CHARACTERIZATION AND IMMUNOGENICITY OF INFLUENZA VIRUS TYPE A ISOLATED FROM DUCKS IN KERALA

By

M. MINI

THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
Mannuthy, Trichur

1988

DECLARATION

I hereby declare that this thesis entitled CHARACTERIZATION AND IMMUNOGENICITY OF INFLUENZA VIRUS TYPE A ISOLATED FROM DUCKS IN KERALA is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title, of any other University or Society.

Signature of the candidate : Mini

Mannuthy,
4-8-1988.

Name of the candidate : M.MINI

CERTIFICATE

Certified that this thesis, entitled CHARACTERIZATION AND IMMUNOGENICITY OF INFLUENZA VIRUS TYPE A ISOLATED FROM DUCKS IN KERALA is a record of research work done independently by Kumari. M.Mini, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Name of the Guide: Dr.S.Sulochana,
(Chairman, Advisory Board)
Designation : Professor & Head
Department of Microbiology

Mannuthy,
4-8-1988.

To my parents

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to Dr.(Mrs) S.Sulochana, Ph.D. (Guelph), Professor and Head, Department of Microbiology for encouragement, meticulous guidance and advice throughout this work.

I express my sincere thanks to the members of the Advisory Committee, Dr.K.T.Punnoose, Professor, Department of Microbiology, Dr.P.C.James, Associate Professor, Department of Microbiology and Dr.G.Reghunathan Nair, Associate Professor of Poultry Science for their valuable suggestions and continued interest rendered throughout this study.

I am gratefully indebted to Dr.G.Krishnan Nair, Dr.R.Madhusoodanan Pillai, Dr.V.Jayaprakasan, Dr.M.C.George for their esteemed help and encouragement.

I wish to thank the staff members of the Department of Microbiology for their help and co-operation during this study.

My sincere thanks to Dr.K.Madhavan Pillai, Professor, Department of Parasitology for the interest and encouragement.

I am grateful to Dr.K.Radhakrishnan, Dean-in-charge, College of Veterinary and Animal Sciences, Mannuthy for providing facilities to carryout this study.

I am thankful to Dr.(Miss) Usha Narayanapillai, Dr.(Miss) Sangeeta Nair, and other friends for their constant help and moral support.

I am grateful to my beloved parents, sister and brother for their love, blessings and constant encouragements for the successful completion of this work.

The financial assistance in the form of Junior Research Fellowship given by the Indian Council of Agricultural Research, New Delhi is gratefully acknowledged.

(M.MINI)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Introduction

INTRODUCTION

Duck rearing is a popular rural occupation in India, particularly in the states of West Bengal, Assam, Andhra Pradesh, Orissa, Kerala, Tripura and Jammu and Kashmir. The topography, climatic conditions and the presence of brackish water areas in Kerala are highly congenial for the rearing of ducks. Consequent to the implementation of various developmental plans for the improvement of poultry industry, better layers and meat producers have been evolved among the duck population also. It is well established that this industry could also contribute to the rural economy to a considerable extent. Due to the managemental and rearing practices followed by majority of the duck farmers like the out door rearing, movement of the flocks during harvesting seasons and summer months to paddy fields and available perinneal water sources of different areas of the state, the ducks get exposed to infectious agents and thus easily infected with various pathogens especially viruses. Much emphasis has not so far been given to the study of viral diseases in ducks except for duck viral enteritis and duck virus hepatitis. Moreover, the information on the susceptibility of ducks to various avian viral infections is scanty.

Avian influenza viruses have been isolated from various species of birds. These reports have also described the

circumstances under which the isolation of these viruses were made and also the clinical aspects of the associated diseases. Ducks are reported to be quite resistant to influenza virus which were virulent for turkeys and chicken, (Slemons and Easterday, 1972; Alexander, 1982a) and they generally showing undetectable inapparent infections.

A distinguishing feature of influenza virus type A is its variability and the capacity to change from relatively harmless virus to one that can cause fatal disease in the same or different species. It periodically causes epidemics in man, pigs, seals, turkeys, chicken and a variety of other species of birds. This virus can undergo frequent antigenic variation and it is possible that a less virulent strain become highly pathogenic as it was reported in 1985 outbreaks of avian influenza in Pennsylvania, United States (Webster, 1984).

A vast reservoir of influenza viruses exists in the wild bird fauna throughout the world. In this ecosystem the virus circulate in various antigenic and pathogenic types and new virus variants most probably arise by the process of recombination. A total of 13 different H subtypes and 9 N subtypes of influenza virus have so far been described (Russell and Edington, 1986) and representative of all these different subtypes have been isolated from birds particularly from aquatic species such as ducks, geese and gulls (Webster, 1984).

There are only few reports on the isolation of influenza virus type A from birds in India (Rao et al; 1979 and Manjunath and Mallick, 1977).

In Kerala during the first half of 1985 and 1987, there was a mortality of 15 to 20% among ducklings of each hatch at Government Duck farm, Niranam, during their first six weeks of life. These birds exhibited respiratory distress, droppiness, oculonasal discharge, swollen eyelids and oedema of head.

Materials collected from ailing as well as dead birds revealed the presence of influenza virus type A from both 1985 and 1987 outbreaks (Sulochana et al; 1987). Sera collected from the recovered birds also had HI antibodies when tested against one of the isolates.

Though ducks are considered as resistant to influenza virus type A infections, Higgins (1971) reported three outbreaks in ducks associated with this virus where in the could observe 20 to 100% mortality. Influenza virus have also been reported to induce poor immune response both under natural and experimental infections as these birds failed to show rise in HI antibodies, though McNulty et al. (1985) observed development of HI antibodies in the absence of clinical symptoms. Thus the reports on the pathogenicity and immunogenicity of influenza virus type A in ducks are conflicting. The observations made by Sulochana et al. (1987)

indicated that this virus was associated with the outbreaks of respiratory tract infection causing 90 to 100% morbidity and 15 to 20% mortality among ducklings below six weeks of age, during 1985 and 1987 at Nranam Duck farm, Kerala. Hence it was felt worthwhile to take up a detailed study on the characterisation, pathogenicity and immunogenicity of some of the influenza virus type A isolates from the above outbreaks so as to get a better understanding about this infection and to suggest suitable control measures.

Review of Literature

REVIEW OF LITERATURE

Avian influenza virus infections occur in a variety of wild and domestic birds. The first avian influenza described was the fowl plague by Perroncito in Italy in 1878 (Stubbs, 1959). The filterable nature of the infectious agent of this disease was reported in 1901 by Centanini and Savonuzzi and it was Schaffer in 1955 who identified fowl plague virus as a type A influenza virus.

The virulence of avian influenza virus is extremely variable. Some virus can be nonpathogenic as to produce no readily detectable signs of illness in a flock, while others may cause slight drop in egg production, sign of respiratory disease, sinusitis, diarrhoea, oedematous head and cyanotic combs and wattles or mortality upto 100% as in the case of fowl plague virus; A/Chicken/Scotland/59; A turkey/England/65; and A/turkey/Ont/7732/66 (Beard, 1976).

1. Incidence

a) Chicken.

Avian influenza causing fowl plague in chicken was first reported in 1927 in Dutch East Indies. It was studied in detail by Moses et al. (1948). In 1949 a virus designated as N virus was isolated from chickens in Germany and was considered to be a variant of fowl plague virus but did not cause high mortality (Dinter and Baker, 1950; Rott and Schaffer, 1960).

Since 1959, infections of domestic fowls with influenza virus have been rare. In 1959 an outbreak of a virulent disease was reported in chicken in Scotland and the causative agent was isolated and designated as A/chicken/Scotland/59 (Pereira et al; 1965). Isolated outbreaks of avian influenza causing mild clinical signs in chicken had been reported in France in 1979 and 1980 - subtype H9N2, Italy in 1980 - subtype H5N2 and Belgium in 1978 - subtype H11N6 (Meulemans et al; 1979; Bennejean, 1982; Petek, 1982). An outbreak of fowl plague was reported in Australia during 1975 (Turner, 1976). The influenza A virus isolated from this outbreak was subtyped to be H7N7. Similarly two outbreaks of influenza infections in chicken have been recorded from Alabama in 1975 by H4N8 subtype (Johnson and Maxfield, 1976; Johnson et al; 1972) and Minnesota in 1979 of the H6N1 subtype (Halvorson et al; 1980).

Osidze et al. (1979) reported influenza outbreaks at two poultry farms in USSR with a hitherto unknown type of avian influenza virus which had neuraminidase of human influenza virus. They have also demonstrated antibodies to human influenza strains (A/Hongkong/68; A/Port Chalmers/73; A/Tokyo/75 and A/Victoria/75) indicating that the birds contracted the infection from man. Similar observations on the transmission of influenza virus infections from farm personnel to chicken was also made by Shablouskaya et al.(1985).

The reported infections of poultry since 1967 in USSR were identified to be due to haemagglutinin subtypes H3, H4, H6 and H7 (Osidze et al; 1979).

A lethal outbreak of avian influenza with subtype H5N2 was recorded in April 1983 in Pennsylvania (Buish et al; 1984) which was controlled by slaughter of affected flock and strict quarantine in the nearby flocks.

An acute avian influenza characterized by sudden onset of depression, sinusitis, cyanosis and moist rales among broiler breeds in a multi-age chicken farm was reported by Barr et al. (1986). The mortality was about 75% in one of the four sheds. Bacteria resembling Haemophilus paragallinarum was isolated from sinuses. There was a positive reaction to Mycoplasma and Newcastle disease virus (NDV) antibodies as well as high Haemagglutination inhibition (HI) titers to infectious bronchitis virus. Tests for HI antibody to NDV and avian influenza confirmed the presence of avian influenza antigen.

b) Turkey.

The first influenza type A virus infections in turkeys was recognized in Canada in 1963 (Lang et al; 1965). The birds from which the isolations were made were showing respiratory and other signs of disease with marked drop in egg production. The virus isolate A/turkey/Canada/63 was

identified to have the antigenic subtype H6N8 (Lang and Wills, 1966). A highly virulent disease of turkeys in Norfolk was reported in the same year by Wells (1963) and the isolate from the outbreak was designated as A/turkey/England/63 (H7N3).

Since then influenza virus infections of turkeys have been reported from many countries. The viruses were mainly associated with respiratory disease, egg production problems and elevated mortality rate (Olesiuk et al; 1967; Lang et al. 1968a; 1968b, Papparella et al; 1969; Smithies et al; 1969; Allan et al; 1970; Kendal et al; 1971; Alexander and Allan, 1982; Pomeroy, 1982; Lipkind et al; 1982). The antigenic subtypes reported from turkeys in England were H6N2 (A/turkey/England/66); H3N2 (A/turkey/England/69); H7N7 (A/turkey/Scotland/70); H5N2 and (A/turkey/England/N28/73); H6N2 (A/turkey/England/110/77) (Alexander, 1982). The most frequently isolated combinations in turkeys in United States were H1N1, H4N9, H5N?, H5N1, H6N1, H6N2, H6N8, H6N?, H7N7 and H9N2; H6N1 being the most frequently isolated combination (Bahl et al; 1979).

In the history of avian disease in Minnesota, the outbreak of turkey influenza involving 140 flocks and 2,000,000 birds was the most extensive and costly (Bahl et al; 1979).

Influenza virus infections in Italy were first reported during 1966-67 and the viruses isolated from these outbreaks

belonged to subtype H10N2 (Franciosi et al; 1981). Isolates between 1973 and 1979 were of H6N2 subtype, the one isolate of 1977 was of H10N2 (Franciosi et al; 1981).

McNulty et al. (1985) isolated an influenza virus subtype H5N8 from turkeys with disease showing 30% mortality.

c) Captive and free flying birds other than water fowls.

In an epidemic involving several common terns (Sterna hirundo) in South Africa in 1961 a virus was isolated and later on identified as influenza A virus, A/tern/S.Africa/1961 (Becker, 1966). It had antigenic relationship with chicken/Scot/1959 and was considered as a variant of the same strain. These observations indicated the possibility of spread of infection between sea birds and domestic poultry.

Mandelli et al. (1969) reported an outbreak of type A influenza virus infection in domestic quail with a mortality rate of 45%. Similarly Nardelli et al. (1970) reported the isolation of 24 strains of influenza A virus from an outbreak of respiratory disease in 13 quail flocks in Northern Italy with 15 to 80% mortality during 1965-68. During this outbreak young birds were mainly affected. In many outbreaks of influenza in domestic poultry, spread from wild birds have been considered as the most likely mehanism of primary infections (Higgins, 1971; Hahl et al; 1979).

Slemons et al. (1973) reported isolation of 15 type-A

influenza viruses from 12 different species of exotic birds imported to USA. All the isolates were antigenically related to A/Mynah/Mass/71 (Hav4Neq2).

Influenza virus isolations were reported from Psittacine birds such as parrots, cockatoos and parakeets (Mc Ferran et al; 1974; Alexander et al; 1974) and starlings (Lipkind et al; 1979).

In 1980 Chernetsov et al. isolated a new influenza virus A/tern/Aktyubins/77 (Hav4Neq2) from black terns while moulting. Five of the 86 serum samples tested also had antibodies to the isolate.

From an epidemic of depression, diarrhoea and anorexia in a flock of 60 day-old budgerigars Imada et al. (1980) isolated influenza A virus from all the three specimens unsed for virus isolation.

Lipkind et al. (1981) reported isolation of avian influenza virus from rockpartridges (Alectaris graeca). The isolates were designated as A/rockpartridge/Beit/70/81 (H7N2) and A/rockpartridge/Biet/9+1/81 (H7N1). These isolates did not kill chick embryos in 96 hours.

Daulbaeva et al. (1981) isolated 66 haemagglutinating agents from 357 wild birds belonging to 55 species, of which 19 were antigenically related to influenza type A. Two strains were H1N1 subtypes and the remaining 17 were Hav2 Nav5 subtype.

During 1979 in Southern Kazakhstan, 36 influenza type A viruses were isolated from 750 wild birds (Sayatov et al; 1981). Twenty nine of the 36 strains had the antigenic formula Hav2 Nav5.

Tautaswasdi et al. (1984) isolated avian influenza virus for the first time in Thailand from an ornamental silver Thaipheasant which had died suddenly without showing clinical symptoms though the other birds were showing dullness, anorexia, and greenish diarrhoea.

d) Ducks and other water fowls.

Walker and Bannister (1953) isolated a virus from ducklongs with central nervous system disease in 1952 in Mannitoba, Canada. The virus was later on identified as influenza type A (H10N7)by Mitchell et al. (1967).

Koppel et al. (1956) described a fatal mass infection in ducks aged 10 to 21 days. The birds were showing severe sinusitis with the involvement of lower respiratory tract. During this outbreak out of 3,000 ducklings 1,250 succumbed to the disease. An influenza type A virus (Duck/Czechoslovakia/56) was isolated from this outbreak.

Franco et al. (1958) isolated a strain of influenza A virus from lungs, nasal discharge and brain of ducks died of acute respiratory disease in Slovakia. This isolate was studied in detail and designated as A/anatis/Kosice/56 (Blaskovic et al; 1959).

In Great Britain Roberts (1964) reported isolation of influenza virus type A, A/duck/Eng/56 (H11N6) from the sinus and airsac material of ducklings showing respiratory distress and oedema of the head. During this outbreak though the morbidity rate was very high mortality was very low and the affected birds recovered without any treatment. Again in the same farm, six years later a similar outbreak occurred but with a different H subtype; A/duck/Eng/62 (H4N6). Similar association of influenza virus with chronic respiratory disease in duck was also reported in USA, Czechoslovakia and Britain (Vrtiak et al; 1966).

Prokofeva and Tsimokh (1966) studied in detail the viruses isolated from duck influenza between 1960 and 1964 in Ukraine and reported that the isolates were related to type A influenza virus but distinct from the Kosice 1956 duck strain and English strain of duck origin.

The first outbreak of duck influenza in Yugoslavia was reported by Paukovic et al. (1969) in a farm near Zagreb. The influenza type A virus isolated (duck/Yugoslavia/446/66) was serologically related to an influenza virus isolated from quail in Italy and to virus N from fowls in Germany.

Hwang et al. (1970) reported an outbreak of respiratory disease in domesticated muscovy ducks in which 10% of the 10 week-old birds died. The main sign of the disease was sneezing. The influenza type A virus isolated was distinct

from the fowl plague virus, English and Czechoslovakian duck strains (A/FP; A/duck/England/62 and A/duck/Czech/56). Sera collected from this flock two months after the outbreak and those from other species such as turkeys and geese had antibodies to the isolate. However, turkeys and geese did not show any clinical signs. The authors suspected wild ducks and geese which mixed freely with the domesticated ducks as the main source of the epornitic of influenza A outbreak.

In an outbreak of illness in a large duck farm in Germany Schettler (1970) reported about 20% mortality among ducklings of 2 to 3 weeks of age. Influenza type A virus, isolated from this outbreak was less virulent and hence it was concluded that additional factors might have involved in the outbreak of the disease.

First report of isolation of influenza A virus from domestic ducks in Hongkong was made by Higgins (1971). From nine outbreak of the disease affecting ducks, influenza A viruses were isolated from three cases. The outbreaks were acute and characterized by sudden onset, rapid spread, high morbidity and mortality. The symptoms reported were malaise, anorexia, diarrhoea, wasting, occulonasal discharge, paraplegia and decrease in egg production with increased proportion of soft shelled eggs. The course of the disease was 2 to 3 weeks from the onset of symptoms until complete recovery of survivers.

Tanyi (1972a) isolated influenza A virus designated Duck/Hungary/1/70 which cross reacted with serotype 6, A/turkey/Wisconsin/66, from respiratory systems of ducklings dying at 2 to 4 weeks of age in a flock of 20,000 birds. From a similar outbreak another strain of the virus designated Duck/Hungary/2/70, antigenically related to Duck/England/56 and Duck/England/62 was also isolated (Tanyi, 1972b).

Eisengarten et al. (1973) isolated haemagglutinating agents which were later on didentified as influenza A virus, from three weeks-old ducks in a fattening flock. The birds were sick and were exhibiting swelling of the cranial sinuses which contained yellowish cheesy secretion and gelatinous greyish exudate and respiratory disease.

Cloacal and tracheal swabbing from 159 migratory water fowls of different species shot by hunters in Delaware and Maryland, United States revealed influenza A virus in four cases (Rosenberger et al; 1974).

Five avian influenza virus strains isolated in the USSR were characterized antigenically using specific antisera to the isolated subunits (Webster et al. 1974). Three of the five isolates had the characteristics of A/duck/Ukraine/63 (Hav7 Neq2), and the remaining two viruses possessed Hav7 Nav2 surface antigens - a combination that has not been reported previously. The authors speculated on the origin of the latter subtype as a recombination of A/duck/Ukraine/63

(Hav7 Neq2) and A/tern/S. Africa/61 (Hav5 Nav2).

Bahl et al. (1975) during their attempts to isolate type A influenza viruses from migratory waterfowls among Mississippi flyway reported that four of the 60 tracheal swabs from migratory mallard (Anas platyrhynchos) yielded type A influenza viruses though none of the 124 serum samples from either mallards or wood duck (Aix sponsa) contained detectable precipitating antibodies to Nucleoprotein (NP) antigens of influenza type A virus. Tanyi et al. (1976) reported unilateral/bilateral sinusitis in a group of 1 to 7 weeks old ducklings and oedema of the head in adult birds. The incidence was 15 to 50% with a mortality rate of 10.6% among the ducklings. Influenza A viruses were isolated from the sinus discharges of the affected ducklings. Cloacal swabs from 829 feral ducks were screened for the presence of influenza A virus by Webster et al. (1976) and reported isolation of three strains.

Shortridge et al. (1977) during their studies on isolation and characterization of influenza viruses from avian species in Hongkong screened 588 samples including 304 cloacal and 284 tracheal swabs. They reported thirteen different influenza A viruses from 24 isolates. Viruses possessing five different avian haemagglutinin subtypes (H10, H4, H5, H6 and H3) in combination with seven different neuraminidase subtypes (N1, N5, N6, N8, N2, N3 and N9) were isolated from

ducks (6 nos) and chicken (1). Haemagglutinin and neuraminidase combinations of seven of the isolates (H1ON1, H1ON5, H4N2, H3N2, H3N6, H3N3 and H3N9) were not reported previously. All these new combinations were from ducks except H3N9 which was from chicken.

Twenty four isolations of influenza virus A reported by Bahl et al. (1977) were from the tracheal swabbings of apparently healthy mallard ducks. None of the 60giant Frank gulls (Larus pipixan) and 65 giant geese (Branta cardadensis) gave any positive isolations.

Nerome et al. (1978) isolated 22 strains of influenza A virus from caged birds that had been imported to Japan from India and Thailand and had died during transportation to Tokyo. These isolates were grouped into two subtypes - H3N8 and H4N6. The former was related to A/duck/Ukraine/1/63 and the latter to A/duck/Czech/56.

Webster et al. (1978) reported replication of influenza virus A, isolated from the cloaca of naturally infected feral ducks in the lungs and in the cells lining the intestinal tract of feral and domestic ducks and were found in high concentration in the faeces. They have also seen that the virus retained its infectivity in faecal material for atleast 30 days at 4°C and seven days at 20°C. Duck influenza virus was more stable to low pH than human strains and was viable for 30 days in nonchlorinated river water at 0°C and for

four days at 22°C. The susceptibility of ducks to infection with human and avian strains and the possibility of transmission to animal species through water supply made the authors to think that ducks are important in the ecology of influenza viruses.

Haemagglutinating agents isolated from the cloaca and/ trachea of all the 15 wild mallard ducks found dead at Tanahim, Israel were studied in detail and identified as influenza type A virus A/mallard/Tanahim/79 (Hav1N2) (Lipkind et al; 1979). The isolate was antigenically identical to the virus isolated from an outbreak on a nearby turkey farm. Hence wild birds were suspected in the dissemination of influenza virus.

Kida and Yanagawa (1979) isolated influenza virus subtype Hav7N2 from 20 wild free flying ducks in Hokkaido in Japan which cross reacted with Asian influenza virus H2N2.

In a study involving cloacal and tracheal swabs from 235 resident and 396 migratory ducks in Miyagi perfecture, Japan during 1977-78, 26 influenza A viruses were isolated. Of these 26 isolates, 12 were antigenically related to avian strain A/duck/Alberta/35/76, though neuraminidase antigens were different. The neuraminidase antigens in these isolates were N2 and N3. It was suggested that avian influenza A viruses among feral ducks may be isolated in various combinations of haemagglutinin and neuraminidase. Weisser (1979)

isolated 15 haemagglutinating agents from 1027 cloacal swabs from 957 birds of various species. Eight of these isolates were identified as influenza A virus and were from mallard and tufted ducks. Yamane et al. (1979) opined that the various subtypes in wild ducks are due to genetic recombination.

Hinshaw et al. (1979) studied in detail the method of transmission of influenza A virus, their extent of antigenic diversity and continued circulation from year to year. Cloacal samples from 2046 waterfowls trapped on 16 lakes in Vermillion River country, Alberta, Canada; ducks trapped 30 to 60 meter from the shore in water 0.6 to 1.0 meter and water collected three meter of the trap and the faecal samples collected from the shore were screened for the presence of influenza A virus. All the three types of samples revealed influenza A virus, though most frequently (26%) from the cloaca of healthy mallard ducks. Unconcentrated water samples and droppings from the shore also yielded the virus. Their isolates comprised 18 different antigenic combinations of H and N. The predominent subtypes were the one which were also isolated from the water and faeces and included HswlNl, Hav7 Neq2 and Hav4 Navi. All the major H and N subtypes of human strains were represented among these ducks isolates. Antigenic counterparts of these duck viruses (Hav1 Nav2; Hav7 Nav1; Hav4 Neq2 and Hav8 Nav4) have been associated with disease

outbreaks in domestic avian species in North America. From their observations the authors considered wild ducks as the natural reservoir of most if not all influenza A viruses disseminating the infection through water. Contamination of water supplies with faeces of infected yet healthy feral ducks offers a mechanism for maintaining a wide variety of influenza viruses within the duck population and for introducing these viruses into other species (Hinshaw et al; 1979). From cases of respiratory disease of ducks aged 5 to 30 days in which 64% of the affected ducks died. Saidov (1979) reported isolation of new hybrid variant of human influenza virus (A/duck/Tashkent/207/77).

Shortridge (1980) reported isolation of orthomyxovirus from domestic poultry in Hongkong between 1977 and 1979. He observed an overall frequency of isolation of influenza virus 10.3% and 2.3% for poultry originating from Southern China and Hongkong respectively. Out of the 141 influenza virus isolations, 135 were from ducks and the isolation rate in ducks was twice as high from cloaca (12.1%) as from trachea. The surface antigens of these isolates were in 39 different combinations of neuraminidase and haemagglutinin and were related to seven reference haemagglutinin and six neuraminidase subtypes.

Kocan et al. (1980) isolated nine type A influenza viruses from migratory and wintering ducks in Oklahoma

during 1976-77. These isolations were grouped into three (Hav1 Nav2; Hsw1 N1 and Hav6 N2) by subtyping. Proof for transmission of influenza virus from wild ducks to sentinel birds on the same lake could not be obtained by the authors. Between 1976 and 1979, 106 strains of Haemagglutinating viruses were isolated from cloacal swabs taken from wild ducks in the Bay of Somme River in France (Hannoun and Devaux, 1980). Most of these isolates were influenza viruses belonging to four haemagglutinin (Hav1, Hav6, Hav7 and Hsw1) types and five neuraminidase (N2, Nav2, Nav4, Nav5 and Neq2) types. On analysing the migrating habits of some of the species of birds the authors opined the annual exchanges of virus strains. From the high rate of virus isolation and the recovery of more than one subtype from a single specimen, it was thought that recombination had actually occurred under natural conditions.

Hinshaw et al. (1980a) during their survery of feral ducks for orthomyxovirus and Paramyxovirus from 1976 to 1978 in the Vermillion area of Alberta, Canada, have shown that influenza A viruses are present year after year in apparently healthy ducks. It was most frequently isolated from mallards, pintails and blue winged teals each year, though not restricted to these species. They have recorded 1262 isolates of influenza virus A from 4827 ducks, but the incidence was lower in ducks migrating through Tennesee. The virus isolates

belonged to 27 different combinations of H and N subtypes. It was also stated that the virus subtypes in ducks varied from year to year but six of the 27 subtypes were present every year. The predominent subtype changed from Hav7 Neq2 in 1976-77 to Hav6 N2 in 1978 indicating antigenic drift in avian influenza.

Hinshaw et al. (1980b) demonstrated genetic reassortment between influenza A viruses both in natural and experimental infections in ducks. They observed that seven per cent of the cloacal samples collected from Canadian feral ducks contained two or more antigenically distinguishable influenza virus indicating mixed infection.

Eighteen strains of avian influenza type A virus were isolated from cloacal swabs from water birds and song birds at four different sites in Hungary and tracheal and cloacal swabs from imported birds. Out of the 18 isolates, one was from a mallard and other 17 from birds imported from Senegal. Nine of these 18 subtypes corresponded to A/duck/England/62 five to duck/Eng/56 and one to A/duck/England/56 in HA and to chicken/Brescia/1902 in NA. Two others to duck/Eng/62 in HA and Quail/Italy/65 in NA (Stunzer et al;1980).

Influenza A virus antigenically related to swine influenza(Hsw1 N1) was reported to have isolated from adult mallard in Southern Germany (Ottis and Bahmann, 1980), which was serologically related to A/duck/Alberta/35/76.

During an investigation on respiratory diseases and high mortality on a commercial duck fattening farm between August 1979 and March 1980, ten influenza A viruses were isolated by Alexander et al. (1981). These isolates were characterized as Hav6 N2; Hav4 Nav1, Hav4 N1 and Hav4 Neq2 subtypes by HI and NI tests. It was also reported that all these isolates were nonpathogenic to six week-old chicken.

During a surveillance of ortho and paramyxoviruses of lower animals and birds to elucidate the natural history and ecology of influenza viruses, Smithka and Maassab (1981) reported isolation of an avian influenza A virus A/duck/ Michigan/77(Hsw1 Nav2) from 100 cloacal samples collected from migrating water fowls during 1977 hunting season in Michigan. Though the virus could be isolated from the respiratory and intestinal tract of experimentally infected chicks, the virus caused only subclinical infection in them.

From December 1979 to April 1980 migratory waterfowls of several species wintering in San-in District of Western Japan were surveyed for influenza A virus(Tsubokura et al; 1981). A total of 27 influenza A viruses of two subtypes Hav1 Neq1 (11) and Hav6 Neq3 (16) were isolated from 90 faecal samples from whistling swans. They had also isolated 13 similar viruses 11 of them with surface antigens Hav1 Neq1; and one Hav6 Nav5, from 245 faecal samples of black tailed

gulls. Two viruses of Hav1 Neq1 were from four tufted ducks. These authors also observed a correlation between sampling date, incidence, and antigenic subtypes of the virus.

Boudreault and Lecomte (1981) isolated 357 influenza viruses from 2293 cloacal samples collected from ducks and other wild birds. Seven H and six N subtypes in 18 different combinations were found and a comparative study on these isolates with the previous isolates indicated a change in subtype from year to year and place to place. These authors speculated that the large reservoir of influenza A virus circulating at the same time in ducks may be involved in the appearance of new subtypes in other species including man.

Markwell and Shortridge (1982) during their monthly examination for influenza viruses for a period of one year on two farms in Hongkong reported that the incidence was high of subtype H3N2 a virus antigenically related to pandemic Hongkong strain. They could isolate this virus monthly throughout the year from faeces or pond water or both indicating a cycle of water borne transmission. They have also reported isolation of the same subtype one to two years after last sampling thus indicating the persistence of the virus in the flock.

From 14 of the 278 migrating wild ducks in Hokkaido between 1978-80, 11 influenza A viruses were isolated

(Mikami et al; 1982). Seven of the 11 isolates had the surface antigen H10N3 and three of their isolation had no antigenic relationship to the 26 previously known strain of avian, swine, equine and human influenza virus.

Shandhu and Hinshow (1983) reported the isolation of 126 influenza A viruses of 11 different antigenic subtypes from two to five week-old ducklings and the water used by the ducks for drinking and swimming.

Sinnecker et al. (1983) isolated 351 influenza viruses from the trachea/cloaca of 3344 apparently healthy ducks, gulls, swans, terns and geese and from unconcentrated water. They were of 14 subtypes. The haemagglutinins were mainly related to avian HA-H4, followed by human H2 and swine Hsw1. The neuraminidases were identified as avian, equine and human types. The isolation rates were 10.7% in feral ducks, one per cent in other feral birds and 38% in Pekin ducks. The isolation ratio was a little higher from cloacal (75%) than from tracheal swabs (70%). It was also reported that 8.2% of the Pekins had dual infection.

Turek et al. (1983) reported isolation of eight influenza A viruses from 269 cloacal swabs from wild ducks shot at their autumn migration in Czechoslovakia during 1978-81. The most frequently isolated subtype was $H4^{N}6$. Repeated isolation of this subtype during their five year period of study suggested its permenant circulation in

wildlife. One of their isolates was identified as subtype (H3N8N6 HaV7 Neq2 Nav1) which is considered as a result of mixed infection with two subtypes with an identifical H but different N.

Halvorson et al. (1983) isolated a total of 213 influenza viruses and identified them to consist of 26 subtypes from ducks. The weekly influenza virus isolation rate varied between 0 to 24.4%. It was also noticed by the authors that the weekly infection rate of ducks at the monitoring sites was well correlated directly with the arrival of wild ducks at these sites.

Turek et al. (1984) investigated the possible circulation of influenza A virus in sentinel domestic ducks during October 1981 to May 1982 during which they isolated a strain of influenza A virus subtype H4N6.

Tracheal and cloacal swabs from apparently healthy mallards, gulls, shear waters and terns in Newzealand were screened by Austin and Hinshaw (1984) for the presence of influenza A virus. Seven influenza A viruses belonging to three antigenic subtypes H4N6; H1N3 and H11N3 were isolated from mallards. They considered importation of poultry and game birds as the source of infection in Newzealand.

Influenza type A virus which had the haemagglutinin (H4) of a subtype that had high pathogenicity for chickens in USA

was isolated from whistling swan in San-in District, Western Japan (Otsuki et al. 1984). On experimental infection of four week-old SPF chicken inoculated intraperitoneally it was found to be nonlethal for chicken.

Halvorson et al. (1985) studied the effect of season on the incidence of influenza A virus in sentinel ducks and domestic poultry in Minnesota. From their four year observation they concluded that the onset of infection among ducks was similar each year occurring in late July or early August. Though the incidence in turkeys was also seasonal, usually the onset was six to eight weeks after the detection of influenza in sentinel ducks. They attributed this to the increased waterfowl activity associated with fledging and congregation in the later summer and early fall presence of vectors, low temperature, cooler environmental temperature and prolonged reservoir through ground water contamination. They have also opined that ducks are not only a natural reservoir of influenza virus but also have a seasonal infection that appears to be related to the seasonal outbreak in domestic turkey, and in migrating waterfowls and in other species including humans.

Lu et al. (1985) reported isolation of influenza A virus, A/duck/Taiwan/72 (Hav6 N1) from a severe outbreak of respiratory disease in a farm with 800 ducks in the spring of 1972 in which 600 of two to four week-old ducklings

died. Because the farm was near a river where migratory waterfowls gather, these birds were suspected as the source of infection for domestic ducks.

Seroprevalence of influenza A virus in Indonesia had been studied in detail (Ronohardjo et al; 1985). Thirty eight per cent of the 410 domestic fowls, 18% of 656 domestic ducks, 19.8% of 101 cockatoos, 9.3% of 86 native fowls and 3.8% of 26 muscovy ducks were found to have antibodies to type A influenza virus.

Aini and Ibrahim (1986) isolated 20 haemagglutinating agents from 96 cloacal swabs taken at random from apparently healthy broiler ducks. Fourteen of them were identified as influenza A virus. Nine of this 14 had H3N6 as their surface antigens while the remaining H4N6 as their HA and NA. Influenza A virus subtype H4N6 was also isolated from two to five week-old mallard ducklings which were suffering from the upper respiratory tract infection and sinusitis. The affected birds were reported to show sneezing and conjunctivitis along with massive swelling of the infra orbital sinuses. The authors also reported that they could isolate duck hepatitis virus from these birds and the mortality was attributed to a mixed infection with this virus and influenza virus.

Fleury et al. (1986) reported an acute disease in ducks in two separate but closely situated farms. On one of the

farms the disease was seen in three week-old duckling and in the other in 13 week-old ducks. In both cases the clinical signs consisted of conjunctivitis, tracheitis and sinusitis. The subtype of influenza A viruses isolated was H11 N9. Experimental infection of newborn duck with this isolate did not induce any detectable clinical effects or histopathological changes. They have also isolated duck plague virus from some of the affected birds. Alexander and Gough (1986) reported a similar association of influenza virus and duck enteritis virus.

Hinshaw et al. (1986) isolated influenza A virus H5N2 from wild birds primarily ducks in the same geographical area where there was an earlier lethal H5N2 avian influenza outbreaks. There were 13 antigenic combinations of which only one belonged to H5N2 subtype. Though this isolate was antigenically related to the lethal H5N2, genetic and antigenic analysis revealed that it was quite distinct from the virulent H5N2. None of their isolates though capable of replicating in chicken produced any disease.

Alexander and Gough (1986) analysed the conditions underwhich influenza virus isolations were made in Great Britain during January 1982 to July 1985. Of the 12 avian influenza viruses with low virulence for chicken 10 were obtained during the periods of migration of wild birds. Six of them were from commercial ducks reared in Norfolk,

two from turkeys and the other two isolates from chicken. Six of the seven isolates from Norfolk were H4 subtype which was indicative of maintenance of the virus on commercial duck farms. The difference in the neuraminidase subtype in combination with the same H type (H4) suggested repeated introduction of the same H type by migratory waterfowls.

Cloacal swabs collected from 166 wild free flying ducks shot by hunters on the Pacific flyway on the Ishikari river in Central Hokkaidido revealed 10 haemagglutinating agents (Mikami et al; 1987). Four of the 10 agents were from each of the mallard and teal and the remaining two from Shoveller species. Six of the 10 were subtypes H3N8, H4N6 and H6N2 while the remaining four were paramyxovirus PMV-1 and PMV-4, two each.

Otsuki et al. (1987a) reported isolation of eight influenza A virus from 354 faecal samples of whistling swan from 1982 to March 1983. None of the faecal samples from 261 black tailed gulls, 113 pintails and 10 mallards were positive for virus isolation. Of the eight isolates from whistling swans five belonged to human pandemic subtype H2N2, two isolates to fowl plague subtype H7N7 and the remaining one to subtype H4N6. In a similar study during November 1983 to March 1984, Otsuki et al. (1987b) screened a total of 1052 faecal samples from 450 whistling swan, 362 pintails and 240 blacktailed gulls. These birds were

winter migratory birds flying from the USSR or Northern China and staying in Shimane and Tottori Perfectures in San-in District of West Japan. A total of 40 influenza virus A was isolated 11 from whistling swan, 28 from pintails and one from black tailed gulls. The isolates from whistling swan belonged to subtype H5N3 and H10N4, from pintails H2N2 and H10N4 and that of black tailed gulls to H13N6. From November 1980 to April 1981 only two strains - H13N1 and H11N6 subtypes were isolated from 465 faecal samples from pintails. All the 255 samples from whistling swan and 625 black tailed gulls were negative. During the winter of November 1981 to March 1982, 17 viruses were isolated from 1156 faecal samples. Fourteen of them were from 459 faecal samples of whistling swans (10 H4N3, 2 H1N6 and 2 H3N8). Two viruses H13N3 and H13N6 subtypes were isolated from 425 faecal samples from black tailed gulls. A strain belonging to H1N3 subtype was isolated from 30 faecal samples from mallards but all the 242 samples from pintails were negative (Otsuki et al. 1987c).

Otsuki et al. (1987d) reported that four strains of influenza A virus subtypes H7N7, H2N2 and H5N3 isolated from whistling swans and pintail ducks replicated in the respiratory tract of mouse and produced antibodies without causing clinical symptoms or gross lesions following intranasal infection.

e) Incidence in India.

Reports of influenza A virus isolations in India have been rare. Rao et al. (1979) isolated this virus from ducks in Tirunelveli area of Tamil Nadu. They reported that some of the ducks of the flock died suddenly about a week before their collection of specimen from the remaining birds. However, their isolation was from an apparently healthy bird. The isolate was designated as duck/India/78/1114 (Hav4N2). Manjunath and Mallick (1977) also reported isolation of influenza virus from birds in India.

Sulochana et al. (1987) isolated influenza A virus from two to six weekold ducklings during an outbreak of respiratory disease in Government Duck farm, Niranam, Kerala characterized by droopiness, malaise, occulonasal discharge, swollen eyelids and oedema of the head. Some birds also had paralysis. The mortality rate ranged between 15 to 20% though in one hatch and it mounted upto 80%. Of the 31 isolates during early 1985 outbreak 10 were subtyped and found to belong to subtypes H9N2(9) and H9N3(1). From a similar outbreak in the same age group of ducklings in the same farm isolations were also reported during early 1987. The subtypes identified during the 1987 outbreak were H3N2 and H9N7 (Sulochana, 1988. Personal communication).

2. Characterization

a) Thermostability.

Influenza A viruses are considered to be temperature sensitive. They get inactivated at 56°C for 30 minutes (Merchant and Packer, 1967; Papparella et al; 1969). Lang et al. (1968) reported that the infectivity and haemogglutinating activity of A/turkey/Ontario/6213/66 were destroyed rapidly by heating at 56°C but at lower temperatures the HA activity was more stable than infectivity. They have also found that their strain was relatively thermostable under laboratory conditions. Buxton and Fraser (1977) reported that influenza viruses are inactivated at a temperature of 55°C for one hour or in 10 minutes at 60°C. However, Homme and Easterday (1970) found that influenza A/turkey/Wisconsin/66 took six hours at 56°C for inactivation. According to Jawetz et al. (1981) infectivity of influenza virus is destroyed by heating at 50°C for a few minutes unless stabilized by 1M $Mgso_4$.

b) pH stability.

pH stability is another criteria used for grouping viruses. Lang et al. (1968) observed that the infectivity and haemagglutinin of A/turkey/Ontario/6213/66 was labile at low pH (pH 3.0) but were unaffected in the alkaline range of pH seven to eight. Similar observations were made by Jawetz et al. (1981). Influenza viruses were found to be

labile to pH 3.0 when held at room temperature for a period of one hour (Papparella et al. 1969; Buxton and Fraser, 1977) but were relatively stable at pH seven to eight (Buxton and Fraser, 1977). Webster et al. (1978) found that duck influenza viruses were more stable to low pH than human strain and thus can multiply in the intestinal tract.

c) Haemagglutination.

It was Hirst in 1941 who first showed that influenza virus can agglutinate chicken red cells. Later on it has been found that all strains of influenza virus agglutinate erythrocytes of chicken, guinea pigs, human and many other species. Haemagglutinating property of an influenza A virus isolated from ducks with respiratory disease was studied in detail by Frano and Kapitancik (1959). They have reported that their isolate agglutinated human, guinea pig, horse, dog, cattle, sheep, rabbit, cat, mouse, hedghog, ground squirrel, fowl, duck, goose and pigeon RBC at 4°C, 20°C and 37°C.

Safonov et al. (1964) have reported that fowl plague virus agglutinated erythrocytes of sheep, horse, cattle, man, fowl, pigeon, guinea pig, rat and mouse. Formina and Sokkar (1966) while studying the efficacy of various methods for differentiation of Newcastle disease virus and fowl plague virus have reported that the latter agglutinated red cells from guinea pig, horses, guinea fowls, fowls, ducks,

turkeys, white mice, sheep, horses, rabbits, cattle and dog but not pigeon.

d) Propagation in cell cultures.

Because of the extensive use of embryonated eggs, cell cultures have not been routinely used for the study of influenza viruses particularly avian influenza viruses.

Negroni and Tyrrell (1959) studied the morphological changes on tissue cultures of epithelial cells infected with influenza A virus. In cell cultures of calf kidney cells infected with various strains of influenza A virus the cells appeared rounded and vacuolated. They have also reported that the cytoplasm of the infected cells were disintegrated or deformed by the presence of bubbles followed by the fragmentation of nucleus and cytoplasm. The percentage of cells that were destroyed varied upon the concentration of the virus. In high multiplicity of infection many of the infected cells survived well, while in low concentration rounding and fall out of cells were such faster. Cytopathic changes were also rapid and extensive when serum was omitted from the medium and when the cultures were rolled and rocked. Degenerative changes such as granulation, vacuolation and disintegration of the cytoplasm of infected cells in which the nucleus shrink to become Pyknotic followed by complete disintegration of the cells was also reported by Jennings (1967). Cytopathic changes produced by avian

influenza virus A/turkey/Ontario/7732/65 in chicken fibroblasts have been described by Narayan et al. (1969). He could observe complete disintegration of cell layer within 24 hours. Increased granulation of the cytoplasm with rounding and opacity of cells due to avian influenza virus A/turkey/Ontario/6213/66 was reported by Rouse (1967).

Niven et al. (1962) and Campans and Dimmock (1969) reported that the cytopathic effects of influenza virus in culture consisted of rounding and eventual detachment of cells. While Kopp et al. (1968) and Compans and Dimmock (1969) observed dense cytoplasmic inclusions with periodic striations under electron microscope. Cytoplasmic inclusions which appeared to contain RNP have also been described by Ter Meulen and Love (1967).

Influenza type A virus was propagated in mallard duck tracheal organ cultures by Kocan et al. (1978). They have reported cytoplasmic vacuolation, nuclear swelling and sloughing of the epithelial cells following infection.

Gharagozlou and Samadiesh (1980) studied the pathogenicity of two avian influenza viruses T/California / Meleagriuza/64 (Hav6 Nav5) and T/California/5142/66 (Hav4 Nav2) in different cell cultures such as chicken embryo fibroblast, lamb embryo kidney and calf embryo kidney cells. Chicken embryo fibroblast cells were found to be the most suitable for both the viruses followed by lamb embryo kidney cells.

They have also observed a close correlation between the virus concentration in the inoculum and CPE in all cultures and T/Calif/5142/66 more cytopathic than T/Calif/Meleogriuza/66.

Rott (1985) used cell cultures-chicken embryofibrolasts-to differentiate pathogenic strain of avian influenza virus. from non pathogenic ones. He observed that non pathogenic strains produced HA in cell cultures only in the presence of trypsin. Similarly plaques were also produced only when trypsin was incorporated in the overlay medium.

3. Experimental infection

Moses et al. (1948) studied the pathogenicity of a fowlplague virus isolated from an outbreak of natural disease in chicken in Dutch East Indies. Following experimental infection the virus attained high titers in blood which persisted until death. During the period of clinical disease, the virus was recovered from the liver, spleen, kidney and brain. They have also evaluated the effect of various routes of inoculation of adult birds on the infectivity and lethality of the virus. The minimum lethal dose was essentially the same for all routes of inoculation (I/V, S/C, I/P, I/C). However, larger amounts of the virus possibly 10 times as much were required to produce disease by intradermal or intratracheal route and about 1000 times by instillation in to the conjunctiva. For effective

infection it was found to be necessary to introduce the virus beyond the outer epithelial barriers of the chicken and minimum lethal dose for chicken and chick embryos were the same.

Prokofeva et al. (1963) in their studies on experimental infection of ducks with influenza type A virus reported that the infected birds excreted the virus in the faeces for two weeks and had serum neutralizing and HI antibodies for four to six months. Similarly, ducklings from eggs laid by the infected birds also had specific HI antibodies upto 1:160 when they were two days of age which later on declined.

Belitzkii (1966) studied the aetiology of respiratory disease of ducklings by simultaneous inoculation of five to ten day-old ducklings with influenza virus and mycoplasma. He observed that when these two organisms were given simultaneously there was typical symptoms of respiratory disease with high mortality but the proportion of infected and dead birds reduced and the time taken for the appearance of the disease was also delayed when only one of the two agents was given.

Experimental infection of chicken with tern virus (A/tern/South Africa/61) and chicken virus (A/chicken/Scot/59) by Becker and Uys (1967) have shown that the former produced acute clinical disease with a higher mortality rate by intranasal, conjunctival or intramuscular infection. It was

also reported that in spite of the close antigenic relationship between these two subtypes they could differentiate the tern virus infection and chicken virus infection from the duration and symptoms of clinical disease the tissues of choice for virus isolation.

Narayan et al. (1969) reported that influenza virus - A/turkey/Ontario/7732/66 which was antigenically related to chicken/Scotland/59 and tern/South Africa/61 viruses was highly pathogenic for chicken and turkeys but did not produce any clinical disease to ducks, geese or pigeons.

Experimental infection of turkeys with A/turkey/ Wisconsin/66 have shown that both exposed and incontact birds excreted the virus for a period of 23 and 31 days respectively (Homme et al; 1969). It was also possible to isolate the virus routinely in the presence of circulating HI antibodies. A consistent correlation between cold stress and clinical disease was also observed by them. They found that the birds subjected to low ambient temperatures developed much more severe disease that became chronic with low antibody titers and shedding the virus for a long period. It was also reported that when the disease was inapparent, acute and self limiting, there was an early high level of HI antibodies, where in, the clinical symptoms were milder and the birds recovered rapidly.

Slemons and Easterday (1972) studied the host response

differences among turkeys, ducks, pheasants and pigeons to influenza A virus (Hav5N1). The pheasants and ducks were resistant to infection while turkeys manifested severe signs of disease that terminated fataly. Though considerable antibody response was noticed in pheasants and quails the response was poor in ducks. They have also noticed that the case and regularity of virus recovery varied between species. Very high HI antibody levels to influenza A virus in pheasants and quails with low levels in ducks and geese have also been reported by Easterday (1975).

Bahl and Pomeroy (1972) studied the Pathogenicity of a turkey influenza A virus (A/turkey/Minnesota/BF/72, Hav6 Neq2) by experimentaly infecting Franklin gulls (Larus pipixan) and mallards (Anas platyrhychos). Both the species did not show any clinical signs of the disease but tracheal shedding of the virus persisted for 24 days after infection in gulls and six days in mallards. Though HI antibodies were demonstrated in the inoculated gulls the levels were low and erratic in ducks.

Alexander et al. (1978) carried out a detailed study on the pathogenicity of four avian influenza virus for two week old fowls, turkeys and ducks by intranasal administration. The strains used were A/fowl/Germany/34 (Hav1N1) the Rostock; A/FPV1/Dutch/27(Hav1 Neq1)-Dutch; A/fowl/Victoria/75 (Hav1 Neq1)-Australian and A/parrot/Ulster/73 (Hav1 N1)-Ulster.

They have found that the Rostock, Dutch and Australian strains were pathogenic for fowls, turkeys and ducks. Though the clinical symptoms manifested in these three species were identical in most cases, ducks had milder inflammatory reactions. Paralysis was also noticed in them. The duration of illness due to these strains were also different. Infection with Rostock strain took only 24 to 48 hour to kill the birds while Dutch and Australian strains took 6 and 15 days respectively. Differences between these strains were also observed in the duration of virus excretion. Ulster strain was non pathogenic to all the three species of birds. However, virus could be isolated from them till the 22nd day following infection. They have observed that virulence of influenza A virus to any specific host has not related to the surface antigens of the virus as, of the four strains studied the most pathogenic was Rostock virus which had identical HA and NA antigens (HavlN1) of the completely avirulent ulster virus. Similar observations were made by Webster et al. (1976). Allan et al. (1977) while pathotyping 13 avian influenza virus isolates have opined that virulence may be the only reasonable criterion for assessing the seriousness of any influenza isolate. Alexander et al. (1978) considered extreme variations in results between species of birds, strain of virus and mode of infection as the difficulties encountered in assessing virulence of a

particular isolate.

Westbury et al.(1979) studied the pathogenicity of three Australian fowl plague viruses for chicken, turkeys and ducks. Strain FPV-1 and 2 were pathogenic for chicken and turkey as it induced clinical disease and mortality upto 25%. The morbidity and mortality in chicken and turkeys infected with these two strains were essentially the same. However, no clinical disease or death were recorded in ducks. Strain FPV-3 was apathogenic for all the three species. From the infected birds viruses were isolated from pharynx, cloace and heart blood. It was also noticed that transmission of the virus from exposed to the contact chicken, turkey and ducks and rate of transmission varied between species. Ducks were more readily infected than chicken and turkeys. They have also found that the virus excretion through cloaca persisted for a longer period exphasizing the importance of cloacal swabbing for virus isolation attempts particularly in birds that do not develop clinical disease. The serological response of chicken and turkeys to infection with the three strain was greater in chicken and turkeys than in ducks, some even failing to develop detectable levels of antibody until day 33 of infection when all the chicken and turkey developed significant titers within 21 days and persisted upto day 85. From the results obtained the authors emphasized the usefulness of serological screening of chicken and turkey as a

system of surveillance of influenza virus infection and opined that in ducks it must be combined with virus isolation.

White Pekin ducks were experimentally infected by various routes with five subtypes of influenza A virus. Of these, only influenza virus of duck (A/duck/Hokkaido/5/77 Hav7N2) and Budgerigar (A/budgerigar/Hokkaido/1/77-Hav4Nav1) origin replicated in the intestinal tract of ducks and shed the virus in high titers for a period of six to seven days and rarely up to 21 days in the faeces (Kida et al; 1980). However, there was no clinical signs of the disease and scarcely produced any detectable levels of serum antibodies. It was also reported that the exposed ducks became resistant to secondary infection but became susceptible from the 46th day onwards. This secondary infection was usually followed by rapid appearance of high titers of antibody.

Podchernyaeva et al. (1981) studied the pathogenicity of influenza virus and their recombinants for ducks by experimentally infecting one to two month-old ducklings intranasally with 10 avian and mammalian influenza viruses and nine recombinants. Though the infected birds were excreting the virus through cloaca none of them showed any clinical symptoms. They have also established contact transmission of the virus as there was an increase, though low,

in HI antibody titers. Similarly Shandhu and Hinshaw (1983) also reported that none of the ducklings that were experimentally infected with two different antigenic subtypes of influenza virus (Hav3Nav1 and Hav6Neq2) produced any clinical signs.

Differences were demonstrated in the transmissibility of two strains of avian influenza virus possessing the same surface antigens (Westbury et al; 1981). They found that A/duck/Victoria/76 (H7N6) spread quickly and infected all incontact chicken where as A/chicken/Victoria/75 (H7N6) spread slowly and failed to infect all the incontact chicken though both strains were isolated from the same locality about the same time.

Austin and Hinshaw (1984) infected ducks with two of their influenza virus isolates from mallards and reported virus excretion in their faeces for a period of 12 days following infection. They have also observed failure in reinfecting the ducks with the homologous (H4N6) strain but not with a different subtype (H11N3).

McNulty et al. (1985) inoculated turkey influenza virus A/turkey/Ireland/83 (H5N8) in to juvenile and adult turkeys, chicken and ducks and reported 100% mortality in chicken and turkeys. No clinical symptoms were observed in inoculated ducks, though they picked up infection as evidenced by the development of HI antibodies against the virus.

Forman et al. (1986) examined the ability of an influenza virus (H7N7) isolated from an outbreak of disease in chicken in Victoria, to cause disease in chicken, turkeys and ducks by experimental inoculation of five to six week-old broilers by intravenous and intranasal routes; and five week-old turkeys and ducklings by intravenous route. The virus was highly pathogenic to chicken and turkeys inducing 100% mortality with or without showing clinical symptoms such as oedema and cyanosis of skin of the head including comb and wattles and paralysis in some cases. However, none of the ducks showed signs of illness and cloacal samples taken three weeks after inoculation showed no evidence of virus. The HI antibody levels were only 1:20 even after four weeks of infection. Though transmission of infection occurred from inoculated chickens to those in direct contact, chicken seperated by a distance of three meter developed neither clinical disease nor antibody to the virus.

Alexander et al. (1986) assessed the pathogenicity of eight avian influenza virus of H5 subtypes for chicken, turkeys, ducks and quails. Clinical signs, death, virus excretion and immune responses were the parameters included in their study. Among the strains utilized A/chicken/Scotland/59 (H5N1); A/tern/South Africa /61 (H5N3); A/turkey/Ontario/7732/66 (H5N9); A/chicken/Pennsylvania/1370/83 (H5N2); A/turkey/Ireland/83 (H5N8) and A/duck/Ireland/113/84 (H5N8) were highly pathogenic for chickens and turkeys while

A/turkey/Italy/Za/80 (H5N2) and A/chicken/Pennsylvania/1/83 (H5N2) were of low pathogenicity. A/chicken/Scotland/59 was more pathogenic for chicken than turkeys while A/turkey/Ontario/7732/66 was more pathogenic for turkeys than chicken. Other strains showed little difference in their pathogenicity for these two hosts. None of the viruses were found to be pathogenic to ducks. Only A/duck/Ireland/84 and turkey/Ireland/83 produced consistent serological responses in ducks. However, intra-muscular infection of ducks with A/tern/South Africa/61 and turkey/Italy/80 produced HI antibodies in some birds. Only these strains could be reisolated from the ducks. Quails were comparatively resistant to strains which were highly pathogenic to chicken and turkeys. It was also observed that the rate of contact transmission varied considerably with both the hosts and the virus and various combinations of these.

Tashire et al. (1987) have found that when a strain of influenza A virus nonpathogenic to quails (A/turkey/Ontario/7732/66) was passaged several times in quails it produced a fatal generalized infection. This was thought to be due to a change in proteolytic clevability between the original and adapted viruses leading to a faster multiplication of the virus at the site of infection.

Materials and Methods

MATERIALS AND METHODS

1. Influenza virus

Influenza type A viruses, isolated by the Department of Microbiology during the first halves of 1985 and 1987 from ducklings stationed at Government Duck farm, Niranam and showing oculonasal discharge, swollen eyelids, oedema of head and respiratory distress were utilized for the present study. The isolates used for detailed investigation were A/duck/India/1/85 (H9N2); designated as CDN from 1985 outbreak, A/duck/India/2/87 (H3N?), A/duck/India/7/87 (H3N2) and A/duck/India/14/87 (H9N?). The latter three isolates were designated as DT3; T19 and lg1 respectively and were from the 1987 outbreak. These isolates were from the cloaca; throat swab; tracheal swab and lung tissue respectively. Isolate number one, two and four were from dead birds while T19 was from an ailing bird. All these isolates were maintained at -20°C in wet state at their 8th, 3rd, 4th and 4th passage levels respectively. On receipt they were passaged once in nine day embryonated eggs by the allantoic route of inoculation. The allantoic fluid collected from these infected embryos and distributed in small quantities in screw capped vials and stored at -20°C, were used throughout this study.

2. Hen eggs

Hatching eggs from white leghorn/Australwhite breeds

received from the University poultry farm were incubated in a bacteriological incubator at 38.5°C till the 9th day for allantoic route of inoculation. For preparation of chicken embryo fibroblasts they were incubated at this temperature till the 11th day.

3. Chicks

Day-old unvaccinated male chicks received from the University Poultry farm were used for assessing intracerebral pathogenicity index or reared till the 6th week for intravenous pathogenicity index.

4. Duck eggs

Duck eggs purchased from the local market and incubated for 10 days as in the case of chicken eggs were used for allantoic route of inoculation to study their susceptibility to the above isolates.

5. Ducklings

Ducklings (Desi/White Pekin) used for this study were received from the ICAR Duck Scheme, Mannuthy.

6. Medias and Buffers

a) Tryptose Phosphate Broth (TPB).

Ready-made media purchased from HI media, Bombay was reconstituted as per the manufacturers instructions and sterilized by autoclaving at 15 lbs pressure for 10 minutes.

Antibiotics such as Penicillin (500 IU/ml) and Streptomycin (500 µg/ml) were added to the medium before it was used for collection/dilutions of specimens for virus isolation (TPB-A).

b) Hanks Balanced Salt Solution 10X.

(i) Sodium chloride	80.0 gm
Potassium chloride	4.0 gm
$Na_2HPO_4 . 2H_2O$	0.6 gm
$MgSO_4 . 7H_2O$	2.0 gm
KH_2PO_4	0.6 gm
Glucose	10.0 gm

The ingredients were dissolved completely one by one in 800 ml of double glass distilled water.

(ii) $CaCl_2$	0.7 gm
Double glass distilled water	100 ml
(iii) $NaHCO_3$	3.5 gm
Double glass distilled water	100 ml

(iv) Phenol red - 0.4 gm of Phenol red (BDH) mixed with small quantity of N/20 NaOH to form a paste and then diluted to 150 ml using double glass distilled water. The pH was adjusted to seven with N/10 NaOH and finally made up the volume to 200 ml.

The solutions were autoclaved separately at 10 lbs pressure for 45 minutes when cool 100 ml of (iv) was added to (i) and the (ii) was added to the mixture to make 1000 ml

and stored at 4°C with one ml of chloroform.

c) Calcium Magnesium Free Phosphate Buffer (CMF-PBS) pH 7.5 10 X.

Sodium chloride	80.0 gm
Potassium chloride	2.0 gm
$Na_2HPO_4 . 2H_2O$	8.5 gm
KH_2PO_4	2.0 gm

All the components were dissolved one by one in 1000 ml double glass distilled water, distributed in 100 ml quantities and sterilized at 10 lbs pressure for 45 minutes cooled and stored at 4°C.

d) Trypsin.

A 0.2 per cent trypsin (1:250 Difco) was prepared in calcium Magnesium free buffer. Sterilized by filteration using seitz filter pads.

e) Antibiotic solution.

A mixture of Benzyl penicillin and streptomycin sulphate was prepared in sterile double glass distilled water. One ml of this solution when added to 100 ml of cell culture medium/buffer gave a final concentration of 200 IU of penicillin and 200 µg of streptomycin/ml. This mixture was stored at -20°C.

f) Citrate phosphate buffer pH 3.2.

The stock solutions A (0.1 M solution of citric acid)

and B (0.2 M solution of dibasic sodium phosphate) were prepared in double glass distilled water. Then 37.7 ml of stock solution A was mixed with 12.3 ml of solution B and diluted to make a total volume of 100 ml.

g) Phosphate buffer pH 7.2 and pH 9.0.

0.2 M solution of monobasic sodium phosphate (Solution A) and 0.2 M solution of dibasic sodium phosphate (Solution B) were prepared as stock solution. 87.7 ml of solution A was mixed with 12.3 ml of solution B and diluted to make a total volume of 200 ml for pH 7.2. For pH 9.0, 8.5 ml of phosphate buffered saline was mixed with 1.5 ml of 0.1 N sodium hydroxide. Both the buffers were sterilized at 15 lbs pressure for 15 minutes.

h) Tris-Barbiturate buffer pH 8.6.

Barbitone sodium	9.9 gm
Tris (hydroxy methyl amino methane)	17.7 gm
Sodium azide	0.3 gm
Distilled water	2000 ml

pH adjusted to 8.6 with 1N Hcl.

i) Cell culture growth medium.

Hank's balanced salt solution, 1X was supplemented with 0.5% lactalbumin hydrolysate and 0.15% yeast extract and five to seven per cent calf serum. Antibiotics at the rate of 200 IU of penicillin and 200 µg of streptomycin/ml were also

added. Before adding serum and antibiotics the medium was sterilized by autoclaving at 10 lbs pressure for 45 minutes.

j) Maintenance medium.

Same as above except that the serum concentration was reduced to one to two per cent.

7. Serum

Blood collected aseptically from cross bred bull calves was allowed to clot at room temperature in a slanting position and then transferred to 4°C for better separation of serum. Next day, serum was transferred into a clean sterile flask, filtered through seitz EK filter pads, inactivated at 56°C for 30 minutes on a water bath, checked for bacterial contamination and stored at -20°C until used.

8. Red blood cells for haemagglutination

a) Chicken RBC.

Blood from the wing vein of cockrels was collected in Alsever's solution. It was centrifuged at 1000 rpm for 10 minutes. The suspended RBCs were washed twice in normal saline and resuspended in fresh normal saline to get a 0.5% concentration for plate haemagglutination test. For rapid spot test a higher concentration (1 to 2%) of red cells was used.

b) Red cells from other species.

Blood from the jugular vein in the case of bovine, horse, goat and sheep; the earvein of rabbit. the heart of guinea pig; the inner canthus of eye in mouse and rat and the cubital vein of man was collected in Alsever's solution. The other steps were the same as in the case of preparation of chicken RBC.

9. Collection of specimens

a) Cloacal and throat swabs.

Cloacal and throat swabs were collected from 20 ducklings showing clinical symptoms similar to as reported during the 1985 and 1987 outbreak at the duck farm, Nirnam. These swabs were brought to the laboratory on the day of collection itself on ice and then soaked in TPB-A at the rate of one ml per swab. In the laboratory they were stored at -20°C until they were used for chick embryo inoculation.

b) Tissues.

Tissues such as liver, spleen, lung and brain from four dead birds were collected under aseptic conditions in to sterile vials. They were brought to the laboratory immediately and cultured on various bacteriological media and then soaked with TPB-A and stored at -20°C until used.

c) Serum for antibody titration.

Blood was collected into small test tubes from the

ischiatic vein of ducklings and allowed to clot at room temperature. Then it was transferred to 4°C for better separation of serum.

10. Processing of specimens

a) Cloacal and throat swabs.

Swabs soaked in TPB-A and stored at -20°C were allowed to thaw at room temperature. The swabs were then squeezed against the wall of the test tubes with sterile pipettes. Separate pipettes were used for each specimen to avoid cross contamination. The fluid expressed from the swab were centrifuged at 2000 rpm for 10 minutes. The clear supernatent fluid was separated and incubated at 37°C for one hour before it was inoculated in to the allantoic cavity of nine day embryonated chicken eggs.

b) Tissues.

At the time of use the tissues were emulsified in TPB-A with the help of a Tenbroeck tissue grinder and sterile silica gel to obtain a 10 to 15% (W/v) suspension. This tissue homogenate was then cleared, separated, incubated and used for chick embryo inoculation as in the case of cloacal and throat swabs.

11. Chick embryo inoculation

The viability of nine day-old embryos was checked and the aircell marked. The air cell region was sterilized with

tincture of iodine and a hole was made using a dental drill, about 0.5 to 1 cm from the margin of the aircell towards the centre.

One in ten (1/10) dilution of the virus was prepared in TPB-A and 0.2 ml was inoculated in to the allantoic cavity with a tuberculin syringe fitted with a 20 gauge needle. The hole was then sealed with paraffin and incubated at 37°C. Candling was done at every 24 hours and the embryos that died after 24 hours and those did not die after five days were chilled at 4°C to avoid contamination with red cells while harvesting.

12. Harvesting of allantoic fluid

The air cell of the embryonated eggs were disinfected with 70% alcohol. The air cell region was cut and removed with a sterile scissors. The shell membrane and chorio-allantoic membrane were removed and allantoic fluid was collected aseptically with sterile pipettes. The fluid from each embryo was tested for haemagglutinating activity, using 1 to 2% chicken RBC by spot agglutination test. The fluid which gave positive haemagglutination test was stored at -20°C in five ml quantities.

13. Characterization

a) <u>Identification of NP antigen</u>.

Preparation of Nucleoprotein (NP) antigen.

Nine day-old embryonated eggs were inoculated with the

four strains of influenza A virus mentioned above. The chorioallantoic membranes were collected 24 hours after infection. They were rinsed in Phosphate buffered saline (pH 7,2) drained to remove excess fluid and ground in a pestle and mortar with some sterile sand. It was frozen and thawed three times and centrifuged at 1000 rpm for 10 minutes. The supernatent formed the NP antigen.

1Preparation of antiserum to NP antigen.

Antiserum to one of the strain (CDN -A/duck/India/1/85-H9N2) was prepared in rabbit by subcutaneous inoculation with one ml of the antigen mixed with equal quantity of Freund's complete adjuvant. A second dose was given 10 days later but without adjuvant. Fifteen days following the second injection serum was collected from the rabbit and used to identify the NP antigen from other three strains.

2Immunodiffusion.

0.7% agarose was prepared in eight per cent sodium chloride buffered to pH 7.2. Three ml of this was poured on to precoated microscopic slides. After the agarose was set, wells having four mm. diameter were cut in a circular pattern about four mm. apart. The central well was filled with antiserum against NP antigen of strain CDN. While peripheral wells were filled with the various NP antigens. The slides were incubated in a humid chamber at room temperature and the results were read after 48 hours.

a) Immunoelectrophoresis.

Electrophoresis was performed in a Toshniwall electrophoresis chamber. Three ml of 0.8% Agarose in Trisbarbiturate buffer, pH 8.6 was poured on to microscopic slide. On settling of agarose, 3 mm diameter wells were cut slightly towards the cathode. After initial electrophoresis antiserum trough was cut in between the antigen wells and filled with NP antiserum. The antibodies in the serum will react with electrophoresed viral protein showing specific lines of precipitation.

b) Embryo Infective Dose 50 (EID 50).

Serial ten fold dilutions of the viruses were made in TPB-A. Each dilution was then inoculated at the rate of 0.2 ml each into nine day embryonated eggs using three eggs per dilution. The eggs were incubated at 37°C, candled daily and examined as described above. The allantoic fluid from each embryonated egg was tested for haemagglutinating activity. The embryo infective dose was calculated as per the method of Reed and Muench (1938).

c) Haemagglutination (Poultry Biologics, 1963).

Two fold dilutios of the viruses were made in normal saline in perspex haemagglutination plates. Equal quantity of 0.5% washed chicken RBC was added to each dilution. RBC control was prepared simultaneously. The plates were incubated at room temperature for 30 minutes. The readings were recorded after the red cells in the control wells had settled.

The haemagglutination tests using red cells from bovine, horse, goat, sheep, rabbit, guinea pig, mouse, rat and human were done as described above.

d) Thermostability.

Chickembryo propagated virus was distributed in one ml volumes into sterile screw capped vials and were submerged in a waterbath at 56°C. One vial each at various intervals of 5, 10 and 30 minutes were transferred to -20°C. The untreated and sample that was kept at 56°C for 30 minutes were assayed for infectivity as described above for EID 50.

The virus samples exposed to 56°C at various intervals were tested for its haemagglutinating activity and compared to that of the untreated sample.

e) pH Stability.

One in ten dilutions of the viruses were made in citrate phosphate buffer (pH 3.2) and phosphate buffer (pH 7.2) and 9.0 and kept at room temperature for one hour. After this period virus titrations were made in nine day-old embryonated chicken eggs, in 0.2 ml quantities using three eggs per dilution. The untreated samples were also titrated simultaneously. Post inoculation incubation, candling and harvesting were done as described earlier.

The viruses treated with citrate phosphate buffer (pH 3.2) and phosphate buffer (pH 7.2 and 9.0) were also tested for HA activity and they were compared to that of the untreated

sample.

f) Chloroform sensitivity (Feldman and Wang, 1961).

Two ml of the allantoic fluid from chick embryos inoculated with the various strains of influenza A virus was mixed with 0.1 ml of chloroform and kept at room temperature for 10 minutes with intermittent shaking. After this period it was centrifuged at 800 rpm for 10 minutes. The chloroform then appeared at the bottom of the tube, above this was an opaque interphase layer covered by the clear supernatent fluid. This supernatent fluid was removed and used for titration of HA activity and infectivity as described above.

g) Propagation in chicken embryo fibroblasts.

1) Chicken embryo fibroblast cultures (Cunningham, 1966).

Eleven day embryonated chicken eggs were selected. After cleaning the shell at the air cell region it was cut with a sterile scissors, the shell membrane and chorio allantoic membrane were torn and the embryo was lifted with a forceps and transferred into a sterile Petridish containing CMF-PBS with antibiotics. The head, limbs and visceral organs were removed and the embryos were transferred into large centrifuge tubes and chopped into small pieces. It was washed twice with CMF-PBS and transferred into a trypsinization flask and washed again in the CMF-PBS containing 0.2% trypsin. Fresh trypsin was added at the rate of 25 ml per embryo. Teflon coated magnetic stirring bar was added

and stirred on a magnetic stirrer for five minutes. The supernatent was poured off, and washed with trypsin. Fresh trypsin was added and then kept for trypsinization for 20 to 30 minutes. The resulting cell suspension was filtered though a double layered muslin cloth. The filterate was centrifuged at 800 rpm for 8 to 10 minutes, discarded the supernatent, resuspended the cells in growth medium and recentrifuged. Likewise washing of the cells was repeated thrice and finally resuspended in growth medium containing five to seven per cent serum to get a final concentration of 5×10^5 cell/ml.

The above cell suspension was seeded into test tubes at the rate of one ml per test tube, closed tightly with rubber stoppers and incubated at 37°C in slanting position. When a satisfactory monolayer was obtained (usually within 24 to 48 hours) it was used for studying the cytopathic effect.

2) Virus inoculation.

Tubes with satisfactory monolayers were selected, poured off the growth medium and the monolayer was washed with maintenance medium. To this a 1:10 dilution of the virus was added at the rate of 0.2 ml per tube and incubated at 37°C for one hour to facilitate adsorption. At the end of this period the inoculum was poured off, washed with maintenance medium and incubated at 37°C. Control tubes

were similarly treated except that instead of the inoculum, 0.2 ml of the maintenance medium was added. At twenty four hour interval the tubes were examined for CPE under an inverted microscope.

After six days of incubation or when the CPE was evident the monolayers were frozen at -20°C and the cells were disrupted by rapid thawing at 37°C. This rapid freezing and thawing was repeated for two more cycles and then centrifuged at 2000 rpm for 10 minutes. The HA activity and the infectivity of the supernatant was determined.

h) Pathotyping.

1) Mean death time at terminal dilution (MDT).

Serial ten fold dilutions upto dilution $10^{9.0}$ of the viruses were prepared in TPB-A. The last five dilutions were inoculated into nine-day embryonated eggs by allantoic route at the rate of 0.2 ml per embryo. Ten embryos were used for each dilution. The eggs were incubated at 37°C and candled at 12 hour intervals and the death time of the embryos were noted. The mean death time was calculated using the formula given in poultry biologics, 1963.

2) Intracerebral Pathogenicity Index (ICPI).

Ten, day-old chicks were inoculated intracerebrally with 0.05 ml of 1/10 dilution of viruses in normal saline. Five, day-old chicks were kept as control, after inoculating 0.05 ml of sterile normal saline intracerebrally. The

chicks were observed for 20 days for the development of disease or death. The neuropathic index was calculated by scoring factor zero for normal, one for diseased and two for dead. The sum total of all the factors was divided by the total number of observations.

3. Intravenous pathogenicity index (IVPI).

Eight, six week-old chicks were inoculated with 0.1 ml of 1/10 dilution of the embryo propagated viruses subcutaneously. They were observed for 15 days for the development of clinical signs, paralysis or death. The factors for each observation was zero for normal, one for clinical signs, two for paralysis and three for death.

14. Pathogenicity and Immunogenicity

Influenza A virus strains CDN (A/duck/India/1/85-H9N2) from 1985 outbreak and lg1 (A duck/India/14/87-H9N?) from 1987 outbreak were examined for their pathogenicity to ducklings of various age groups.

1. Experiment I.

A total of 25, day-old ducklings were used for this study. Twenty of them were divided into four groups of five each. Group A received $10^{8.5}$ EID 50 of the influenza virus strain CDN both by oral and cloacal route. Group B and C received the same dose of the virus by oculonasal

and subcutaneous routes respectively. Ducklings in group D served as uninfected control. To study the transmissibility of the virus five age matched uninfected ducklings were introduced in to group A, two hours following infection.

2. Experiment II.

The number of birds, their groupings, strain of the virus, routes and dose per bird were the same as in experiment I except that one week-old ducklings were used for this experiment.

3. Experiment III.

Same as in experiment I and II but two week-old ducklings were used to study the pathogenicity. The contact infection studies were carried out by keeping ducklings along with group C.

4. Experiment IV.

A total of 43 one-week-old ducklings were used for this study. Twenty eight of them were divided into four groups of seven each. Birds in group A infected with influenza A virus strain lg1 at the rate of $10^{8.0}$ EID 50/ bird by oral route. Group B and C were infected with the same dose of the virus by oculonasal and cloacal routes respectively. Birds in group D served as uninfected controls. Transmissibility of this strain was studied by

keeping five, age matched uninfected ducklings each, along with all the three group of infected ducklings.

In all the experiments, following infection different groups were kept separately and extreme care was taken to avoid cross infection.

They were observed twice daily for the development of clinical symptoms or death. Cloacal and throat swabs were collected at 2, 5, 7, 10, 14, 21 and 28 days of infection, for virus isolation as described previously. Sera from the birds in all the experiments were collected at weekly intervals till the 4th week and subjected to haemagglutination inhibition test for detection of antibodies.

5) Antibody titration.

Immune response to the above strains was detected by HI test using the corresponding virus as antigen.

Beta procedure of HI test was employed throughout the study. After ascertaining the HA titer of the virus, four HA units of the virus was prepared in normal saline. Serial double fold dilutions of the serum were prepared in normal saline. Each of these dilutions was mixed with 0.2 ml of four HA units of the virus and incubated at room temperature for 30 minutes. After this time 0.4 ml of 0.5% of suspension of washed chicken RBC was added to each well and mixed. Simultaneous virus and RBC controls were also set. The HI

antibody titer was taken as the highest dilution of the sera in which there was complete inhibition of HA.

Table 1. Influenza A virus strains used for the present study.

Sl.No.	Strain.	Antigenic subtype	Passage number	Source/ year of isolation	Infectivity titer.
1.	CDN A/duck/India/ 1/85	H9N2	8	Cloacal swab/1985	$10^{8.5}$/0.2 ml
2.	DT3, A/duck/ India/2/87	H3N?	3	Throat swab/1987	$10^{8.25}$/0.2 ml
3.	T19, A/duck/India/ 7/87	H3N2	4	Tracheal swab/1987	$10^{5.75}$/0.2 ml
4.	lg1, A/duck/India/ 14/87	H9N?	4	Lung tissue/1987	$10^{8.5}$/0.2 ml

Table 2. Treatments of day-old ducklings with influenza A virus strain CDN (A/duck/India/1/85-H9N2).

Group	Number of ducklings used	Virus strain	Route	Dose per bird
Group A	5	CDN	O + CS	$10^{8.5}$ EID 50
(Five uninfected ducklings were introduced 2 hours following infection for contact infection)				
Group B	5	CDN	ON	$10^{8.5}$ EID 50
Group C	5	CDN	SC	$10^{8.5}$ EID 50
Group D	5	..	Control	..

O+CS - Oral and cloacal swabbing
ON - Occulonasal
SC - Sub cutaneous

Table 3. Treatments of one week-old ducklings with influenza A virus strain CDN (A/duck/India/1/85 - H9N2).

Group	Number of ducklings used	Virus strain	Route	Dose/bird
Group A	5	CDN	O+CS	$10^{8.5}$ EID 50 per
(Five age matched ducklings kept for contact infection)				
Group B	5	CDN	ON	$10^{8.5}$ EID 50
Group C	5	CDN	SC	$10^{8.5}$ EID 50
Group D	5	..	Control	..

O+CS - Oral and cloacal swabbing
ON - Oculonasal
SC - Subcutaneous

Table 4. Treatments of two week-old ducklings with CDN (A/duck/India/1/85 - H9N2).

Group	Number of ducklings used	Virus strain	Route	Dose per bird
Group A	5	CDN	O+CS	$10^{8.5}$ EID 50
Group B	5	CDN	ON	$10^{8.5}$ EID 50
Group C	5	CDN	SC	$10^{8.5}$ EID 50
(Five age matched ducklings kept for contact infection)				
Group D	5	..	Control	..

O+CS - Oral and cloacal swabbing
ON - Oculonasal
SC - Subcutaneous

Table 5. Treatments of one week-old ducklings with influenza A virus strain lg1 (A/duck/India/14/87-H9N?).

Group	Number of ducklings used	Virus strain	Route	Dose per bird
Group A	7	lg1	O	$10^{8.0}$ EID 50
Group B	7	lg1	ON	$10^{8.0}$ EID 50
Group C	7	lg1	CS	$10^{8.0}$ EID 50
Group D	7	..	Control	..

To group A, B, C week old ducklings (five each) were introduced two hours following infection for contact infection.

O - Oral

ON - Oculonasal

CS - Cloacal swabbing

Results

RESULTS

1. Virus isolation

None of the cloacal and throat swabs (20 each) collected from ducklings showing respiratory disease during the early half of 1988 revealed any viral isolations by allantoic route of inoculation of nine day-old embryonated chicken eggs. The tissues collected from four of the dead birds were also negative.

On bacteriological examination though organisms such as Coliforms, Staphylococci and Streptococci were isolated from some of the samples, No further studies were carried out on these isolates.

2. Chick embryo propagation

Influenza virus strains CDN, DT3, T19 and lgl multiplied very well in nine day embryonated chicken eggs by allantoic route of inoculation, killing the embryos in two to four days time. The HA titers of the infected allantoic fluids were 1:64, 1:128, 1:64 and 1:256 respectively for CDN, DT3, T19 and lgl (Table 8). The infected embryos were usually congested. Some of them also showed petechial haemorrhages with cranial ecchymosis. No specific lesions were seen in the internal organs except that the liver was dark red in colour. The chorioallantoic membranes though appeared slightly oedematous and congested were free from any pock lesions.

Infectivity titers of the infected allantoic fluid of the four viruses were CDN - $10^{8.5}$ EID 50/0.2 ml, DT3 - $10^{8.25}$ EID 50/0.2ml; T19 - $10^{5.75}$ EID 50/0.2 ml and lg1 - $10^{8.5}$ EID 50/0.2 ml when calculated by the Reed and Muench (1938) method (Table 6).

3. Propagation in duck embryos

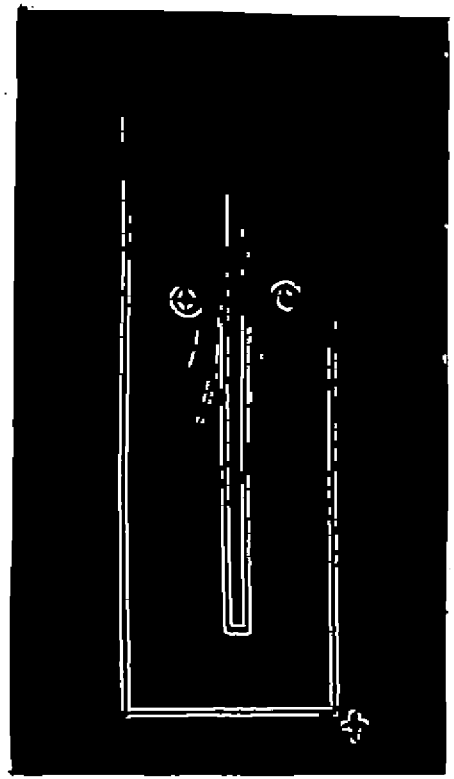
The four strains of influenza virus could be well propagated in 10 day embryonated duck eggs too by allantoic route of inoculation.

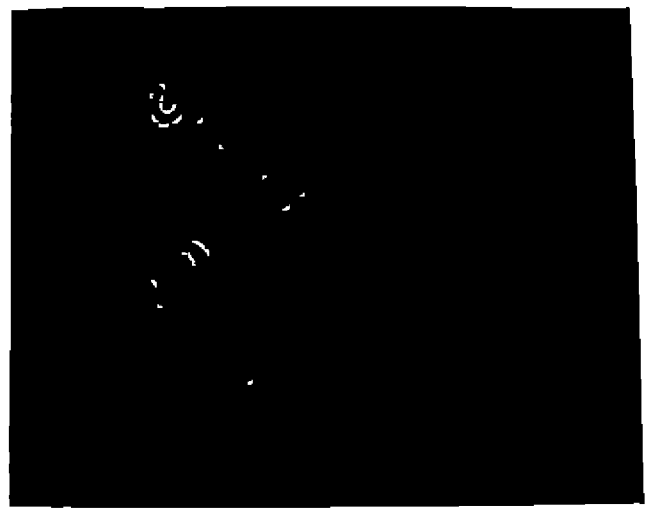
The virus killed the embryos in two to four days time as in the case of chicken eggs. There was no specific lesions either on the embryos, chorioallantoic membranes or in the internal organs. The HA titers of the allantoic fluids from infected duck embryos were 1:32, 1:64, 1:32 and 1:64 respectively for CDN, DT3, T19 and lg1; while the corresponding infectivity titers were $10^{7.5}$, $10^{7.25}$, $10^{5.0}$ and $10^{7.0}$ EID 50/0.2 ml (Table 7).

4. Identification of NP antigens

a) Agar gel precipitation test.

When antiserum to NP antigen of strain CDN, produced in rabbit and absorbed with normal CAM extract was diffused against NP antigens of CDN, DT2, T19 and lg1, a clear and thick and another faint Precipitin lines were formed against all the antigens. Both the lines of different strains joined each other indicating identify of the respective antigens- the NP antigens and MP antigens thus confirming that they all belong to the influenza virus type A (Fig. 1).





One additional line each was also seen against the homologous antigen CDN and strain lgl. This line was very faint and not well defined as in the previous case.

b) Immunoelectrophoresis.

Immunoelectrophoresis of NP antigen of the four isolates reproducibly gave a characteristic tripple line pattern as shown in Fig.2. The lines were seen near the point of application slightly towards the anode.

5. Thermostability

Data on thermoinactivation of the various strains are presented in table 6. It was observed that the infectivity of all the four strains were completely destroyed by heating at 56°C for 30 minutes. There was a gradual reduction in the HA titer when tested after five minutes and 10 minutes exposures followed by a complete loss in 30 minutes time.

6. pH Stability.

Results of stability of the viruses at various pH levels are shown in table 6. It is seen from this table that the infectivity and haemagglutinins were unaffected at pH 7.2. At pH 3.2 all the four strains lost their infectivity eventhough the HA property was not considerably affected except in the case of strain lgl where the HA titer was brought down to 1:16 from 1:256. At pH 9.0 there was marked reduction in HA activity and slight reduction in infectivity.

Table 6. Thermostability, pH stability and chloroform sensitivity of influenza A virus strains CDN, DT3, T19 and lg1.

Strain	Effect of temperature 56°C for 30 minutes				Effect of pH						Chloroform sensitivity	
	HA titer		Infectivity titer		pH 3.2		pH 7.2		pH 9.0			
	C	T	C	T	HA	infectivity	HA	infectivity	HA	infectivity	C	T
CDN(A/duck/India/1/85-H9N2)	64	0	$10^{8.5}$	0	32	0	64	$10^{9.5}$	32 (256)	$10^{6.5}$	$10^{8.5}$	0
DT3 (A/duck/India/1/87-H3N?)	64	0	$10^{8.25}$	0	16	0	64	$10^{9.5}$	4 (32)	$10^{6.5}$	$10^{9.25}$	0
T19 (A/duck/India/7/87- H3N2)	128	0	$10^{5.75}$	0	32	0	64	$10^{5.7}$	8 (64)	$10^{4.5}$	$10^{5.75}$	0
lg1 (A/duck/India/14/87 - H9N?)	512	0	$10^{8.5}$	0	16	0	256	$10^{9.5}$	8 (64)	$10^{5.75}$	$10^{9.0}$	0

C - Control
T - Treated

Table 7. Pathotyping of Influenza A virus strains.

Sl. No.	Strains	HA titer		Characteristics				
		Chicken embryo propagation	Duck embryo propagation	ICPI	IVPI	MDT	EID 50/0.2ml Chick embryo	EID 50/0.2ml Duck embryo
1.	CDN (A/duck/ India/1/85-H9N2)	64	32	0.325	0	78 hours	$10^{8.5}$	$10^{7.5}$
2	DT3 (A/duck/ India/1/87-H3N?)	128	64	0.66	0	76.8 ,,	$10^{8.25}$	$10^{7.25}$
3.	T19 (A/duck/ India/7/87-H3N2)	64	32	0.00	0	72 hours	$10^{5.75}$	$10^{5.0}$
4.	lg1 (A/duck/ India/14/87-H9N?)	256	64	0.163	0	76 hours	$10^{8.5}$	$10^{7.0}$

7. Haemagglutinating property

All the four strains agglutinated human O, A and B, cattle, sheep, goat, rat, mouse, horse, rabbit, guinea pig, and chick red blood cells.

8. Chloroform sensitivity

All the four strains of influenza A virus studied were sensitive to chloroform when treated at five per cent level for 10 minutes at room temperature (table 6) indicating that these viruses are enveloped.

9. Propagation in chicken embryo fibroblasts

Primary chicken embryo fibroblasts were grown in Hank's balanced salt solution supplemented with 0.5% lactalbumin hydrolysate, 0.15% yeast extract and five to seven per cent calf serum. Satisfactory monolayers were formed in 24 to 48 hours. Morphologically the cells were more or less spindle shaped and in areas of high density were oriented with their long axis parallel to one another. It was also noticed that when the cell density was high the monolayer pealed off very easily and were unsuitable for virus inoculation.

Infection with the four strains of the influenza A virus did not produce any marked morphological changes of the cells even after six days of incubation. However, monolayers infected

with strain CDN produced rounding of cells randomly in 48 hours which further extended throughout the monolayer by 96 hours. Cytopathic changes were minimal even after three passages of the other strains.

The cell culture fluids from the infected monolayers collected after repeated freezing and thawing were positive for haemagglutination test and when inoculated into nine day embryonated eggs, embryo mortality was noticed indicating virus multiplication. The infectivity titer of the cell culture fluid was also low for all the strains. It was $10^{6.5}$, $10^{5.5}$, $10^{4.0}$ and $10^{5.5}$ EID 50 /0.2 ml for CDN, DT3, T19 and lg1 respectively. On further passage in CEF cultures the titer of CDN increased to $10^{7.75}$ EID 50/02 ml.

10. Pathotyping

a) Mean death time.

The mean death time for strains CDN, DT3, T19 and lg1 were 78 hours, 76.8 hours, 72 hours and 76 hours respectively (Table 7).

b) Intracerebral Pathogenicity Index.

Intracerebral pathogenicity index was studied by inoculating 10, day-old chicks with 0.05 ml of 1/10 dilution of each strain. The results obtained are presented in table 7.

Day-old chicks inoculated intracerebrally with influenza A virus strain CDN were normal during the 1st day. On the 2nd day

two chicks showed clinical signs and were dead by the 3rd day. All the remaining eight chicks were normal throught the period of observation. The ICPI was calculated to be 0.325.

Three chicks inoculated with strain DT3 were dead on the 1st day itself followed by another one on the subsequent day. The remaining seven chicks were normal till the 6th day when one chick became sick and started showing clinical signs and died on the 7th day. All the other chicks remained normal till the end of the observation period of 20 days. The ICPI calculated for this strain was 0.66.

All chicks inoculated with strain T19 remained apparently normal throughout the period of observation and thus the ICPI was 0.00.

Chicks that received strain lgl were normal during the 1st day. One chick became sick with clinical signs of drowsiness, weakness and was in sleeping posture. Another bird was also found dead on the 3rd day. All other remained normal till the end of the observation period and the ICPI was calculated to be 0.163 (Table 7).

c) Intravenous Pathogenicity Index.

This was studied in six week-old chicks. None of the chicks inoculated with the above strains showed any clinical symptoms or death and appeared normal till the end of the

observation period. However, HI antibody response was noticed in chicks receiving all the four isolates indicating virus infections. The IVPI was calculated to be zero (Table 7).

11. Experiment I

Day-old ducklings infected with strain CDN by oral route did not show any specific clinical symptoms. Virus isolations were made regularly from 5th day onwards till the 10th day. The isolation rate was 50% on the 5th, 33.33% on 7th and 16.67% on 10th day. Two of the birds died suddenly on the 4th day. Specific lesions were not seen on postmortem examination. However, virus could be isolated from the tissues of both the birds. On the 12th day two more birds died with signs of droopiness and discharge from the eyes. Petechial haemorrhages were noticed in the internal organs. The bird that died on the 13th day also showed the same clinical signs. Tissues from all the three birds revealed incluenza A virus (Table 8). The pathogenicity index was calculated to be 1.19 (Table 20).

The ducklings that were kept along with this infected group to study the rate of transmission remained negative for virus excretion till the 4th day. On the 5th day tracheal samples from one of the five birds was positive for the virus. Tracheal excretion of the virus continued till the 10th day while in one of them cloacal excretion was evidenced on the 12th day also. The incontact ducklings died at different

periods - one each on 7th, 10th, 12th, 13th and 14th day. None of these birds revealed any clinical symptoms. However, virus isolations were made from the tissues of the birds died on 7th, 12th and 13th day, but not from the one each died on the 10th and 14th day.

Birds in group B picked up infection as virus could be isolated from the 3rd day of infection onwards. One bird was found dead on the 3rd day of infection. Droopiness was the only clinical symptom shown by this bird. Though post mortem could not reveal any specific lesions virus could be isolated from the tissues. The remaining birds died on the 4th, 8th, 10th and 11th day. They were showing droopiness, diarrhoea, discharges from the eyes and ruffled feathers. Except from the one which died on 11th day, virus could be isolated from all these birds. However, cloacal and throat samples collected from all these ducklings before their death did not reveal the presence of the virus (Table 9). Pathogenicity index was found to be 1.15 (Table 20).

In group C sudden death without any symptoms or lesions was observed in two birds on the 4th day. The remaining birds died on the 5th (1 no), 7th day (1 no) and 9th day (1 no) of infection. These ducklings were showing symptoms such as respiratory distress, nasal and eye discharge and were in sleeping posture. Virus isolation were made from all the birds except from the one which died on 9th day. Postmortem

Table 8. Experimental infection of day-old ducklings with CDN (A/duck/India/1/85-H9N2) by oral route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No.examined.	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	ND
	5*	0/5	0/5	..	..	..	0/10	ND
3	5	0/5	0/5	..	..	..	0/10	ND
	5*	0/5	0/5	..	..	..	0/10	ND
4	..	..	..	2/2	No specific symptoms/ lesions	2 birds died	2/2	ND
	..							
5	3	2/3	1/3	..	..	..	3/6	ND
	5*	0/5	1/5	..	..	..	1/10	ND
7	3	2/3	0/3	..	..	..	2/6	0
	5*	0/5	1/5	1/1	No specific symptoms	1	2/11	0
10	3	1/3	0/3	..	..	..	1/6	ND
	4*	1/4	1/4	0/1	No specific symptoms	1	2/9	ND
12	..	..	..	2/2	Discharge from eyes, droopiness	2	2/2	ND
	3*	1/3	0/3	1/1	..	1	2/7	ND
13	..	..	..	1/1	Discharge from eyes droopiness	1	1/1	ND
	..	..	..	1/1	..	1	1/1	ND
14	..							
	1*	..	..	0/1	No specific symptoms	1	0/1	ND

* contact birds CS - Cloacal swab TS - Throat swab ND - Not done

Table 9. Experimental infection of day-old ducklings with CDN by occulonasal route.

Time of collection/ observation (in days)	No. of birds screened.	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No. examined.	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	ND
3	5	0/5	0/5	1/1	Droopiness	1	1/11	ND
4	..	..	..	1/1	Slight diarrhoea and ruffled feathers	1	1/1	ND
5	3	0/3	0/3	..	..	..	0/6	ND
8	..	..	..	1/1	Droopiness diarrhoea and discharge from the eyes	1	1/1	0
10	..	..	..	1/1	Same as above	1	1/1	ND
11	..	..	..	0/1	No specific clinical symptoms	1	0/1	ND

CS - Cloacal swab TS - Throat swab ND - Not done

Table 10. Experimental infection of day-old ducklings with CDN by subcutaneous route

Time of collection/ observation in days	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation /total No. examined	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	ND
3	5	2/5	1/5	..	..	..	3/10	ND
4	..	..	..	2/2	Sudden death No specific symptoms were shown	2	2/2	ND
5	3	0/3	1/3	1/1	Respiratory distress, nasal and eye discharge, sleeping posture	1	1/7	ND
7	2	1/2	1/2	1/1	Same as above	1	2/5	0
9	..	..	..	..	Died suddenly, No specific symptoms were shown	1	0/1	ND

CS - Cloacal swab TS - Throat swab ND - Not done

lesions revealed by these birds were minimal (Table 10). Pathogenicity index was 1.51 (Table 20).

None of the birds in all the four group that survived even beyond seven day of infection did reveal any specific HI antibodies.

12. Experiment II

One week-old duckling infected with influenza A virus strain CDN by oral route did not show any clinical symptoms or death till the 4th day. One of the five ducklings died on the 4th day of infection and was showing droopiness, ruffled feathers and slight diarrhoea. Postmortem examination did not show much of the lesions except mild petechiae of the proventriculus. The virus could be isolated from the tissues of this bird. All the remaining four birds were apparently healthy throughout the observation period. Virus isolations from the tracheal and cloacal samples collected from these birds are shown in table 11. The virus recovery from these two samples was made at regular intervals from day 3 to 14. On the 3rd and 5th day, the isolation rate was 100% from both cloacal and tracheal samples. However, the number of isolations decreased thereafter as isolations could be made only from 50% of the cloacal swabs by day seven. Cloacal shedding of the virus continued till the 14th day and all the birds were negative by the 21st day. Isolations from the tracheal samples could be made only for a short period up to

Table 11. Experimental infection of one week-old ducklings with influenza A virus strain CDN (A/duck/India/1/85-H9N2) by oral route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms.	Mortality	Total No. of isolation/total No.examined	HI antibody titer range
		CS	TS	Tissues (pooled)				
0	5	0/5	0/5	..	..	..	0/10	0
	5*	0/5	0/5	..	..	..	0/10	0
3	5	5/5	5/5	+	..	one bird died on 4th day with droopiness, ruffled feathers diarrhoea	10/10	ND
	5*	5/5	3/5	..	..	..	8/10	ND
5	4	4/4	4/4	..	..	..	8/8	ND
	5*	5/5	0/5	..	..	..	5/10	ND
7	4	2/4	0/4	..	..	..	2/8	1:4-1:8
	5*	3/5	0/5	..	..	..	3/10	1:4-1:8
10	4	1/4	0/4	..	..	..	1/8	ND
	5*	0/5	0/5	..	..	..	0/10	ND
14	4	2/4	0/4	..	..	..	2/8	1:16-1:64
	5*	0/5	0/5	..	..	..	0/10	1:64-
21	4	0/4	0/4	..	..	..	0/8	1:4 -1:8
	5*	0/5	0/5	..	..	..	0/10	1:16
28	4	0/4	0/4	..	..	..	0/8	1:4-1:8
	5*	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

* Contact birds

the 5th day and all the samples were negative thereafter. Pathogenicity index was 0.36 (Table 20).

All the pre inoculation serum samples were negative for HI antibodies. In infected birds, though low an antibody response was noticed from the 7th day onwards and the range was 1:4 to 1:8. An increase in titer ranging between 1:6 to 1:64 was noticed by 14th day. But by the 21st day the antibody titer showed a marked reduction and remained steady till the 28th day (Table 11).

The virus spread from the orally infected ducklings to the incontact birds was evidenced by the virus isolation from these birds. However, neither clinical symptoms nor mortality could be observed. Virus could be regularly isolated between day three and seven. The rate of isolation was 80% on day three which was reduced to 50% by the 5th day and only 37.5% on the 7th day. Positive isolation could be made from the tracheal samples only on the 3rd day, though cloacal isolations were possible till the 7th day. Afterwards virus isolation attempts were not successful. Like the infected ones specific HI antibodies could be demonstrated from day seven onwards. The titer gradually increased and peak titers were obtained by the 14th day followed by a gradual decline.

Birds infected by the occulonasal route showed no clinical signs or death throughout the observation period. But virus

Table 12. Experimental infection of one week-old ducklings with influenza A virus strain CDN (A/duck/India/1/85-H9N2) by oculonasal route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No. examined	HI antibody titer range
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	0
3	5	3/5	4/5	..	..	..	7/10	ND
5	5	3/5	1/5	..	..	..	4/10	ND
7	5	0/5	1/5	..	..	..	1/10	1:4 - 1:8
10	5	0/5	0/5	..	..	..	0/10	ND
14	5	0/5	0/5	..	..	..	0/10	1:32-1:64
21	5	0/5	0/5	..	..	..	0/10	1:8 -1:16
28	5	0/5	0/5	..	..	..	0/10	1:8

CS - Cloacal swab TS - Throat swab ND - Not done

Table 13. Experimental infection of one week-old ducklings with influenza A virus strain CDN (A/duck/India/1/85-H9N2) by subcutaneous route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No.examined	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	0
3	5	3/5	3/5	..	..	..	6/10	ND
5	5	3/5	5/5	..	..	..	8/10	ND
7	5	5/5	5/5	+	..	one bird died.	10/10	1:16
10	4	2/4	0/4	..	..	..	2/8	ND
14	4	0/4	0/4	..	..	..	0/8	1:32
21	4	0/4	0/4	..	..	..	0/8	1:8
28	4	0/4	0/4	..	..	..	0/8	0

CS - Cloacal swab TS - Throat swab ND - Not done

could be isolated between day three and seven following infection. The isolation rates were 70 % on the 3rd day; 40% on 5th day and 10% on 7th day. Specific HI antibodies were first observed on day seven with a range of 1:4 to 1:8. By the second week on increase in HI titer could be observed (1:32 to 1:64). This was followed by a decline in the titer and it was only 1:8 by the end of the 4th week (Table 12). Pathogenicity index was 0 (Table 20).

From the subcutaneously infected week-old ducklings virus isolations were made from the 3rd to 10th day. The isolation rate increased from 3rd to 7th day and 100% isolations could be made on the 7th day. Cloacal and tracheal samples collected on day 14, 21 and 28 were negative. One bird that died on the 7th day with signs of respiratory distress, nasal discharges and rales had petechial haemorrhages in the proventriculus and myocardium. Similar changes were also noticed in the subcutis. Virus was isolated from the tissues of this bird. Pathogenicity index was 0.27 (Table 20).

The HI antibody titer of pooled serum was 1:16; 1:32 and 1:8 respectively for day 7, 14 and 21. It was absent on day 28 (Table 13).

13. Experiment III

Experimental infection of two week-old ducklings with influenza A virus strain CDN by oral route did not show any clinical symptoms or mortality throughout post inoculation observation period of 28 days. Virus isolations were made from the cloacal samples between 3rd to the 7th day of infection. However, the isolation rate decreased from 60% on the 3rd day to 20% on the 7th day. Tracheal isolations were possible only on day five and seven and isolation rate was low compared to cloacal samples. It was 40% and 20% respectively for day three and five. The antibody response was low compared to the week-old ones that received the virus by the same route, only 1:8 on the 7th day 1:4 on 14th and 21st day and absent by day 28 (Table 14).

Ducklings in group B that were infected with the virus by occulonasal route behaved the same way as in the case of group A. Cloacal sample from three of the five ducklings were positive for the virus on day five and seven. Tracheal isolations were possible from the 3rd day onwards till the 7th day. Later on the birds were found to be negative. Sera collected and pooled from the birds in this group had titers 1:16; 1:8 and 1:8 on day 7, 14 and 21 respectively. No antibodies could be detected on the 28th day (Table 15).

The results of infection of birds in group C are presented in table 16. It is seen from the table that none of

Table 14. Experimental infection of two week-old ducklings with CDN by oral route

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No. examined	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	0
3	5	3/5	2/5	..	..	..	5/10	ND
5	5	4/5	1/5	..	..	..	5/10	ND
7	5	1/5	0/5	..	..	..	1/10	1:8
10	5	0/5	0/5	..	..	..	0/10	ND
14	5	0/5	0/5	..	..	..	0/10	1:4
21	5	0/5	0/5	..	..	..	0/10	1:4
28	5	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

Table 15. Experimental infection of two week-old ducklings with CDN by oculonasal route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No. examined	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	0
3	5	0/5	2/5	..	..	..	2/10	ND
5	5	3/5	0/5	..	..	..	3/10	ND
7	5	2/5	2/5	..	..	..	4/10	1:16
10	5	0/5	0/5	..	..	..	0/10	ND
14	5	0/5	0/5	..	..	..	0/10	1:8
21	5	0/5	0/5	..	..	..	0/10	1:8
28	5	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

Table 16. Two week-old ducklings with CDN by subcutaneous route

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No.examined	HI antibody titer
		CS	TS	Tissues				
0	5	0/5	0/5	..	..	..	0/10	0
	5*	0/5	0/5	..	..	..	0/10	0
3	5	1/5	4/5	..	..	..	5/10	ND
	5*	3/5	0/5	..	..	..	3/10	ND
5	5	2/5	1/5	..	..	..	3/10	ND
	5*	1/5	1/5	..	..	..	2/10	ND
7	5	0/5	3/5	..	..	..	3/10	1:4-1:16
	5*	1/5	1/5	..	..	..	2/10	1:4
10	5	0/5	0/5	..	..	..	0/10	ND
	5*	0/5	0/5	..	..	..	0/10	ND
14	5	0/5	0/5	..	..	..	0/10	1:4 - 1:8
	5*	0/5	0/5	..	..	..	0/10	1:4
21	5	0/5	0/5	..	..	..	0/10	0
	5*	0/5	0/5	..	..	..	0/10	0
28	5	0/5	0/5	..	..	..	0/10	0
	5*	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

* Contact birds

the birds showed either clinical symptoms or died of infection. However, virus could be isolated from the cloacal samplings from the third to the 5th day. Tracheal samplings gave positive isolations upto the 7th day and thereafter both tracheal and cloacal samples were negative. The immune response of the birds to the virus was very low. HI antibody titers were very low between 1:4 to 1:16 on 7th day which declined by the 2nd week (1:4 to 1:8) and became undetectable by the 3rd week onwards.

None of the ducklings that were kept along with these infected birds to study transmissibility of the virus did showed any clinical symptoms or mortality. However, contact infection was evidenced from the positive virus isolation from the cloacal sampling from day three to day seven and from tracheal sampling from day five and seven. None of the incontact birds were positive after this period. Antibody response was also very poor as the HI antibody titers never increased more than 1:4.

14. Experiment IV

One week-old ducklings infected with influenza A virus strain lg1 (H9N?) either by oral, occulonasal, and cloacal routes did not show any clinical disease and remained apparently normal throughout the period of observation. However, virus isolation were made from the cloacal as well as tracheal samples from day three and seven. None of the samples collected after this period showed the presence of virus. The HI antibody titers in group A were also low compared to the age matched ducklings infected with strain CDN and the titers were 1:4; 1:8 and 1:4 respectively on day 7, 14 and 21 and were negative on day 29. HI antibodies could not be demonstrated in group B and C (Table 17, 18 and 19).

None of the incontact birds kept along with group A, B and C either had clinical symptoms or any indication of infection as none of the samples collected from them gave positive virus isolations. In addition, the sera from these birds were also negative for HI antibodies throughout the period of observation.

Table 17. Experimental infection of one week-old ducklings with lg1 (A/duck/India/14/87-H9N7) by oral route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No. examined	HI antibody titer
		CS	TS	Tissues				
0	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
3	7	2/7	1/7	..	..	..	3/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
5	7	4/7	3/7	..	..	..	7/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
7	7	0/7	2/7	..	..	..	2/14	1:4
	5*	0/5	0/5	..	..	..	0/10	0
10	7	0/7	0/7	..	..	..	0/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
14	7	0/7	0/7	..	..	..	0/14	1:8
	5*	0/5	0/5	..	..	..	0/10	0
21	7	0/7	0/7	..	..	..	0/14	1:4
	5*	0/5	0/5	..	..	..	0/10	0
28	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

* contact birds

Table 18. Experimental infection of one week-old ducklings with lg1 (A/duck/India/14/87-H9N?) by occulonasal route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No.examined	HI antibody titer
		CS	TS	Tissues				
0	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
3	7	3/7	2/7	..	..	..	5/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
5	7	2/7	1/7	..	..	..	3/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
7	7	1/7	0/7	..	..	..	1/14	0
	5*	0/5	0/5	..	..	..	0/10	0
10	7	0/5	0/5	..	..	..	0/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
14	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
21	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
28	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

* Contact birds

Table 19. Experimental infection of one week old ducklings with lg1 (A/duck/India/14/87-H9N?) by cloacal route.

Time of collection/ observation (in days)	No. of birds screened	Virus isolation			Clinical symptoms	Mortality	Total No. of isolation/total No.examined	HI antibody titer
		CS	TS	Tissues				
0	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
3	7	2/7	0/7	..	..	..	2/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
5	7	2/7	0/7	..	..	..	2/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
7	7	1/7	0/7	..	..	..	1/14	0
	5*	0/5	0/5	..	..	..	0/10	0
10	7	0/7	0/7	..	..	..	0/14	ND
	5*	0/5	0/5	..	..	..	0/10	ND
14	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
21	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0
28	7	0/7	0/7	..	..	..	0/14	0
	5*	0/5	0/5	..	..	..	0/10	0

CS - Cloacal swab TS - Throat swab ND - Not done

* contact birds

Table 20. Pathogenicity indices in various routes of infection.

Experiment	Strains	Oral	Occulonasal	subcutaneous	cloacal
I	CDN	1.19	1.15	1.51	..
II	CDN	0.36	0	0.27	..
III	CDN	0	0	0	..
IV	lg1	0	0	0	0

Discussion

DISCUSSION

Influenza A virus has been isolated from domestic ducks as early as 1953 in Canada (Walker and Bannister, 1953) and 1956 in Czechoslovakia and England (Koppel et al; 1956). Since then large number of influenza A viruses have been isolated from domestic ducks in other countries. Though ducks are generally regarded as refractory to influenza virus many isolations are reported from apparently healthy birds. Hence, they can be a source and possible reservoir of avain influenza (Lang, 1982; Alexander, 1982b; Lipkind et al; 1982). With the increasing importance of the duck industry their role in disseminating the infections to other avian species is of interest and it is supposed that they are the origin of the new pandemic human influenza virus (Profeta and Palladino, 1986).

1. Virus isolation

Influenza virus could not be isolated from the cloacal and throat samplings from 20 ducklings between the age group of two to six weeks suffering from respiratory disease. Sulochana et al. (1987) and Sulochana (1988) could isolate influenza A virus from 31 out of 88 and 17 out of 54 samples respectively during 1985 and 1987 outbreaks. These isolations were from the same age group of ducklings and from the same farm and manifesting similar respiratory disease as reported

during 1988 outbreak from which the present specimens were collected. Successful isolation of the causative agent depend on various factors. Lang et al. (1972) reported that during their studies on an outbreak of influenza virus infection in turkeys they could isolate only a single strain even after several attempts. Moreover, during the present study the specimens as were not collected repeatedly at intervals during the outbreak; it was done in 1985 and 1987. This is quite important as infected ducks excrete the virus generally for a short period of six to eight days as shown by experimental infection studies (Alexander et al; 1978). The time of collection of the specimen was also not ideal for virus isolation. The information about the outbreak of the disease was received too late and by the time the samples were taken, two weeks have elapsed. By this time the virus shedding might have decreased considerably. Another possibility is the cyclical emergence of the virus as indicated by the failture in isolating the virus during 1986 (Sulochana, 1987). Moreover, slightly deviating from the procedure of Sulochana et al. (1987) and Sulochana (1988), the samples were passaged only once in chickembryos using a single embryo per sample. According to Sulochana et al. (1987) a sample can be considered negative only after three blind passages. Fazekas de StGrowth and White (1958) opined that selection of chickembryos for influenza virus isolation is quite important as it has got the disadvantage of being

nonhomogenous and embryos with an allantoic pH below six will not support the multiplication of influenza viruses. The collection of samples only during the terminal phase of the outbreak, nonhomogenous nature of chickembryos, lesser number of chickembryos used per sample and single passage of the samples might be the probable reasons attributable for the failure in the present attempt to isolate the virus.

2. Propagation in embryonated chicken and duck eggs

Influenza virus strains CDN, DT3, T19 and lg1 multiplied very well in nine day embryonated eggs killing the embryos in two to four days time. The HA titers of the allantoic fluids were 1:64, 1:128, 1:64 and 1:256 respectively. The HA titers of the chorioallantoic membrane extracts were almost same as that of the allantoic fluid.

The lesions noticed in the embryos were congestion and petechiel haemorrhages with cranial ecchymosis. Lang et al. (1965) reported similar observations in chickembryos infected with an influenza A virus isolated from turkeys. Lang et al. (1968b) also observed dark red skin of the embryos, petechial haemorrhages on the head, congestion of the muscles and punctiform haemorrhages particularly on the sternum. In the present study lesions were mild or absent probably because of the low virulence of the strains used in this study compared

to the highly virulent virus (A/turkey/Canada/7732) of Lang et al. (1968 b). However, similar to their findings the CAM of infected embryos were slightly oedematous and congested without any pock lesions.

Except with strain T19 the infectivity titers were high being $10^{8.5}$ EID 50/0.2ml; $10^{8.25}$ EID 50/0.2ml; $10^{5.75}$ EID 50/0.2ml and $10^{8.5}$ EID 50/0.2 ml respectively for CDN, DT3, T19 and lgl. It is seen from the table 7 that though DT3 and T19 were isolated from the 1987 outbreak and having the same HA type, the infectivity titers of the latter were always low. This type of heterogeneity within the same antigenic subtypes have also been observed with human strains (Luzyanina et al; 1986).

Infected duck embryos did not show marked lesions except slight congestion of the whole embryo. As against this Lu et al. (1985) reported extensive haemorrhages of the duck embryos inoculated with duck influenza virus (A/duck/Taiwan/72). Their isolates were also from cases of respiratory disease among ducks. It is difficult to attribute this to the high virulence of the isolate as they have not mentioned about the virulence index of their isolate; but probably due to strain variation.

3. Identification of NP antigens

Influenza virus possess two antigenically stable type specific internal antigens the nucleoprotein and the matrix

protein (Pereira et al; 1965; Hana and Hoyle, 1966; Schild and Pereira, 1969; Schild and Dowdle, 1975). Influenza A virus form a homogenous group sharing common NP and MP antigens. Typing of new influenza virus isolates are being done either by complement fixation or agar gel diffusion test. Since complement fixation test is complex and time consuming Agar gel precipitation test is usually employed using extracts of infected chorio-allantoic membrane (Beard, 1970) or acid precipitate of infected allantoic fluid (Dowdle et al; 1974) as the antigen against type specific NP antisera.

In this study the NP antiserum was prepared against NP antigens of isolate CDN which was identified earlier as a type A influenza virus (Sulochana et al; 1987). The formation of a well defined line of precipitation by strains DT3, T19 and lg1 which was identical to that of the homologous strain CDN confirm the identity of all the four strains as influenza A virus. The second line which was also common for all the four isolates probably represents MP antigens as separation and purification of NP and MP antigens were not done during this study. Dowdle et al. (1974) reported that CAM extract from infected embryos contains both NP and MP antigens. The possibility of non specific lines was avoided by adsorbing the NP antiserum with normal CAM. Moreover no precipitin line was seen when NP antiserum was diffused against extracts from normal CAM. Beard (1970) reported that extracts of CAM from

influenza virus infected embryos as an excellent source of NP antigen for serological tests with avian and mammalian sera. Beard and Helfer (1972) also opined Agar gel precipitation test as a suitable method for typing influenza virus. But Dowdle et al. (1974) considered anti MP test as more sensitive than anti NP due to the abundance of this antigen in the virion. Moreover the time required for MP precipitin line is much less as this antigen is of higher molecular weight and migrate more rapidly than NP antigen in agarose gels. The additional line seen with CDN and lgi probably represents the H antigens as these two strains possessed the same H antigen.

Immuno-electrophoresis of CAM extracts of influenza A virus infected eggs reproducibly gave a characteristic tripple line pattern. This shows that there are atleast three cationic antigens that migrate towards the anode. These three antigens showed only slight difference in their rate of migration. Freidlin et al. (1985) immunoelectrophoresed influenza antigen prepared in a similar manner, reported in this study against antisera from naturally infected turkeys which were positive by Agar gel precipitation test. In contrast to Tris-barbiturate buffer used in this study these workers used phosphate buffered system pH 7.7. They have reported excellent resolution of viral proteins in this system and reported various advantages such as conveience, cheapiness and easy availability compared to the barbital buffer system.

4. Thermostability

Infectivity and HA property of all the four strains studied were lost by heating at 56°C for 30 minutes suggesting that these two properties are thermolabile. Similar findings of inactivation of influenza A virus were also reported by Merchant and Packer,(1967), Papparella et al. (1969) and Lang et al. (1968a, 1968b). Lang et al (1968a) also observed that at lower temperatures the HA activity was more stable than infectivity. Incontrast to this, Homme and Easterday (1970) reported that their isolate of influenza A virus from turkeys (A/turkey/Wisconsin/66) took six hours at 56°C to get inactivated, while Jawetz et al. (1981) reported loss of infectivity at 50°C in a few minutes time when not stabilized by 1M $MgSO_4$. Marked reduction in both HA and infectivity titers of duck influenza virus at 56°C for 30 minutes was also reported by Lu et al. (1985), but unlike the findings in the present study there was no complete loss of HA or infectivity. Differences in thermostability of haemagglutining of different strains of influenza A virus has also been reported by Lang et al. (1968b) and according to them this quality is more characteristic of an individual strain than a differential feature between pathogenic and non-pathogenic strains.

5 pH Stability

The observations made during this study indicated that

the infectivity of all the four strains was lost when they were exposed to pH 3.2 for a period of one hour at room temperature. Though only a slight reduction in HA activity was noticed with strains CDN, DT3 and lg1 a marked reduction in titer was a feature with T19. Papparella et al. (1969), Buxton and Fraser (1977) and Lu et al. (1985) also recorded similar findings. Lang et al. (1968a) observed that both HA and infectivity of A/turkey/Ontario/6213/66 were labile at pH 3.2 but not affected in the alkaline range of pH seven to eight. In this study also it is seen from table 6 that the infectivity and HA property were unaffected at slightly alkaline pH 7.2. A marked reduction in HA and slight reduction in infectivity was noticed at pH 9.0. Unlike the observations made during this study Webster et al. (1978) reported that duck influenza viruses were more stable at low pH than other influenza virus isolates particularly human strains. This discrepancy in the observations made in this study and that of Webster et al. (1978) could not be explained as they have not mentioned about the pH level at which they have seen a better stability of duck influenza virus, but can be attributed to strain variation.

6. Haemagglutinating property

Red cells from a variety of species such as cattle, sheep, goat, guinea pig, rabbit, rat, mouse, chicken and human O,A and B were agglutinated by all the four strains studied. The

ability of influenza A virus to agglutinate red cells from a variety of species have also been reported by Frano and Kapitancik (1959); Safonov et al. (1964), Formina and Sokkar (1966) and Lang et al. (1968a)

7. Chloroform sensitivity

Chloroform sensitivity is usually done to find out whether a virus is enveloped or not. Envelopes are lipoprotein in nature and are sensitive to lipid solvents like ether and chloroform, in which case infectivity is completely lost or greately reduced.

The chloroform sensitivity of influenza viruses had already been demonstrated by Papparella et al (1969) and Lang et al. (1968a) and various other workers. Complete inactivation of the strains when treated with five per cent chloroform indicate that all these strains are enveloped.

Chloroform was preferred to ether because of its polarity as a lipid solvent. Since it is heavier than ether and can be easily separated by centrifugation. Moreover, chloroform is as effective as ether (Feldman and Wang, 1961).

8. Propagation in chicken embryo fibroblast

Of the four strains propagated in monolayer cultures of chicken embryofibroblasts, only strain CDN produced marked cytopathic effect characterized by rounding of cells which

extended to the whole of the monolayer by 96 hours. The other three strains DT3, T19 and lg1 did not produce marked CPE even after six days of incubation. Multiplication of the virus in cell cultures was evidenced from the HA activity and infectivity of the cell culture fluid after repeated freezing and thawing. However, the HA and infectivity titers were low compared to those with chick embryo passaged virus. Rott (1985) used chicken embryofibroblasts to differentiate pathogenic and non-pathogenic strains of avian influenza virus. It was reported that non pathogenic strains produced HA in CEF cultures only in the presence of trypsin while with the virulent strain trypsin produced a rise in the HA titer. During this study effect of trypsin on HA activity of infected cell culture fluid was not attempted and hence cannot be commented.

Narayan et al. (1969) observed complete disintegration of cells in 24 hours of infection by avian influenza A virus (A/turkey/Ontario/7732/65) in CEF cultures, while increased granulation of cytoplasm with rounding and opacity of cells was reported by Rouse (1967) with the same strain. Rapid CPE in CEF cultures by this virus compared to the virus strains used in the present study could probably be due to the highly virulent nature of the virus A/turkey/Ontario/7732/65 used by Rouse (1967) and Narayan et al. (1969).

Negroni and Tyrrell (1959) while studying the CPE in

tissue cultures of epithelial cells infected with influenza virus observed that the percentage of cells destroyed following virus infection varied upon the concentration of the virus. At high multiplicity of infection many of the cells survived, while in low concentration the CPE was much faster. In contrast Gharagozlou and Samadiesh (1980) observed a close correlation between virus concentration in the inoculum and production of CPE. Failture of CEF monolayers to show marked CPE following infection with duck influenza virus isolates could probably due to high multiplicity of infection used in this study. However, swelling and rounding of cells were observed with one of the strains - CDN. Lu et al. (1985) observed rapid CPE in 24 hours of inoculation of a duck influenza virus in chicken embryo kidney cells where the cells began rounding and pealed off within 30 hours of inoculation. They have also demonstrated multiplication of the duck influenza virus in various cell lines of epithelial origin. Lavrentieva et al. (1986) reported that two influenza A viruses of the same antigenic makeup and indices of virulence for humans showed different characteristics in their replication in human embryo kidney (HEK) cells, human embryo lung (HEL) and chicken embryo kidney (CEK) cell cultures. The highly virulent strain A/Victoria/35/72 (H3N2) reproduced intensively in HEK and HEL cells irrespective of the dose of inoculum while the moderately virulent virus A/Bangkok/1/79 (H3N2) replicated well only in the presence of trypsin. However, in

CEK cells the rate of multiplication of both the virus remained the same even after addition of trypsin. These authors also observed a marked reduction in the virus titers when the infected monolayers were incubated for more than 72 hours from $10^{6.0}$ EID 50/ ml at 48 hours to $3.2 \times 10^{3.0}$ EID 50/ ml at 72 hours. HA activity was also demonstrated by them in 48 to 72 hours of infection. The above reports show that influenza A virus prefers epithelial cultures compared to fibroblast cells for their multiplication and different strains differ in the degree of cellular changes produced following infection.

9. Pathotyping

Classification of influenza A virus is based on the type and subtype specific antigen (WHO 1980). In this classification it is seen that several virulent strain are placed in different antigenic subtypes and isolates placed in the same antigenic subtype may be virulent or avirulent. This shows that there is no relationship between antigenicity and virulence. Hence it has become necessary to adopt some measures by which the virulence of the isolates can be detected and measured. Allan et al. (1977) suggested the use of intracerebral Pathogenicity index (ICPI) and intravenous Pathogenicity Index (IVPI) in day-old and six week-old chicks respectively for assessing the virulence of avian influenza A virus isolates, as in the case of NDV.

Based on these criteria NDV is grouped into lentogenic, mesogenic and velogenic strains. Lentogenic strains rarely kill day-old chicks, mesogenic strain kills all the chicks within a period of 8 days and velogenic strain kills all the chicks at a faster rate. The ICPI for the above types of NDV are 0.1 to 0.2, 0.8 to 1.5 and $\angle$1.5 respectively. In this study the ICPI for CDN, DT3, T19 and lg1 were 0.325, 0.66, 0.00 and 0.163 respectively. If these values are analysed based on the criteria given for NDV, it is seen that none of the four strain is virulent or possess lentogenic character. When the values of ICPI were compared DT3 seems to be most virulent of the four followed by CDN, lg1 and T19. CDN and lg1 have the same H antigen (H9) while DT3 and T19 possess distinct but same H antigen (H3). Between CDN and lg1, CDN is more virulent and in the second group DT3 is more virulent than T19. This is in aggrement with the findings of Beard and Easterday (1973) and Allan et al. (1977) that strains having the same H type may differ in their virulence. Allen et al. (1977) opined that though virulence indices may be a better guide in determining the seriousness of an influenza virus isolate than antigenic relationship with other influenza A virus, the wide variation in host response to infection and the possible contribution of other organisms to the disease should also be considered.

Mean death time, another criteria used for pathotyping

NDV was also used in this study. In the case of NDV the values for velogenic, mesogenic and lentogenic strains were 24 to 50 hours, 60 to 90 hours, and over 100 hours respectively. On this basis, the influenza virus strains studied are to be grouped with the medium virulence type (72 to 78 hrs.) as against the evaluation based on ICPI. The mean death time reported for highly virulent A/turkey/Ontario/7732/65 was 28.2 and that for moderately virulent A/turkey/Canada/6213 was 51.8 hours (Lang et al; 1968b). In their study the lethal end points were identical to infectivity end points as virus could not be demonstrated from those survived after six days. In contrast to this, in this study the embryo did not die at higher dilution but showed the presence of virus by HA activity indicating that the lethal end point and infectivity end points were not the same. According to Lang et al. (1968b) the practice of applying the method of Pathotyping of NDV to influenza A virus will not work as one of the highly virulent strain A/turkey/Ontario/7732 behaved as a velogenic strain in chick embryos while another strain (A/turkey/Canada/6213) though behaved like a velogenic strain in chick embryos was more a lentogenic strain in its pathogenicity to chicken. Thus the method of pathotyping cannot be transposed directly to avian influenza virus.

10. Pathogenicity and Immunogenicity

Number of repots have indicated a significant variation

in the pathogenicity of avian influenza virus and variation in the susceptibility to this virus by different breeds/ species (Narayan et al; 1969; Slemons and Easterday, 1972; Allan et al; 1977; Alexander et al; 1979). Ducks have been shown to be remarkably resistant to influenza A viruses which were virulent for turkeys or chicken (Alexander et al; 1978; Westbury et al; 1979; Forman et al; 1986).

During the present study pathogenicity of influenza A virus - one strain each from 1985 and 1987 outbreaks of respiratory disease among ducklings below six weeks of age was studied in ducklings of various age groups. Different routes of inoculations were also attempted. Both the strains used in this study had the same H antigen (H9).

Strain CDN (A/duck/India/1/85-H9N2) was found to be pathogenic to day-old ducklings. Hundred percent mortality was observed in those received the virus either by oral, occulonasal or subcutaneous routes. The mortality was noticed in 3 to 13 days time. The time taken for death of all the birds was only nine days in group C that received the virus by subcutaneous route followed by 11 days in group B and 13 days in group A. In all the three groups sudden death was noticed in most cases without showing marked clinical symptoms except discharges from the eyes and nostrils, droopiness, slight diarrhoea, ruffled feathers and mild respiratory distress in some cases.

In orally infected birds virus isolations were made from the cloacal and tracheal samplings before death. Cloacal shedding was noticed till the 10th day while tracheal shedding was evident only upto seven days. The percentage of isolation from the cloacal samplings was also higher compared to the tracheal samples. These findings emphasizes the importance of cloacal samplings for influenza virus isolation particularly when the birds are not showing clinical signs. Similar observations were also made by Westbury et al. (1979) and Shortridge (1980).

Virus isolations were made from all those ducklings died with or without showing clinical symptoms except in one case each from group B and C. Lack of marked clinical symptoms in most cases might be due to the acute nature of the disease indicating high susceptibility of day-old ducklings to this virus.

Among the three routes of inoculation, subcutaneous route was more effective as all the infected ducklings died in nine days time compared to 11 and 13 days by occulonasal and oral routes respectively. The pathogenicity indices calculated for various routes of infection were 1.19; 1.15 and 1.51 respectively for group A, B and C (Table 20). This again shows the high susceptibility of day-old ducklings particularly when given subcutaneously. Moses et al. (1948) evaluated the effect of various routes of inoculation of

adult birds on the infectivity and lethality of the virus. They found that though the minimum leathal dose was essentially the same for all routes of inoculation (I/V, S/C, I/P, I/C), larger amounts of the virus (about 10 times) were required to produce the disease by intradermal or intratracheal route and 1000 times by occular route and for effective infection it was necessary to introduce the virus beyond the epithelial barriers of the chicken.

The age matched ducklings kept along with group A to study the transmissibility of the virus also died in 7 to 14 days time. As in the case of the infected group, clinical symptoms were milder or even absent. However, virus could be isolated from cloacal and throat samplings before death and from the tissues after death. This shows that influenza A virus strain can be transmitted horizontally from the infected ones to the incontact birds and suggest that oral and or respiratory infection might be the natural route of infection.

One week-old ducklings were quite resistant to the same virus when given at the same dose rate and by the same routes. No clinical symptoms were noticed till the 4th day. One of the five ducklings that died on the 4th day of infection and was showing ruffled feathers, droppiness and slight diarrhoea. Virus could also be isolated from the tissue of this bird. The remaining birds were apparently normal throughout the period of observation. However, virus shedding was evident

from both cloacal as well as tracheal routes from day 3 to 14 in group A; 3 to 7 in group B and 3 to 10 in group C. Virus isolation from the cloacal and tracheal samples indicates that the virus multiply in the lungs and cells lining the intestinal tract as suggested by Webster et al. (1978). It is also seen that cloacal samples were positive for a longer period compared to tracheal ones indicating the persistance of the virus in this system. This indicate the possibility of its transmission through contaminated water.

The low pathogenicity index of 0.36; 0.00 and 0.20 respectively for group A, B and C also indicate the resistance of week-old ducklings to strain CDN.

The infected ducklings showed an antibody response to the virus from day seven onwards. The initial titers were low ranging between 1:4 to 1:8 only. The titer increased later on by the 14th day it ranged between 1:16 to 1:64. This peak titer later declined and was only 1:4 to 1:8 by the end of the 4th week. Similar range and pattern of antibody response was also noticed in the incontact birds. It is seen from table 11 that virus isolations were possible even when a satisfactory level of HI antibodies were detected in the serum. Isolation of influenza A virus in the presence of antibody has also been reported by Homme et al. (1969).

Neither clinical symptoms nor mortality was observed in two week-old ducklings that received the virus by different

routes. However virus infection was established from the positive virus isolations from day three to seven from both cloacal and tracheal samplings. The antibody response detected was low compared to the week-old ones. Absence of clinical symptoms and low antibody titers in the serum of ducks experimentally infected with A/duck/Hokkaido/5/77 (Hav4N2) and A/budgerigar/Hokkaido/1/77 (Hav4Nav1) was observed Kida et al. (1980). Similar findings were also reported by Podchernyaeva et al. (1981) and Shandhu and Hinshaw (1983).

The contact ducklings kept along with group C birds behaved the same way as the infected ones as virus could be isolated from both cloacal and tracheal materials. Similarly antibody response was also detected in them.

The results obtained during this study indicate that immunogenicity of influenza A virus is very poor. Moreover, the antibody response when it occurred was only for a short period and declined very rapidly. Poor immune response of ducks to influenza virus has also been reported by other workers. Slemons and Easterday (1972) reported that when considerable antibody response was noticed in pheasants and quails the response in ducks to the same strain was very poor. Similarly Easterday (1975) also reported high HI antibody levels in pheasants and quails with low levels in ducks and geese. Absence of clinical signs of the disease

with tracheal shedding for a period of six to eight days after infection with low and erratic levels of HI antibodies have been observed in ducks by Bahl and Pomeroy (1977); Westbury et al.(1979); McNulty et al. (1985); Forman et al. (1986) and Alexander et al.(1986)

Because of this poor immune response of ducks to influenza A virus, Westbury et al. (1979) opined that the system of surveillance of influenza virus infection in ducks must be combined with virus isolation.

One week-old ducklings infected with strain lg1 having the same H antigen but isolated during 1987 outbreak did not produce any clinical symptoms or death. The infected birds remained apparently healthy throughout the period of observation. However, virus isolations were made from the cloacal as well as tracheal samplings from day 3 to 7. Antibody response was observed till 21st day in those birds which had received the virus by oral route only while birds in other groups remained negative.

None of the incontact ducklings kept along with the infected groups picked up infection as neither virusnor antibody could be detected in them. This shows the poor transmissibility of strain lg1 compared to CDN. Westbury et al. (1981) also reported differences in the transmissibility of strain of influenza A virus possessing the same surface

antigen. The variation in the virulence of strains of influenza A virus with the same surface antigen observed in this study is supported by the observation made by Beard and Easterday (1973) and Allan et al. (1977).

Summary

SUMMARY

Influenza virus strains isolated from ducklings showing respiratory disease were characterized and their pathogenicity and immunogenicity were studied. The strains used were A/duck/India/1/85 (H9N2)-CDN; A/duck/India/2/87 (H3N?)-DT3; A/duck/India/7/87 (H3N2)-T19 and A/duck/India/14/87 (H9N?)-lg1. All four strains multiplied very well in nine day embryonated chicken eggs. The embryos died in two to four days time with congestion, petechial haemorrhages and cranial ecchymosis. The infectivity titers were $10^{8.5}$ EID 50/0.2ml; $10^{8.25}$ EID 50/0.2ml; $10^{5.75}$ EID 50/0.2 ml and $10^{8.5}$ EID 50/0.2ml respectively for CDN, DT3, T19 and lg1 while the corresponding HA titers were 1:64; 1:128; 1:64 and 1:256.

Infected duck embryos did not show marked lesions and the infectivity titers were $10^{7.5}$, $10^{7.25}$, $10^{5.0}$ and $10^{7.0}$ EID 50/0.2 ml respectively for CDN, DT3, T19 and lg1.

All the strains were thermolabile and got inactivated at 56°C in 30 minutes. The infectivity and haemagglutinating property were unaffected at pH 7.2, but at pH 3.2 complete loss of infectivity was noticed. However, HA property was not considerably affected except in the case of lg1 where in the HA titer was brought down from 1:256 to 1:16. At pH 9.0 there was slight reduction in infectivity and marked reduction in HA activity.

All the four strains were sensitive to chloroform at five per cent level indicating the enveloped nature of these strains. All of them agglutinated red cells from a variety of species such as cattle, sheep, goat, guinea pig, rabbit, horse rat, mouse, chicken, and human O,A and B at room temperature.

In chicken embryo fibroblasts marked cytopathic changes were not seen when infected with strains DT3,T19 and lgl. In CDN infected monolayers marked cytopathic effect characterized by rounding of cells extending to the whole of the monolayer was observed by 96 hours.

Agar gel precipitation test was conducted with NP antiserum to NP antigen of CDN and NP antigens of DT3, T19 and lgl. Two lines of precipitation which were identical to the lines formed by the homologous strain indicated withe identity of all the four strains as influenza A virus.

Similarly immunoelectrophoresis of CAM extracts of influenza A virus infected embryos produced a tripple line pattern with all the strains confirming the observations made by Agar gel precipitation test.

The intracerebral pathogenicity indices were 0.325, 0.66, 0.00 and 0.163 respectively for CDN, DT3, T19 and lgl while the intravenous pathogenicity indices were 0.00 for all the strains. The mean death time of the strains varied between 72 to 78 hours, being 78 hours, 76.8 hours, 72 hours and 78 hours respectively.

Pathogenicity and immunogenicity studies were carried out in day-old, one week-old and two week-old ducklings with strain CDN and in one week-old ducklings with strain 1g1.

Strain CDN was found to be highly pathogenic to day-old ducklings both by oral, occulonasal and subcutaneous routes causing hundred per cent mortality. The pathogenicity indices were 1.19, 1.15 and 1.51 respectively for Group A, B and C. Among the routes of inoculation, subcutaneous route was more effective as evidenced from the pathogenicity index. Contact infection was also established in this case.

In one week-old ducklings the pathogenicity indices were 0.36, 0.00 and 0.26 respectively for group A, B and C indicating the resistance of this age group of ducklings. Here also the incontact ducklings picked up infection as evidenced from virus isolation and antibody estimation.

Though low, both infected and incontact birds showed HI antibodies from 7th day onwards. By 14th day of infection the titers ranged between 1:16 to 1:64 and then declined and remained steady by the end of the 4th week.

In two week-old ducklings neither clinical symptoms nor death was observed. However, infection was established by virus isolation and antibody titration. The HI titers ranged between 1:4 to 1:16. The incontact ducklings were also positive for virus isolation and antibody response.

One week-old ducklings infected with strain lgl did not produce clinical signs or death. Virus isolations were made from cloacal and tracheal samples from 3rd to 7th day. But no antibody response was observed except in birds that received the virus by oral route. None of the incontact birds picked up infection as they did not reveal virus excretion or antibody response.

References

REFERENCES

Aini, I. and Ibrahim, A.L. (1986). Isolation of influenza A viruses from domestic ducks in Malaysia. Vet. Rec. 118 (5) : 130.

Alexander, D.J. (1982a). Avian Influenza-recent development. Vet. Bull. 52 (6) : 341-359.

Alexander, D.J. (1982b). Current situation of avian influenza in poultry in Great Britain. Proc. 1st Int. Symp. Avian influenza, Beltsville, Maryland, USA, 1981. pp. 35-45.

Alexander, D.J. and Allan, W.H. (1982). Cited by Alexander, D.J. (1982a).

Alexander, D.J., Allan, W.H., Harkness, J.W. and Hall, S.A. (1974). Isolation of influenza virus from psittacines. Res. Vet. Sci. 17 (1) : 125-127.

Alexander, D.J., Allan, W.H., Parson, D. and Parsons, G. (1978). The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. Res. Vet. Sci. 24 (2) : 242-247.

Alexander, D.J., Allan, W.H. and Parsons, G. (1979). Characterization of influenza Viruses isolated from turkeys in Great Britain during 1963-1977. Res. Vet. Sci. 26 : 17-20.

Alexander, D.J. and Gough, R.E. (1986). Isolation of avian influenza virus from birds in Great Britain. Vet. Rec. 118 (9) : 537-538.

Alexander, D.J., Parsons, G. and Manwell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chicken, turkeys and ducks. Avian. Path. 15 (4): 647-662.

Alexander, D.J., Spackman, D., Gough, R., Borland, E.D. and Stuart, J.C. (1981). Isolation of influenza A viruses from commercial ducks on a farm in Norfolk between August 1979 and March, 1980. Avian. Path. 10 : 263-272.

Allan, W.H., Alexander, D.J., Pomeroy, B.S. and Parsons, G. (1977). Use of virulence index tests for avian influenza viruses. Avian. Dis. 21 (3) : 359-363.

Allan, W.H., Wicht, J.V. and Kendal, A.P (1970). The isolation of a strain of influenza A viruses from turkeys. Vet. Rec. 86 : 43-44.

Austin, F.J. and Hinshaw, V.S. (1984). The isolation of influenza A viruses and paramyxoviruses from ducks in Newzealand. Aust. J. Exp. Biol. Med. 62 (3) : 355-360.

Bahl, A.K., Langston, A., Deusen, R.A.Van., Pomeroy, B.S. Newman, J., Karunakaran, D. and Halvorson, D. (1979). Prevention and control of avian influenza in turkeys. Proc. 83rd Ann. Meet. US. Anim. Hlth. Ass. pp. 355-363.

Bahl, A.K. and Pomeroy, B.S. (1977). Experimental exposure of Franklin gulls (Larus pipixan) and mallards (Anas platyrhynchos) to a turkey influenza A virus A/turkey/Minn/BF/72 (Hav6Neq2). J. Wildl. Dis. 13 (4) : 420-426.

Bahl, A.K., Pomeroy, B.S., Easterday, B.C. and Mangundimedjo, S. (1975). Isolation of type A influenza viruses from the migrating waterfowl mong the Mississippi fly way. J. Wildl. Dis. 11 (3): 360-363.

Bahl, A.K., Pomeroy B.S., Mangundimedjo, S. and Easterday, B.C. (1977). Isolation of a type A influenza virus and Newcastle disease virus from migratory waterfowl in the Mississippi flyway. J. Am. Vet. Med. Ass. 171 : 949-951.

Barr, D.A., Kelly, A., Badman, R.T., Campey, A.R., O'Rourke, M.D., Grix, D.C. and Reece, R.L. (1986). Avian influenza on a multi-age chicken farm. Aust. Vet. J. 63 (6) : 195-196.

Beard, C.W. (1970). Avian influenza antibody detection by immunodiffusion. Avian. Dis. 14 : 337-341.

Beard, C.W. (1976). Avian influenza. In Isolation and identification of avian Pathogens. Arnold Printing Corporation; Ithaca, Newyork. pp. 174-180.

Beard, C.W. and Easterday, B.C. (1973). A/turkey/Oregon/ 71. An avirulent influenza isolate with haemagglutinin of fowl plague virus. Avian.Dis. 17 : 173-181.

Beard, C.W. and Helfer, D.H. (1972). Isolation of two turkey influenza viruses in Oregon. Avian. Dis. 16 : 1133-1136.

Becker, W.B. (1966). The isolation and classification of tern virus A/tern/South Africa/1961. J. Hyg. Camb. 64 : 309-320.

Becker, W.B and Uys, C.J. (1967). Experimental infection of chickens with influenza A/tern/South Africa/1961 and Chicken/Scotland/1959 virus. 1. Clinical Pathology and virology. J. Comp. Path. 77 : 159-165.

Belitzkii, A. (1966). Actiology of respiratory disease of ducklings. Leningrad. 2: 76-84. (Vet. Bull. 1968. 38 (5) : Abst. 76-84).

Bennejean, G. (1982). Current worldwide situation of avian influenza - France. Proc. 1st int. Symp. Avian influenza, Beltsville, Maryland, USA, 1981. pp. 280.

Blaskovic, D., Rathova, V. and Borecky, L. (1959). Antigenic relationships to myxoviruses of a virus isolated from a respiratory infection in ducks. Acta. Virol. Prauge. (3) : 17-24. (Vet. Bull. 1960. 30 (3) : Abst. 731).

Boudreault, A. and Lecomte, J. (1981). Isolation of influenza viruses from different avian species in Canada in 1978. Revue. Canadenne. de. Biologie. 40 (1) : 139-140. (Vet. Bull. 1982. 52 (7): Abst. 3837).

Buish, W.W.: Hall, A.E. and Mc Daniel, H.A. (1984). 1983-1984 lethal avian influenza outbreak. Proc. US. Anim. Hlth. Ass. 88 : 430-446. (Vet. Bull. 1985. 55: Abst. 2840).

Buxton, A. and Fraser, G. (1977). Myxoviruses in Animal Microbiology. Blackwell Scientific Publications; Oxford London, Edinburgh, Melbourne. p.513.

Centanni and Savonuzzi (1900) Cited by Stubbs, E.L.(1959).

-----Compans, R and Dimmock, N.S. (1969). An electron-microscopic study of single-cycle infection of chickembryo fibroblasts by influenza virus. Virology. 39 : 499-515.

Chernetsov, Yu. V., Slepushkin, A.N., L'vov, D.K., Braude, N.A., Gavrilov, A.E., Berezovskii, N.G. Egrova, E.N., Manykin, A.A. and Gavrilov, E.I. (1980). Isolation of influenza virus from black terns (Chlidonias niger) and a serological survery of birds for influenza virus antibody (in Kazakhstan) Voprosy Virusologii. (1): 35-40. (Vet. Bull. 1980. 50 (9): Abst. 5636)

Cunningham, C.H. (1966). A laboratory guide in Virology. Burgess Publishing Company: Minneapolis. pp. 50-52

Daulbaeva, K.D., Sayatov, M.K.H., Beismbaeva, P.U., L'vov, D.K., Myasnikov, I.A., Gavrilov, E.I., Bagashcheva, S.S., Daubova, E.A., Khrokov, V.V. and Savi, Yu.U. (1981). Isolation of two subtypes of influenza virus type A from wild birds in the Alma-Ata region of Kazakhstan. Iza. Akad. Naukkazakh. SSR. (3) : 51-54. (Vet. Bull. 1982. 52 (1) : Abst. 112).

Dinter, Z. and Bakos, K. (1950). Ueber de Bezihungen des virus N Zudem virus der Klassichen Gefluegelpest. Berhn. Muench. Tieraerztl. Wochschr. 63 : 101-105. Cited by Easterday, B.C. and Tumova, B. (1972). Advances in Veterinary Science and Comparative Medicine. Academic Press : Newyork and London. 16: pp. 201-222.

Dowdle, W.R, Galphin, J.C., Coleman, M.T. and Schild, G.C. (1974). A simple double immunodiffusion test for typing influenza viruses. Bull. Wld. Hlth. Org. 51 : 213-218.

Easterday, B.C. (1975). The influenza viruses and influenza. Academic press; Newyork, San Francisco, London. pp. 449-471.

Eisengarten, H.J., Erler, M. and Hemmann, D. (1973). Duck influenza in a fattening flock. 1. Disease picture and isolation of the virus. Montashefte. fur. Veterinarmedizin. 28 (14): 551-553. (Vet. Bull. 1974. 44 (1) : Abst. 107)

Fazekas de St Growth, S. and White, D.C. (1958). An improved assay for the infectivity of influenza viruses. J. Hyg. 56 : 151-162.

Feldman, H.A. and Wang, S.S. (1961). Sensitivity of various viruses to chloroform. Proc. Soc. Exp. Biol. Med. 106 : 736-738.

Fleury, H.J.A., Babin, M., Bonnici, J.F., Poveda, J.D., Beyrie, M., Vuillayme, A. and Alexander, D.J. (1986). First simultaneous isolation of influenza A virus and duck enteritis virus from rared ducks in France. Vet. Rec. 119 (9) : 208-209.

Forman, A.J., Parsonson, I.M. and Doughty, W.J. (1986). The pathogenicity of an avian influenza virus isolated in Victoria. Aust. Vet. J. 63 (9) : 294-296.

Formina, A. Ya. and Sokkar, I.M.H. (1966). Efficacy of some methods for serological diagnosis of Newcastle disease and fowl plague viruses. Trudy. Vecs. Inst. ekspret. 32 : 72-81. (Vet. Bull. 1967. 37 (9) : Abst. 3743)

Frano, J., Bystrieky, V. and Vrtiak, O.J. (1958). Influenza virus A anatis isolates from ducks with a respiratory disease. Vet. Cas. 7 : 411-418. (Vet. Bull. 1959. 29 (7) : Abst. 2088)

Frano, J. and Kapitancik, B.C. (1959). Influenza virus A anatis isolated from ducks with a respiratory disease. II. Haemagglutinating properties. Vet. Cas. 8 : 244-254. (Vet. Bull. 1959. 29 (12) : Abst. 3846)

Franciosi, C., D'Aprile, P.N., Alexander, D.J. and Petek, M. (1981). Influenza A virus infection in commercial turkeys in North East Italy. Avian. Path. 10 : 303-311.

Freidlin, P.J., Bock, A., Inbar, A. and Sanberg, A (1985). Immunoelectrophoresis of avian viral proteins in phosphate buffered system. Avian. Dis. 29 (3) :613-616.

Gharagozlou, M.T. and Samadiesh, B. (1980). Cytopathogenicity of two avian influenza A viruses in different cell cultures. Clinica Veterinaria. 103 (3): 132-137. (Vet. Bull. 1981. 51 (6) : Abst. 3123).

Halvorson, D.A., Karunakaran, D. and Newman, J.A. (1980). Avian influenza in caged laying chickens. Avian. Dis. 24 : 469-472.

Halvorson, D., Karunakaran, D., Senne, D.A., Kelleher, C.J., Bailey, C., Abraham, A., Hinshaw, V.S. and Newman, J.A (1983). Epizootiology of avian influenza -simultaneous monitoring of sentinel ducks and turkeys in Minnesota. Avian. Dis. 27 (1) : 77-85.

Halvorson, D.A., Kelleher, C.J. and Senne, D. (1985). Epizootiology avian influenza- effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota. Appl. Envt. Microbiol : 49 (4) : 914-919.

Hana, L. and Hoyle, L. (1966). Cited by Dowdle et al. (1974).

Hannoun, C. and Devaux, J.M. (1980). Permenant circulation of avian influenza virus among wild ducks in the bay Somme - France. Comp. Immun. Microbiol. Infect. Dis. 3 (½) : 177-183.

Higgins, D.A. (1971). Nine disease outbreaks associated with myxoviruses among ducks in Hongkong. Trop. Anim. Hlth. Proc. 3 (4) : 232 -240.

Hinshaw, V.S., Bean, W.J., Webster, R.G. and Sriram, G. (1980a). Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology. 102 (2) : 412-419.

Hinshaw, V.S., Nettles, V.F., Schorr, J.F., Wood, J.M. and Webster, R.G. (1986). Influenza virus surveillance is waterfowls in Pennsylvania after the H5N5 avian outbreak. Avian. Dis. 30 (1) : 207-212.

Hinshaw, V.S., Webster, R.G. and Turner, B. (1979). Waterborne transmission of influenza A viruses?. Intervirology. 11 (1) : 66-68.

Hinshaw, V.S., Webster, R.G. and Turner, B. (1980b). The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can. J. Microbiol. 26 (5) : 622-625.

Hirst, G.K. (1941). Cited by Merchant, I.A. and Packer (1967).

Homme, P.J. and Easterday, B.C. (1970). Avian influenza virus infections - characteristics of influenza A/turkey/Wisconsin/1966. Virus. Avian. Dis. 14:66-74.

Homme, P.J., Easterday, B.C. and Alexander, D.J. (1969). Avian influenza virus infections. II. Experimental epizootiology of influenza A turkey/Wisconsin/1966 virus in turkeys. Avian Dis. 13 : 240-269.

Hwang, J., Leif, F.S., Miller, C.W. and Mallinson, E.T. (1970). An epornitic of type A influenza infection in ducks. J. Am. Vet. Med. Ass. 157 : 2106-2108.

Imada, T., Yamaguchi, S., Kawamura, H. and Nerome, K. (1980). Isolation of an influenza A virus from the budgerigar, Melopsittacus, Undulatus. Natn. Inst. Anim. Hlth. Q. Japan. 20 (1) : 30-31.

Jawetz, E., Melink, J.L. and Adelbery, E.A. (1981). Orthomyxovirus (Influenza) and Corona virus families in Review of Medical Microbiology. Lange Medical Publication ; Maruzin, Asian. 15th ed. p.420.

Jennings, A.R. (1967). Cellular and Tissue Reactions. in Viral and Rickettsial infections of Animals. Academic Press; Newyork and London. pp.223-225.

Johnson, D.C. and Maxfield, B.G. (1976). An occurrence of avian influenza virus infections in laying chickens. Avian. Dis. 20 : 422-424.

Johnson, D.J., Maxfield, B.G. and Moulthrop, J.I. (1977). Epidemiologic studies of the 1975 avian influenza outbreak in chicken in Alabama. Avian Dis. 21(2): 167-177.

Kendal, A.P., Mandely, C.R. and Allan, W.H. (1971). Antigenic relationship in avian influenza A viruses-identification of two viruses isolated from turkeys in Great Britain during 1967-70 J. Gen. Virol. 13 : 95-100.

Kida, H. and Yanagawa, R. (1979). Isolation and characterization of influenza A viruses from wild free flying ducks in Hokkaido, Japan. Zentlbl. Bak. Hyg 1 Org.A 244 : 135-143 (Vet. Bull. 1980.50 (8) : Abst. 4902).

Kida, H., Yanagawa, R. and Mtsuoka, Y. (1980). Duck influenza lackling evidence of disease signs and immune response. Infect. Immun. 30 (2) :547-553.

Kocan, A.A., Daubney, G.A. and Kocan, K.M. (1978). Structural changes associated with type A influenza in mallard duck tracheal organ culture. Avian. Dis. 22 (3) : 534-543.

Kocan, A.A., Hinshaw, V.S. and Daubney, G.A. (1980). Influenza a viruses isolated from migrating ducks in Okbahoma. J. Wildl. Dis. 16 (2) : 281-289.

Kopp, J.V., Kempf, J.E. and Kroeger, A.U. (1968). Cytoplasmic inclusions observed by electronmicroscopy later influenza virus infection of chicken embryo fibroblasts. Virology. 36 : 681-683.

Koppel, Z., Vrtiak, M. and Spiesz, S. (1956). Mass illness of ducklings in Eastern Slovakia with a clinical picture of infectious sinusitis. Veterinarsti. 6 : 267-268.

Lang, G. (1982). A review of influenza in Canadian wild and domestic birds. 1962-1980. Proc. 1st int. Symp. Avian Influenza, Beltsville, Maryland, U.S.A., 1981. pp. 21-27.

Lang, G., Ferguson, A.E., Connell, M.C. and Wills, C.G. (1965). Isolation of unidentified haemagglutinating virus from the respiratory tract of turkeys. Avian. Dis. 9 : 495-504.

Lang, G.,Narayan, O., Rouse, B.T., Ferguson, A.E. and Connell, M.C. (1968b). A new influenza A virus infection in turkeys. II. A highly pathogenic variant, A/turkey/Ontario/7732/66. Can. Vet. J. 9 : 151-160.

Lang, G., Rouse, B.T., Narayan, O., Ferguson, A.E. and Connell, M.C. (1968a). A new influenza virus isolation in turkeys. I. Isolation and characterization of virus 6213. Can. Vet. J. 9 : 22-24.

Lang, G., Tumova, B. and Schild, G.C. (1972). A new subtype of type A influenza A virus isolated from turkeys. Bull. Wld. Hlth. Org. 47 : 515-519.

Lang, G. and Wills, C.G. (1966). Wilmot virus - a new influenza A virus infecting turkeys. Arch. ges. virusforsch. 19 : 81-90.

Lavrentieva, I.N., Medvedeva, I.E. and Golubov, D.B.(1986). Characterization of the reproduction of influenza A epidemic viruses in cell cultures. Acta. Virol. 30: 137-142.

Lipkind, M., Shoham, D. and Shihmanter, E. (1981). Isolation of influenza viruses from rockpartridges in Israel. Vet. Rec. 109 (24): 540.

Lipkind, M., Weisman, Y., Shihmanter, E. and Shoham, D. (1982). Review of the three year studies on the ecology of avian influenza viruses in Israel. Proc. 1st. Int. Symp. Avian influenza, Beltsville, Maryland, USA, 1981. pp. 69-78.

Lipkind, M.A., Weisman, Y., Shihmanter, E., Shoham, D., Yural, C. and Aronovici, A. (1979). Identification of avian influenza virus isolated from wild mallard ducks in Israel. Vet. Rec. 105 : 558.

Lu, Y.S., Sugimura, T., Shich, H.K., Lee, Y.L. and Jong, M.H. (1985). Isolation and identification of an avian influenza A virus in ducks in Taiwan. J. Chinese. Soci. Vet. Sci. 11 (1) : 23-24.

Luzyanina, T, Ya., Crinbaum, E.B., Yukhnova, L.G., Klimov, A.L., Ghendon, Ya. and Gohubey, D.B. (1986). Laboratory characterization of influenza A virus (H3N2)- Pathogenicity of Leningrad 1983 epidemic. Acta. Virol. 30 : 294-302.

Mandelli, G., Rinaldi, A., Nardelli, L., Cervio, G., Cessi, D. and Valeri, A. (1969). An outbreak of type A influenza in domestic quail (Coturnix coturnix japonica). Att. Soc. Ital. Sc. Vet. 22 : 787-792.

Manjunath, L.H. and Mallick, B.B. (1977). Isolation of influenza viruses from birds. Second National Seminar on Zoonosis in India and their control. held at New Delhi/Mukteswar, June, 1977.

Markwell, D.D. and Shortridge, K.F., (1982). Possible waterborne transmission and maintenance of influenza virus in domestic ducks. Appl. Envt. Microbiol. 43 (1) : 110-116.

Mc Ferran, J.B., Connoor, T.J., Collins, D.S. and Allan,G.M. (1974). Isolation of an avirulent influenza virus from a parrot. Vet. Rec. 95 : 466-467.

Mc Nulty, M.S., Allan, G.M., Mc Cracken, R.M., Mand McParland, P.J. (1985). Isolation of highly pathogenic influenza virus in turkeys. Avian. Path. 14 (1) 173-176.

Merchant, I.A. and Packer, R.A. (1967). Veterinary Bacteriology and virology. The Iowa State University Press; Ames, Iowa, USA. 7th ed. p. 686.

Meuleman's G., Froyman, R., Dekegel, D. and Halen, P.(1979). Isolement de virus influenza chezdes vollailles domestique Belgique. Ann. Med. Vet. 123 : 109-114.

Mikami, T., Izawa, H., Kodama, H., Onuma, M., Sato, A., Kobuyashi, S., Ishida, M and Nerone, K. (1982). Isolation of ortho and paramyxoviruses from migrating feral ducks in Hokkaido. Archs. Virol. 74 (2/3): 211-217.

Mikami, T., Kawamura, M., Kondo, T., Murai, T., Horichu, M., Kodama, H., Izawa, H. and Kida, H. (1987). Isolation of ortho-and paramyxoviruses from migrating feral ducks in Japan. Vet. Rec. 120 (7) : 417-418.

Mitchell, C.A., Guerin, L.F. and Robillard, J. (1967). Myxovirus influenza A isolated from ducklings. Can. J. Comp. Med. 31 : 103-105.

Moses, K.F., Brandly, C.A., Elizabeth Jones and Jungherr, E.L. (1948). Isolation of and identification of fowl plague virus. Am. J. Vet. Res. 9 : 314-330.

Narayan, O., Lang, G. and Rouse, B.T. (1969). A new influenza A virus infection in turkeys. IV. Experimental transmissibility of domestic birds to virus strain A/turkey/Ontario/7732/1966. Arch. ges. Virusforsch. 26 : 149-165.; 166-182.

Nardelli, L., Rinaldi, A., Pereira, H.G. and Mandelli, G. (1970). Influenza virus infection in Japanese quails. Arch. Exp. Vet. Med. 24 : 231-249.

Negroni, G. and Tyrrell, D.A. (1959). Morphological observations on tissue culture of epithelial cells infected with influenza a virus. J. Path. Bacteriol. 77 : 497.

Nerome, K., Nakayama, M., Ishida, M., Fukumi, K., Butterfield, W.K., Webster, R.G. and Campbell, C.H. (1978). Isolation and serological characterization of influenza A viruses from birds that were dead on arrival at Tokyo airport. Archs. Virol. 57 : 261-270.

Niven, J.S.F., Armstrong, J.A., Balfour, B.M., Klemperer, H.G and Tyrrell, D.A. (1962). Cellular changes accompanying the growth of influenza virus in bovine kidney cell culturers. J. Path. Bacteriol. 84 : 1

Olesiuk, O.M., Snoeyenbos, DH and Roberts, D.H. (1967). An influenza A virus isolated from turkeys. Avian. Dis. 11 : 203-208.

Osidze, N.G., L'vov, D.K., Syurin, V.N., Smorzhevskaya, L.S., Lenchenko, E.V., Padchuk, L.A., Braude, N.A., Myasnikova, I.A., Podchernyeava, R. Ya., Kiryanova, A.I. and Shaik, Z.T. (1979). New Variants of avian influenza virus isolated from fowls on two Russian Poultry farms. Veterinariya, Moscow. (9) : 29-31. (Vet. Bull. 1980. 50 (3) Abst. 3989).

Otsuki, K., Takemoto, O., Fujimoto, R., Kawaoka, Y. and Tsubokura, M., (1987a). Isolation of influenza A viruses from migratory waterfowls in San-in District, Western Japan in Winters of 1980-82. Zentbl. Bakt. Parasitekde. p. 625. (Vet. Bull. 1987. 57 (1) : abst. 6953).

Otsuki, K., Takemoto, O., Fujimoto, R., Yamazaki, K., Kubota, T. Hosaki, H., Mikami, T., Kawaoka, Y. and Tsubokura, M. (1984). Isolation of H5 influenza viruses from whistling swans in Western Japan in November, 1983. Acta. Virol. 28 (6) : 524.

Otsuki, K., Takemoto, O., Fujimoto, R., Yamazaki, K., Kubota, N., Hosaki, H., Kawaoka, Y. and Tsubokura, M. (1987b). Isolation of influenza A viruses from migratory waterfowls in San-in District, Western Japan, in the winters of 1982-83. Acta. Virol. 31 (5) : 439-442.

Otsuki, K., Takemoto, O., Fujimoto, R., Yamazaki, K., Kubota, N., Hosaki, H., Mikami, T. and Tsubokura, M. (1987c). Isolation of influenza A viruses from migratory waterfowl in San-in District, Westen Japan in the winter of 1983-1984. Res. Vet. Sci. 43 (2) :171-179.

Otsuki, K., Yamazaki, K., Kawaoka, Y. and Tsubokura, M. (1987d). Infectivity of mice of avian influenza A viruses of H7N7, H5N3 and H2N2 subtypes isolated from migratory waterfowls, in San-in District, Western Japan. Jap. J. Vet. Sci. 49 (1) : 199-201.

Ottis, K. and Bahmann, P.A. (1980). Occurrence of HSW1N1 subtype influenza viruses in wild ducks in Europe. Archs. Virol. 63 (3/4) : 185-190.

Papparella, V. Zampino, V., Vaccaro, A. and DeRogatis, A. (1969). Isolation of type A influenza A virus on a turkey farm in Campania. Acta. Med. Vet. Nepoli. 15 : 105-113. (Vet. Bull. 1969. 39: Obst. 1667.)

Paukovic, C., Audi, S. and Aleraj, Z. (1969). First outbreak of duck influenza in Yugoslavia. Vet. Arch. 39 :278-280. (Vet. Bull. 1970. 40. Abst. 2257)

Pereira, H.C., Tumova, B. and Law, V.G. (1965). Avian influenza A viruses. Bull. Wld. Hlth. Org. 32: 855-860.

Petek, M. (1982). Current situation in Italy. Proc. 1st Int. Symp. Avian Influenza, Beltsville, Maryland, USA. 1981. pp. 31-34.

Podchernyaeva, R. Ya., Blinova, K., L'vov, D.K., Alymbaea, D.B., Karas, F.R. and Zhanuzkov, S.H. (1981). Pathogenicity of influenza viruses and their recombinants for ducks Veteinariya. Moscow (10): 27-28- (Vet. Bull. 1982. 52 (7) : Abst. 3838).

Pomeroy, B.S. (1982). Avian influenza in the United States (1964-1980). Proc. 1st Int. Symp. Avian influenza, Beltsville, Maryland, USA. pp. 13-17.

Poultry biologics (1963). Publication No. 1038. National Academy of Science. National Research council, Washington, D.S. pp. 35-39.

Profeta, M.L. and Palladino, G. (1986). Serological evidence of human infections with avian influenza viruses- Brief report. Archs. Virol. 90 : 355-360.

Prokofeva, M.T., Gurova, E.I. and Tsimokh, P.F. (1963). Virus influenza of ducks. Veterinariya, Moscow. 40 (10) : 33-35. (Vet. Bull. 1964. 34 (5): Abst. 1788).

Prokofeva, M.T. and Tsimokh, P.F. (1966). Virus influenza in ducklings. Leningrad. 2: 95-106. (Vet. Bull. 1968. 38 (5) Abst. 1894)

Rao, B.L., Gupta, N.P. and Deshmukh, P.K. (1979). Isolation of duck influenza virus from Tirunelveli town in Tamil Nadu. Indian J. Med. Res. 70 : 687-690.

Reed, L.J. and Muench, H. (1938). A simple method of estimating fifty percent end points. Am. J. Hyg. 27 :493-497.

Roberts, D.H. (1964). Isolation of an influenza A virus and a mycoplasma associated with duck sinusitis. Vet. Rec. 76 : 470-473.

Ronohardjo, P., Hardjoswaro, S., Pratoatmodjo, S and Partadiredja, M. (1985). The identification and distribution of influenza A virus in Indonesia. Penyakit. Hewan. 17 : 249-257. (Vet. Bull. 1986. 56 (6) : Abst. 3645).

Rosenberger, J.K., Krauss, W.C. and Slemons, R.D. (1974). Isolation of Newcastle disease and type A influenza viruses from migratory waterfowl in Atlantic flyway. Avian. Dis. 18 (4) : 610-613.

Rott, R. (1985). Differentiating pathogenic and nonpathogenic avian influenza virus in cell culture. Berl. Munch tierarztl. Wschr. 98 (2) : 37-39. (Vet. Bull. 1985. 55 (7) : Abst. 4155)

Rott, R. and Schafer, W. (1960). Physikalishchemische und biologiche Eigenschaften virus N and Bezienhungen zur influenza A untergruppe der myxovirus. Zentr. Veterinaermed. 7 : 237-245.

Rouse, B.T. (1967). Influenza virus infection in turkeys. A study of influenza A/turkey/Onterio/6213/66. M.Sc. Thesis. University of Guelph, Canada.

Russell, P.H. and Edington, N. (1986). Veterinary viruses. The Burlington Press; Foxton, Cambridge. pp. 146-148.

Safonov, G.A., Ivanova, G.A. and Syurin, V.N. (1964). Comparitive haemaggluting properties of the viruses of classical fowl plague and Newcastle disease. Veterinariya, Moscow. 41 (3) : 13-17 (Vet. Bull. 1964. 34 (4) : Abst. 2549).

Saidov, U.S. (1979). Respiratory disease caused by influenza virus in ducks in the Tashkhent region. Dokl. Vaes. Sel'skokloz. Nauk. (2) : 36-37. (Vet. Bull. 1980. 50 (11) : Abst. 7278).

Sayatov, M.K.H., Beisembaeva, R.O., L'vov, D.K., Daulbaeva, K.D., Myasnikov, I.A., Gavrilov, E.I., Dubova, E.A., Bagasheva, S.S., Yamnikova, S.S., Savin, Yu.G. and Khrokov, V.V. (1981). Influenza viruses isolated from wild birds (In Kazakhstan). Voprosy Virusologii (4) : 466-477. (Vet. Bull. 1982. 52 (5) : Abst. 2282).

Schafer, W (1955). Cited by Alexander(1982a).

Schettler, C.H. (1970). Isolation, characterization and biological properties of a strain of influenza A virus of group 6 isolated from ducks. (Duck/Germany/1868/68. Zentbl. Vet. Med. 17B : 817-827. (Vet. Bull. 1971. 41 (4) Abst. 3330.)

Schild, G.C. and Dowdle, R.W. (1975). In "The influenza viruses and influenza". Academic Press., Newyork. pp. 316-368.

Schild, G.C. and Pereira, H.G. (1969). Characterization of ribonucleoprotein and Neuraminidase of influenza A virus. J. Gen. Virol. 4: 355.

Shablouskaya, E.A., Voronenku, S.G. and D'Yachenka, A.P. (1985). Circulation of influenza virus within poultry farms. Voprosy. Virusologii (3) : 308-310. (Vet. Bull. 1986 56 (1) : Abst 176).

Shandhu, T. and Hinshaw, V.S. (1983). Influenza A virus infection in domestic ducks. Proc. 1st Int. Symp. Avian influenza. Beltsville, Maryland, USA. 1981. pp. 22-41.

Shortridge, K.F. (1980). Isolation of ortho-and paramyxo viruses from domestic poultry in Hongkong between November, 1977 and October, 1978 and comparison with isolation made in the preceeding two years. Res. Vet. Sci. 28 (3) : 296-301.

Shortridge, K.F., Butterfield, W.K., Webster, R.G. and Campbell, C.H. (1977). Isolation and characterization of influenza A subtype from domestic poultry in Hongkong. Bull. Wld. Hlth. Org. 57 : 465-469.

Sinnecker, R., Sinnecker, K., Zilske, E. and Kohler, P. (1983). Surveillance of pelagic birds for influenza A viruses. Acta. Virol. 27 (1) : 75-79.

Slemons, R.D., Cooper, R.S. and Osborn, J.S. (1973). Isolation of type A influenza viruses from imported exotic birds. Avian. Dis. 17 (4) : 746-751.

Slemons, R.D. and Easterday, B.C. (1972). Host response differences among five avian species to an influenza virus A/turkey/Ontario/7732/66 (Hav5 N2). Bull. Wld. Hlth. Org. 47 (4): 521-525.

Smitka, C.W and Maassab, K.F. (1981). Ortho and Paramyxo-viruses in the migratory waterfowl in Michigan. J. Wildl. Dis. 17 : 147-150.

Smithies, L.K., Emerson, F.G., Robertson, S.M. and Ruedy, D.D. (1969). Two different types A influenza virus infections in turkeys in Wisconsin. II. 1968 outbreak. Avian Dis. 13 : 606-610.

Stubbs, E.L. (1960). Fowl plague. In Diseases of Poultry. State University Press., Iowa, 5th Ed. pp. 813-822.

Stunzer, R., Thiel, W., Potsch, F. and Sixl, M. (1980). Isolation of influenza viruses from exotic and central European birds. Zentbl. Bak. Hyg. I Orig. 249 A (1) : 8-17. (Vet. Bull. 1980. 50 (12) : Abst. 8094)

Sulochana, S. (1987). Personal communication.

Sulochana, S. (1988). Personal communication.

Sulochana, S., Sudharma, D., Jayaprakasan, V., Mathew, E.S. and Mini, M. (1987). Isolation of influenza virus type A from ducks in Kerala. Indian J. Poult. Sci. (In press).

Tanyi, J. (1972a). Influenza in guinea fowl. Magy Allatorv Lap. 27 (1) : 5-8. (Vet. Bull. 1972. 42 (5) : Abst. 5211).

Tanyi, J. (1972b). Influenza in guinea fowl. II. Influenza virus of serotype 6 in ducks. Magy. Allatorv. Lap. 27 (1): 9-12 (Vet. Bull. 1972. 42 (5) : Abst. 5211).

Tanyi, J., Romvary, J., Derzsy, D., Temesi, Z and Sari, I. (1975). Severe sinusitis in ducklings caused by influenza A virus. Acta. Vet. Acad. Sci. Hung. 25:265-266.

Tashiro, M., Reinacher, M. and Rott, R. (1987). Aggravation of pathogenisity of an avian influenza virus by adaptation in quail. Archs. Virol. 91 (1/2) : 81-95.

Tautaswasdi, V., Malayaman, A and Shortridge, K.F. (1984). Influenza A virus isolation of a pheasant. Vet. Rec. 119 (15) : 375-376.

Ter Meulen, V. and Love, R. (1967). Virological, immunochemical and cytochemical studies of four HeLa cell lines infected with two strains of influenza virus. J. Virol. 1 : 626.

Tsubokura, M., Otsuki, K., Kawaoka, Y. and Yanagawa, P. (1981). Isolation of influenza A. virus from migratory waterfowl in San-in District, Western Japan, 1978-1980. Zentbl. Bak. Hyg. Orig.A. (Vet. Bull. 1982. 52 (5): Abst. 2281).

Turek, R., Gresikova, M. and Tumova, R. (1984). Isolation of influenza A virus and paramyxo virus from Sentinel domestic ducks. Acta. Virol. 28 (2) : 156-158.

Turek, R., Tumova, R., Mucha, V. and Stumpa, A (1983). Type A influenza virus strain isolated from wild ducks in Czechoslovakia during 1978-1981. Acta. Virol. 27 (2) : 523-527.

Turner, A.J. (1976). The isolation of fowl plague virus in Victoria. Aust. Vet. J. 52 : 384.

Vrtiak, O.J., Frano, J. and Kapitancik, B.C. (1966). Influenza anatum. Bull off. Int. Epizoot. 65 : 1231-1246. (Vet. Bull. 1967. 37 (9) ; Abst. 3692).

Walker, R.V.L. and Bannister, G.L. (1953). A filterable agent in ducks. Can. J. Comp. Med. Vet. Sci. (17): 248-250.

Webster, R.G. (1984). Avian influenza -A vaccine needed. Poult. Intl. 23 (9) 97-110.

Webster, R.G., Isachenko, V.A. and Carter, M. (1974). A new avian influenza virus from feral birds in the USSR, recombination in nature?. Bull Wld. Hlth. Org. 51 (4) : 325-332.

Webster, R.G., Morita, M., Pridgen, C. and Tumova, B. (1976). Ortho and paramyxoviruses from migratory feral ducks. Characterization of a new group of influenza A viruses. J. Gen. Virol. 22 : 217-218.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J. and Murti, K.G. (1978). Intestinal influenza, replication and characterization of influenza viruses in ducks. Virology. 84 : 268-278.

Weisser, H. (1979). Occurrence of influenza type A virus in wild birds and some domestic animals. Inagural Dissertation, Fachberich Tiermedizin, Munchen. pp. 79. (Vet. Bull. 1979. 49 (12) : Abst. 7233).

Wells, R.J.H. (1963). An outbreak of fowl plague in turkeys. Vet. Rec. 75 : 783-786.

Westbury, H.A., Turner, A.J. and Amon, L. (1981). Transmissibility of two avian influenza A virus (H7N6) btween chicken. Avian. Path. (10) : 481-487.

Westbury, H.A., Turner, A.J. and Kovesdy, L. (1979). The pathogenicity of three Australian fowl plague viruses for chickens, turkeys and ducks. Vet. Microbiol. 4 (3) : 223-234.

WHO expert Committee (1980). A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull. Wld. Hlth. Org. 58 : 585-591.

Yamane, N., Odagiri, J., Arikawa, J. and Ishida, N. (1979). Isolation and characterization of influenza A viruses from wild ducks in northern Japan. appearance of Hsw1 antigen in the Japanese duck population. Acta. Virol. 23 (5) : 375-385.

CHARACTERIZATION AND IMMUNOGENICITY OF INFLUENZA VIRUS TYPE A ISOLATED FROM DUCKS IN KERALA

By

M. MINI

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
Mannuthy, Trichur

1988

ABSTRACT

Ducks are generally regarded as resistant to influenza virus infection. During the early halves of 1985 and 1987 influenza A viruses were isolated from cases of respiratory disease causing 15 to 20 per cent mortality in two to six week-old ducklings at Government Duck Farm, Niranam. Four strains of the virus (A/duck/India/1/85 (H9N2)-CDN; A/duck/India /2/87 (H3N?)-DT3; A/duck/India/7/87 (H3N2)-T19 and A/duck/India/14/87 (H9N?)-lg1) isolated from these outbreaks were studied in detail with particular reference to their characteristics, pathogenicity and immunogenicity.

The strains were propagated in nine day embryonated chicken eggs by allantoic route of inoculation. All the four strains multiplied well killing the embryos in two to four days time. Though characteristic lesions were not present, the embryos were slightly congested. The chorioallantoic membranes showed moderate congestion and oedema but no pock lesions. Infectivity titers were $10^{8.5}$ EID 50/0.2ml; $10^{8.25}$ EID 50/0.2ml; $10^{5.75}$ EID 50/0.2ml and $10^{8.5}$ EID 50/0.2ml respectively for CDN, DT3, T19 and lg1. The corresponding HA titers were 1:64, 1:128; 1:64 and 1:256.

The strains also multiplied well in duck embryos but no specific lesions were seen either in the embryo or on the chorioallantoic membranes. The infectivity titers were low compared to the titers obtained in chick embryos.

All the strains were inactivated at 56°C in 30 minutes. They lost their infectivity at pH 3.2 while HA property was not considerably reduced except for lg1. At pH 7.2 both infectivity and HA property were not affected. But infectivity was slightly affected, and HA property was markedly reduced at pH 9.0.

The viruses were chloroform sensitive and agglutinated red cells from cattle, sheep, goat, guinea pigs, horse, rabbit, rat, mouse, chicken and man (O, A and B groups).

Chicken embryo fibroblasts infected with strain CDN produced cytopathic effects characterized by rounding of cells affecting the whole monolayer in 96 hours. The remaining three strains did not produce any CPE. In all the four cases virus infection was evidenced as the cell culture fluid gave haemagglutination. The infectivity titers of these fluids were $10^{6.5}$ EID 50/0.2 ml, $10^{5.5}$ EID 50/0.2 ml, $10^{4.0}$ EID 50/0.2 ml and $10^{5.5}$ EID 50/0.2 ml respectively for CDN, DT3, T19 and lg1.

Two common antigens possibly the type specific MP and NP antigens were detected in the CAM extracts of embryos infected with these strains by agar gel precipitation and immunoelectrophoresis.

Mean death time for these strains calculated according to the method adopted for NDV were 78 hours, 76.8 hours,

72 hours and 76 hours respectively for CDN, DT3, T19 and lgl, while the ICPI in day-old chicks were 0.325, 0.66, 0.00 and 0.16 and IVPI 0.00 in all cases.

Pathogenicity and immunogenicity studies were carried out in day-old, one week-old and two week-old ducklings with strain CDN and in one week-old ducklings with strain lgl.

Strain CDN produced hundred percent mortality in day old ducklings that received the virus by oral, occulonasal and subcutaneous routes. They died with no marked clinical signs except droopiness, discharges from the eyes, slight diarrhoea and ruffled feathers in some of them. The pathogenicity indices were 1.19, 1.15 and 1.51 respectively for group A, B and C. The ducklings that were kept to study contact infection revealed cloacal excretion of the virus till 12th day, though tracheal excretion stopped by 10th day. None of the birds in all the four groups that survived seven day of infection did reveal any specific HI antibodies.

In one week-old ducklings no clinical symptoms were observed. Virus could be isolated from cloacal and throat swabs before death and from tissues of dead birds. The pathogenicity indices were 0.36, 0.00 and 0.20 respectively for group A, B and C. In-contact ducklings picked up

infection as indicated from virus isolation and antibody titration.

Both infected and incontact birds showed HI antibodies from 7th day onwards. By 14th day the titers reached peak level ranging between of 1:16 to 1:64 followed by a decline and remained steady through out the observation period.

The two week-old ducklings did not show any clinical symptoms or death. But they revealed tracheal and cloacal shedding of the virus. The HI antibody titers never increased beyond 1:16. None of the incontact birds either showed clinical symptoms or death. Positive virus isolation could be made till the 7th day. Antibody response was also very poor and HI antibody titers never increased beyond 1:4.

One week-old ducklings infected with strain lg1 remained apparently healthy throughout the period of observation. However, virus isolations were made from cloacal and tracheal samples from 3rd to 7th day. In birds that received the virus by oral route, HI antibody titers were 1:4, 1:8 and 1:4 respectively on 7th, 14th and 21st day and nil by 28th day. In other two groups the birds did not reveal HI antibodies till 28th day.

None of the incontact birds in all three groups showed clinical symptoms, death or any other indication of infection by the virus. In addition the sera from these birds were also negative for HI antibodies.
