proportions, however mild the disease may be in the medical sense. Modern poultry breeding and raising is a highly specialized, highly industrialized, and highly concentrated business serving a highly competitive market. Financial profit or loss are dependent upon narrow limits of feeding and housing costs, rapid rotation schedules, and maximum use of manpower. The lifespan of a turkey breeding flock is about twice that of a broiler flock but the second half of the lifespan of breeding turkeys will not substantially raise their meat value. The cost of maintenance, therefore, has to be balanced with the revenue from the sale of eggs and poults and if the laying performance of a flock does not reach or maintain the established standards, the breeder will invariably lose money.

The losses of the turkey breeding station stricken by the epizootic discussed here were estimated to be $180,000. Thus, though medically moderate, the infection by virus 6213 severely affected the economic value of the infected turkey flocks.

The second interesting aspect of this disease outbreak is the nature of the causative agent. Virus 6213 possesses the biological and serological properties of an influenza A virus. It is related serologically to the avian influenza strains A/Chicken/Scotland/59 and A/Tern/South Africa/61.

The Scottish fowl virus was isolated during a sporadic disease outbreak in a small chicken flock at one farm in Aberdeenshire. The natural infection caused severe mortality, and most diseased chickens presented lesions characteristic of classical fowl plague. Clinical signs and lesions were regularly reproduced in transmission experiments in chickens, but turkeys, ducks, and pigeons underwent subclinical infections only (24).

The Tern virus caused a severe, lethal epizootic among common terns (Sterna hirundo) along the Atlantic coast near Cape Town (3, 18). The disease was reproducible in common terns (4), chickens and turkeys, but not in ducks or several other species of the tern family (5, 24).

Virus 6213 is the first North American member of this serological family. There is no conclusive evidence as to the origin or the port of entry of the agent to this continent. The three related viruses were isolated from three different avian species in widely separated geographic locations and time intervals. Thus, there must be a carrier host which guarantees the survival of the agent and its wide dissemination. The most plausible carriers satisfying these conditions seem to be wild migratory birds. This theory has already been presented to explain the overseas transmission of fowl plague (23) and Newcastle disease (10). It has also been used by Rowan (18) to link the South African epizootic in terns with the outbreak of the "fowl-plague-like" disease in chickens in Scotland. The outbreak caused by virus 6213, however, was observed first at the end of December, i.e. long after the migratory birds had left and before they had returned from their wintering grounds. In addition, the infected turkeys had been kept indoors for six to eight weeks before the disease outbreak. Thus, if the wild bird fauna was the source of infection,

the initial exposure must have taken place much earlier and the infection remained latent until activated by stressing factors. Furthermore, it is possible that the virus had gained entry into the turkey population in a more distant past and was transmitted from one flock to another. The original infection of turkeys may have taken place at any point, and endemic foci may exist in other parts of the North American continent. Only large scale serologic surveys can reveal the actual dissemination of this influenza serotype in the poultry population. The most important place to test for the presence of this infection is at the relatively few, but large, breeding and hatching centers which supply many commercial turkey establishments over wide geographic areas. Serological differences between avian and mammalian influenza strains are sufficient to assume that non-avian hosts are not involved in the transmission cycle of these agents.

The great pathogenic differences of the three viruses are another interesting aspect of this serological family. The Scottish fowl virus is lethal to chickens but not to turkeys, the tern virus is lethal to both species, and virus 6213 is pathogenic to very young turkey poults only and not to older turkeys or to chickens of any age. Again, whereas the two former viruses caused severe systemic diseases with scant respiratory signs the experimental disease by virus 6213 in young poults was clearly dominated by upper respiratory disturbances. Yet sinusitis was never seen in turkeys during the natural infection by this virus, and the laying turkeys that died during the epizootic showed lesions indicative of a generalized disease.

Summary

Virus 6213, isolated during an epizootic in turkeys, was identified as an influenza A virus and was serologically classified with the avian antigenic family 5 (tern-Scottish fowl viruses). The natural disease in turkeys was clinically ill-defined, resulted in moderate death losses, but affected egg production seriously. In transmission experiments, the virus regularly produced sinusitis in turkey poults, but was found apathogenic to turkeys older than four weeks and to chickens of any age. In this respect, virus 6213 contrasts sharply with the other two known members of avian serotype 5, which are highly pathogenic to chickens. This is the first time that infection by an influenza virus of serotype 5 has been recognized in North American birds.

Résumé

Le virus 6213, isolé au cours d'une maladie épizootique de dindons, était identifié comme un virus du groupe influenza A de la famille antigénic 5 (Tern-Scottish fowl virus) d'influenza aviaire. La maladie naturelle des dindons était cliniquement vague, entraînait une mortalité modérée, mais avait de sérieuses répercussions sur la production d'œufs. Expérimentalement, le virus 6213 produisait de la sinusite chez les dindonneaux mais il n'était pas pathogénique pour les dindons plus âgés que quatre semaines, ou pour les poules de tout âge. A cet régard, le virus 6213 contraste avec les deux types connus du sérotype aviaire 5, lesquels sont hautement pathogéniques pour les poules. C'est la première fois qu'une infection par un sérotype 5 a été décelé chez des oiseaux de l'Amérique du Nord.

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ABSTRACT

Schmidt, N. J., Lennette, E. H., and Hanahoe, M. F. (1966). A micro method for performing parainfluenza virus neutralization tests.—Proc. Soc. exp. Biol. Med. 122, 1062–1067 (Viral and Rickettsial Dis. Lab., State Dep. Publ. Hlth., Berkeley, Calif.).

A microneutralization technique is described for the parainfluenza viruses of man and animals. Rhesus monkey kidney cells were used in cups in a disposable plate, virus was detected by the haemadsorption technique, and the results were read with a standard light microscope. The tests were reliable, less cumbersome to perform and to read than conventional tube neutralization tests, and far more economical in terms of cell cultures and reagents. Its use for the identification of viral isolates is limited as certain isolates do not possess sufficiently high titres to produce clear-cut haemadsorption.

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