The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961

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THE EPIZOOTIC IN COMMON TERNS—*STERNA HIRUNDO*

The field observations on the epizootic among Common Terns along the coast of the Cape Province of South Africa in April 1961 have been reported in detail by Rowan (1962). The Common Tern is usually seen in the Republic of South Africa from October to February, but in 1961 many Common Terns delayed their migration to the breeding grounds in the temperate regions of the Northern Hemisphere until May. Several dead terns were reported in the region of Cape Town in the second and third weeks of April, but the epizootic became explosive in the third and fourth weeks and either spread rapidly or was multifocal in origin along the 1000-mile stretch of coast from Port Elizabeth to Lambert's Bay (Fig. 1). The
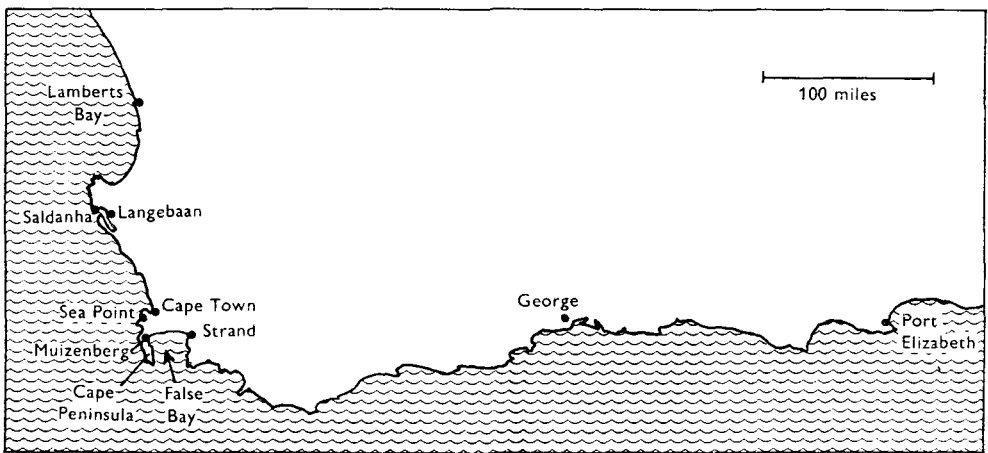

Fig. 1. Places in South Africa where dead Common Terns were found during the epizootic of 1961.

mortality was high as evidenced by the finding of 1300 dead terns in four small areas alone. Only Common Terns were affected; no overt infection of any other species was noted. No more dead terns were found after the second week in May, by which time most of the remaining birds had left, presumably on migration northward.

The causal agent was isolated from a number of afflicted terns and was named Tern virus (Becker, 1963).

MATERIALS AND METHODS
Virus strains

Tern virus was classified as a result of this work as influenza virus A/Tern/South Africa/1961. Influenza virus A/Chicken/Scotland/1959 (Chicken/Scot. virus) was obtained from Dr J. E. Wilson.* Other viruses used were influenza virus A_2/Cape Town/51/1961 (Influenza A_2), influenza virus B/Lee (Influenza B), influenza virus C/JJ/1950 (Influenza C) and Newcastle disease virus strain Komarov (NDV).

Virus propagation

The virus strains were propagated by inoculating 10^3 to 10^5 egg-infective doses (EID)50 allantoically, or amniotically in the case of Influenza C, into several embryonated hens' eggs and subsequently harvesting and pooling the infected fluids which contained between 10^7 and 10^9 EID50 of virus per ml. Some strains were also propagated in chick embryo cell monolayer cultures. Reference stocks were lyophilized and working stocks were stored in ampoules at $-20°$ C.

Embryonated hens' eggs

Leghorn–Australorpe-cross eggs from a commercial source were used and incubated at 36–37° C. after inoculation.

White mice

The highly inbred laboratory strain of white mice was used.

Tissue cultures

Monkey kidney (MK) cells. Standard methods were used to prepare secondary roller-tube cultures of Vervet monkey (*Cercopithecus aethiops pygerythrus*) kidney epithelial cells. The growth medium consisted of Hanks's salt solution containing 0·5 % lactalbumin hydrolysate, 5–10 % filtered calf serum, 0·002 % phenol red, 0·035 % sodium bicarbonate and antibiotics. The maintenance medium contained 0·135 % sodium bicarbonate and 0·5 % fowl serum in place of calf serum.

Strain KB malignant human epithelial cell line (KB). KB cell roller-tube cultures were prepared according to standard methods. The MK cell media were used except that the content of calf serum was 10 % in the growth medium and 10 % of fowl serum was used in the maintenance medium.

Chick embryo (CE) cells. Monolayers of CE cells were cultured in Petri dishes by the method of Porterfield (1960) based on the technique of Dulbecco (1952). Roller-tube cultures were prepared by seeding with approximately 10^6 cells per ml. of growth medium which was also used as maintenance medium.

All tissue cultures were incubated at 36–37° C.

Fixation and staining of cells grown on cover-slips. Cell sheets grown on flying cover-slips in roller tubes were fixed in absolute alcohol or in Bouin's fluid and stained with haematoxylin and eosin following stardard procedures.

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Haemagglutination (HA) and haemagglutination-inhibition (HI) tests

HA and HI tests were done by standard methods on Perspex plates (W.H.O., 1959).

Sea-birds

Species

Virus studies were carried out on three affected Common Terns submitted during the epizootic; on thirteen sea-birds collected in 1961 after the epizootic had ended, of which three were Common Terns, three Arctic Terns (*Sterna macrura*), four Swift Terns (*Sterna bergii*), two Cape Cormorants (*Phalacrocorax capensis*) and one a Hartlaub's Gull (*Larus hartlaubii*); and on a further three Common Terns received in 1963.

Autopsy

Live birds were exsanguinated and blood was obtained from the opened heart of dead birds. Using aseptic techniques, the liver, spleen, heart, lungs, kidneys and brain were removed. Each organ except the brain was washed in three 20 ml. lots of sterile saline, a sample was removed for viral studies and the remainder fixed in 10 % formol-saline for histological examination.

Preparation of tissue emulsions

Serum was separated and stored at $-20°$ C. The blood clots and tissue samples were emulsified (10 %, w/v) in MK cell maintenance medium without serum. The emulsions were frozen and thawed twice, clarified by centrifugation and stored at $-20°$ C. or in a dry-ice cabinet.

Virus titration

Serial tenfold dilutions of suspensions were inoculated allantoically in 0·1 ml. amounts into eggs, four or six per dilution, which were examined after 48–72 hr. for death of the embryo and a positive HA test on the embryonic fluids. Alternatively CE cell cultures were employed and examined for cytopathic effect (CPE) daily for 7–14 days. The infective dose 50 % end-point (ID50) was calculated according to the method of Reed & Muench (1938).

Neutralization tests

The standard test utilizing a constant dose of virus was carried out in CE cell roller tubes. The titre of a serum was expressed as the reciprocal of the highest dilution which neutralized the virus in at least 50 % of the inoculated cultures.

Complement-fixation (CF) tests

Both the small-volume method in tubes and the micro-method on standard Perspex HA plates were used following a procedure similar to that described in the W.H.O. Technical Report Series (1959). Overnight incubation at 4° C. was employed. The diluent was that of Mayer, Osler, Bier & Heidelberger (1946). Complement was preserved by the method of Richardson (1941) and 2 M.H.D. were used in the test.

Complement-fixing antigens

The procedures followed for the preparation of the type-specific nucleoprotein (NP) antigen and the strain specific haemagglutinin (V) antigen were based on the methods of Hoyle (1952) as modified by Lief & Henle (1956a, b) and Fabiyi, Lief & Henle (1958). Influenza C virus was however concentrated and partially purified by differential centrifugation, treated with ether and the resultant mixture of nucleoprotein and haemagglutinin used as antigen.

Sera prepared

Anti-V sera. Strain-specific sera were prepared according to the method of Fabiyi *et al.* (1958).

'*Anti-NP*' *sera.* These sera were prepared using the method of Lief, Fabiyi & Henle (1958) except that the same strain was employed for the initial intranasal inoculation and as the source of the NP for the second inoculation. Consequently, the guinea-pig immune sera were expected to have both anti-NP and anti-V antibodies. In the case of Influenza C the second inoculum consisted of a mixture of V and NP antigens.

Immune ferret sera. The animals were bled out 3–4 weeks after intranasal infection.

Immune chicken sera were obtained from birds (Leghorn–Australorpe cross) convalescing from experimental infection with Tern or Chicken/Scot. virus.

Immune rabbit sera. Rabbits were immunized with a series of four inocula of Tern-virus-infected allantoic fluid administered by the intramuscular (IM), intraperitoneal or intravenous routes over a period of 5 weeks.

Sera acquired

Anti-Fowl-plague virus: strain Brescia, strain Alexandrien and strain 'N'—Plum Island Animal Disease Laboratory. Chicken/Scot. virus immune chicken serum— Dr J. E. Wilson. NDV hyperimmune chicken sera—Onderstepoort Veterinary Laboratory. Human convalescent Mumps sera—C.S.I.R. and U.C.T. Virus Research Unit. Influenza A_2/Asia/57 and B/Johannesburg/33/58 hyperimmune rabbit serum —N.V. Philips-Duphar. Influenza A/Phil./53, A/FM1/47, A/PR 8/34, Mumps virus, Para-influenza 1, 2 and 3 hyperimmune guinea-pig sera; Simian myxovirus (SV 5) hyperimmune rabbit serum—Microbiological Associates.

RESULTS

Virological investigation of sea-birds

Isolation of Tern virus

Tern virus was isolated from three Common Terns (birds 1, 2 and 3, Table 1), which were the only birds received during the epizootic. Two pools were prepared from the organ emulsions of each bird by mixing equal portions of brain and liver, and of heart, lung and kidney emulsions. The pools were inoculated in 0·1 ml. amounts into embryonated eggs allantoically, into both KB and MK cells and into newborn mice intracerebrally.

In the case of bird 1, the embryos of the inoculated eggs died within 48 hr. and presented a rather striking appearance; their external surfaces were congested and showed punctate and frequently large focal haemorrhages. The allantoic fluids harvested from these eggs gave positive HA tests with chicken red cells and contained 80 haemagglutinating units (HAU) per ml. while HA tests on control allantoic fluids were negative. All fluids were bacteriologically sterile. The haemagglutinating agent could be passed serially and showed enzymatic activity similar

Table 1. *Virological data on four of the sea-birds from the Cape Peninsula region*

Bird no....	1*	2*	3*	4†
Species	Common Tern	Common Tern	Common Tern	Hartlaub's Gull
Received	27. iv. 61	27. iv. 61	11. v. 61	6. ix. 61
Condition	Dead 2 days	Dead 2 days	Ill	Alive
Autopsy	27. iv. 61	27. iv. 61	11. v. 61	14. ix. 61
Storage before titration	72 days, $-20°$ C.	78 days, $-20°$ C.	5 days, $-70°$ C.	1 day, $-20°$ C.
Tern virus content‡				
Heart	4·3	1·9	Trace	3·75
Lung	5·1	3·3	Trace	3·5
Liver	3·7	Trace	Trace	5·25
Kidney	3·5	1·7	Trace	4·75
Brain	1·7	—	—	—
Blood	5·1	3·7	—	NT
Serum HI antibody titre	NT	NT	< 5	< 5

* Naturally infected.
† Experimentally infected.
‡ $\log_{10}$EID 50 per g. of tissue.
−, No virus isolated. NT, Not tested.

to that of the influenza viruses. The conclusion that it was a myxovirus was confirmed by subsequent serological and morphological studies and it was named Tern virus. Pass 1 in KB cells was subinoculated after 9 days into fresh KB cells and 7 days thereafter CPE was noted. A second subinoculation resulted in CPE in 6 days and the culture fluid contained 1280 HAU per ml. while control fluids showed no haemagglutinating activity. The results of culture in MK cells and newborn mice were negative.

A similar agent was isolated from birds 2 and 3 in eggs, but not in KB or MK cells nor in newborn mice.

Prototype strain of Tern virus

The strain isolated in eggs from the heart-lung-kidney pool of bird 1 was employed as the prototype strain of Tern virus. HI tests using immune serum prepared against this strain showed that the same virus was isolated from all three birds.

Growth of Tern virus in embryonated eggs

The embryonated egg could be infected via all routes of inoculation with Tern virus, which could be recovered in approximately equal concentrations from all

the tissues of the embryo. The allantoic route was routinely used and the allantoic and amniotic fluids harvested as the most suitable sources of virus. These fluids usually contained $10^{7.5-9}$ EID 50 of Tern virus/ml.

End-points were sharp in titrations in eggs and the results were read within 48–72 hr. of inoculation.

Birds 1 and 2 were received dead. Bird 3 was received alive but ill and sat huddled up with ruffled feathers and was unwilling to move when disturbed. It is noteworthy that the virus content of some of the organs of Tern 1 was moderately high despite the storage of the emulsions at $-20°$ C. for 72 days. Less virus was found in the tissues of Tern 2, and only traces in those of Tern 3 in the serum of which no HI antibody to Tern virus was detected.

Post-epizootic period

In the post-epizootic period the only evidence of natural infection with Tern virus was the presence of specific HI and neutralizing antibodies in the serum of each of two Common Terns shot in July 1961. Two Swift Terns inoculated experimentally with live Tern virus showed no ill effects but developed HI and neutralizing antibodies in their sera.

Bird 4 (Table 1), a Hartlaub's Gull, died on the third day after IM inoculation of Tern virus. Virus was present in moderately high concentration in the tissues.

Histological examination of the sea-birds

Lesions were only found in birds 1, 2 and 3 (Table 1), which had a meningo-encephalitis; no histological lesions were detected in the remaining sixteen birds, including those inoculated experimentally (C. J. Uys, personal communication).

Serological classification of Tern virus
Non-specific inhibitors

No non-specific inhibitors of Tern virus haemagglutination were detected in the following sera which were heated at $56°$ C. for 30 min. and tested at an initial dilution of 1/5 (in the case of birds) or 1/10 against 4 HAU of unheated Tern virus: sera of four Swift Terns, a Common Tern, an Arctic Tern, a Hartlaub's Gull, three ferrets and five rabbits; pooled and individual guinea-pig and chicken sera, pooled human, pooled mouse and pooled rat sera, calf and horse sera.

HI tests

Tern virus haemagglutination was inhibited by homologous and Chicken/Scot. virus immune serum (Table 2). No inhibition occurred with any of the other sera listed under materials and methods.

Cross-neutralization tests in CE cells

The only demonstrable cross-neutralization was between Tern and Chicken/Scot. viruses (Table 3), which showed a close reciprocal antigenic relationship (Table 4).

Table 2. *HI tests with Tern virus antigen and myxovirus strain-specific sera*

Strain-specific serum	Source of serum	HI antibody titre against 4 HAU of Tern virus	Homologous HI antibody titre
Tern/SA/1961	Rabbit	160	160
Chicken/Scot./1959*	Chicken	160	640
Fowl plague			
(1) Strain Brescia	—	< 4	—
(2) Strain Alexandrien	—	< 4	—
(3) Strain N	—	< 4	—
NDV (Komarov)	Chicken	< 5	160

* Serum received from Dr J. E. Wilson.
—, Information not available.

Table 3. *Cross-neutralization tests in chick embryo cell cultures*

			Neutralizing antibodies against			Homo-logous HI antibody titre
Strain-specific serum	Source	Dilution used	Tern virus	Chicken/ Scot. virus	NDV	
Tern/SA/1961	Rabbit	1/5	+	+	−	160
Chicken/Scot./1959*	Chicken	1/10	+	+	−	640
Fowl plague						
(1) Strain Brescia	No data	1/5	−	−	−	
(2) Strain Alexandrien	No data	1/5	−	−	−	
(3) Strain N	No data	1/5	−	−	−	
NDV (Komarov)	Chicken	1/5	−	−	+	160

+, Antibodies present.   −, No antibodies detected.
* Serum received from Dr J. E. Wilson.

Table 4. *Cross-neutralization tests in chick embryo cell cultures*

Serum of chicken convalescent from infection with	Neutralizing antibody titre against	
	Tern virus (100 ID 50)	Chicken/Scot. virus (50 ID 50)
Tern virus	320	320
Tern virus	1280	320
Chicken/Scot. virus	160	2560
Chicken/Scot. virus	1280	5120
Control chicken	< 5	< 5

CF tests

Several batches of antigens and immune guinea-pig sera were prepared for Tern, Chicken/Scot., and Influenza A_2, B and C viruses. The results of cross-CF tests (Table 5) indicated the similarity of the nucleoprotein of Tern, Chicken/Scot. and Influenza A_2 viruses. The strain-specific antigens of Tern and Chicken/Scot. viruses were closely related to each other but not to the Influenza A_2 antigen. There was

no antigenic relationship between Influenza B and C viruses or between these two viruses and Tern, Chicken or Influenza A_2 viruses.

Guinea-pigs immunized with purified Tern virus V antigen and shown to produce strain-specific CF antibodies also developed homologous HI and neutralizing antibodies (Table 6).

Growth in CE cells

Tern and Chicken/Scot. viruses produced CPE in CE cells with the formation of intranuclear inclusions, a feature which has apparently not been recorded before in Influenza A strains. Both strains produced plaques in CE cell monolayers in Petri dishes.

Table 5. *Cross-CF tests*

Virus nucleoprotein antigen	'Anti-NP' sera				
	Tern	Chicken/Scot.	Influenza A_2	Influenza B	Influenza C*
Tern	1024†	256	512	< 8	< 8
Chicken/Scot.	256	256	512	< 8	< 8
Influenza A_2	128	128	512	< 8	< 8
Influenza B	< 8	< 8	< 8	256	< 8
Influenza C*	< 8	< 8	< 8	< 8	256

Virus strain-specific antigen	Strain-specific sera				
	Tern	Chicken/Scot.	Influenza A_2	Influenza B	Influenza C*
Tern	256	64	< 8	< 8	< 8
Chicken/Scot.	256	256	< 8	< 8	< 8
Influenza A_2	< 8	< 8	256	< 8	< 8
Influenza B	< 8	< 8	< 8	512	< 8
Influenza C*	< 8	< 8	< 8	< 8	256

* Influenza C antigen consisted of ether-treated purified virus which was also used to prepare the immune sera.

† All tests were two dimensional. Figures represent the reciprocal of the highest serum dilution giving > 50 % fixation with any of the antigen dilutions tested.

Table 6. *Titration of homologous CF, HI and neutralizing antibodies in the sera of guinea-pigs immunized with purified strain-specific Tern virus antigen*

Strain-specific guinea-pig sera	Serum antibody titres against Tern virus antigen		
	CF	HI	Neutralizing
1	128	80	2560
2	128	40	1280
3	256	160	2560

DISCUSSION

Classification of Tern virus

Serological relationship

Tern virus is an avian strain of influenza A, classified as Myxovirus influenzae A/Tern/South Africa/1961.

Its haemagglutinating properties were similar to those of the influenza viruses.

Serological studies showed that it had type-specific nucleoprotein antigens similar to the Influenza A2 strain with which it was compared. However, the strain-specific haemagglutinin of Tern virus was antigenically quite distinct from that of other influenza strains investigated with the single exception of Chicken/Scot. virus which had an antigenically closely related haemagglutinin. This relationship was confirmed by H. G. Pereira (personal communication), and Pereira, Tumova & Law (1965) who also found no antigenic sharing between Tern virus haemagglutinin and the haemagglutinins of the following viruses: Fowl plague, Duck/England/1956 and 1962, Duck/Czechoslovakia/1956, Turkey/England/1963 and a virus recently isolated from turkeys in Canada.

Electron microscopy

Electron microscopical studies of Tern and Chicken/Scot. viruses (Becker, 1963 1964*a*, *b*) showed that both strains had the same structure as other influenza viruses which have been discussed by Waterson, Hurrell & Jensen (1962). The study of the morphology of Tern virus, however, did raise several new points: in high resolution electron micrographs of ether-fractionated, purified haemagglutinin the radiating rods of haemagglutinin appeared to be delicate tube-like structures linked at their central ends to form the viral envelope; both in ether-fractionated preparations of nucleoprotein and in ultra-thin sections of whole virus particles the nucleoprotein was clearly shown to be a double helix which was enantiomorphous; the double helix appeared to be regularly arranged within the viral particle and to lie adjacent to the viral envelope where, under certain conditions, it simulated a membrane.

Comparison of Tern and Chicken/Scot. viruses

A comparison of Tern and Chicken/Scot. viruses (to be published) has shown that they have many features in common and are probably variants of the same strain.

Epizootiology of Tern virus infection

Tern virus as the causal agent of the Tern epizootic

Tern virus was isolated from all three diseased Common Terns (birds 1, 2 and 3, Table 1) collected during the epizootic, and the same virus was isolated from a further two diseased Common Terns examined at the Onderstepoort Veterinary Laboratories (R. A. Alexander, personal communication). In birds 1 and 2 the virus content of the organs was high considering the long interval of storage at $-20°$ C. before titration. Bird 3 was probably examined at an early stage of infection, before the virus had had sufficient time to establish itself. In addition, serum was obtained from each of two Common Terns collected shortly after the epizootic and both sera contained HI and neutralizing antibodies to Tern virus, providing presumptive evidence that they had recovered from infection with Tern virus. Unfortunately it was not possible to capture live Common Terns but experimentally inoculated chickens developed an acute illness similar to that noted in Terns (Becker, 1964*b*).

It seems reasonable to conclude that during the epizootic there was widespread

infection of Common Terns with Tern virus. It is interesting that two captive Swift Terns which are not migrants, were clinically unaffected by experimental inoculation with live Tern virus, but antibody formation was stimulated. The other bird inoculated experimentally was the gull (bird 4, Table 1) which was not doing well in captivity and died 3 days after IM inoculation of Tern virus. It is not clear if the gull died of Tern virus infection; no histological lesions were found despite the isolation of virus from the tissues.

Sea-birds as a source of infection of domestic poultry

The isolation of Tern virus raises interesting epidemiological possibilities. The outbreak in chickens in Scotland caused by Chicken/Scot. virus preceded the Tern epizootic by about 17 months and occurred during stormy weather which drove sea-birds a little inland to take shelter. Large numbers of Herring Gulls (*Larus argentatus*) were at that time working the farm at which the outbreak in chickens occurred in November 1959 (J. E. Wilson, personal communication). The chickens might have contracted the infection from sea-birds, a viewpoint possibly supported by the preceding mass mortality in Kittiwakes (*Rissa tridactyla*) and Fulmars (*Fulmaris glacialis*) from February to August 1959 (Joensen, 1959) off the coast of Britain and Scandinavia. Unfortunately the aetiology of the last-mentioned outbreak was not investigated, but it is tempting to think it was caused by the Tern virus which was isolated at Cape Town some 18 months later in 1961, from migrant European Common Terns.

One might postulate: that certain sea-birds suffer latent or sporadic infection with avian influenza; that epizootics may be precipitated in them by conditions of stress, e.g. poor feeding under unfavourable weather conditions such as preceded the Tern epizootic; and that spread to other sea-birds or domestic poultry may occur.

Of particular interest is the investigation of Blaxland (1951) into the possible role of sea-birds in the spread of NDV amongst domestic poultry in Scotland, neighbouring off-shore islands and Ireland. NDV was isolated from the bone marrow emulsions of six out of twenty-six shags (*Phalacrocorax aristotelis*) and antibodies to NDV were found in eighteen of fifty-four blood samples of shags and cormorants (*P. carbo*). Blaxland considered sea-birds as a possible means of spread of NDV because of the local habit of shooting shags for the table and throwing the offal to the poultry. Wilson (1950) isolated NDV from a gannet (*Sula bassana*) caught on the Orkney islands. Wells (1963) described an outbreak of fowl plague on a turkey farm on the Norfolk coast and concluded that the infection may have been introduced by wild birds which entered the folds in search of food.

Other epizootics

No report has been found in the literature of any other epizootic in terns or of avian influenza in other than domestic birds, i.e. chicken, turkey, duck (Pereira *et al.* 1965; Wells, 1963; Dinter, 1964), probably because they attract more attention and are more easily investigated.

Mass mortalities or epizootics affecting wild birds, including sea-birds other than

terns, have from time to time been reported but few of these were fully investigated and most of the reports are not very informative. In addition there appears to be too ready a tendency to ascribe these mortalities directly to adverse weather conditions.

Spread of Tern virus infection among terns

The rapid spread and high mortality rate seen in the epizootic in Common Terns were features in common with outbreaks of fowl plague in domestic poultry (Wells, 1963). The mode of spread of the infection could not be established but probably depended upon the close contact between birds at night at their roosting places in shallow water along estuaries, on sandbanks or on the small boats in harbours. The many birds affected during the epizootic had soiled vents and virus might have been spread via infected secretions or excretions. Tern virus was recovered from the nasopharynx and cloaca of experimentally infected chickens (Becker, 1964*b*). The role of ectoparasites is unknown. Ticks, mites and lice are known to harbour NDV for several days but are not known to transmit infection (Stubbs, 1959; Brandly, Moses, Jones & Jungherr, 1946).

SUMMARY

The aetiological agent of an epizootic among Common Terns (*Sterna hirundo*) in South Africa in 1961 was isolated from several sick birds and named Tern virus. It was classified on the basis of antigenic and morphological properties as a strain of avian influenza virus, Myxovirus influenzae A/Tern/South Africa/1961. The strain-specific antigen of Tern virus was unrelated to all known influenza strains with the single exception of Chicken/Scotland/1959 virus and the two viruses may be regarded as variants of the same strain. This relationship raised the interesting epidemiological possibility of the spread of infection between sea-birds and domestic poultry because the Common Tern migrates between Europe and South Africa.

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