ISOLATION AND IDENTIFICATION OF VIRUSES FROM WATERFOWLS SEEN IN KERALA

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**ISOLATION AND IDENTIFICATION OF VIRUSES FROM WATERFOWLS SEEN 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.

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Certified that this thesis, entitled "**ISOLATION AND IDENTIFICATION OF VIRUSES FROM WATERFOWLS SEEN IN KERALA**" is a record of research work done independently by **Bindu, M.S.**, 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.

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Introduction

1. INTRODUCTION

The rural scenario of our country is presently undergoing a rapid change from the traditional concept of farming as a subsistence activity, barely meeting the needs for food, fuel and fibre. Today all efforts are underway to transform agriculture into a vibrant business that would enable farmers to live in dignity and prosperity. Livestock is important for increased productivity in agriculture. Among livestock based vocations, poultry occupies a pivotal position because of its enormous potential to bring about rapid economic growth, particularly benefiting the weaker sections. Further, it needs low capital investment and yet assures quick returns.

The structure of poultry industry has changed dramatically over the past 50 years world wide. The small family-farm flocks of chicken, turkeys, geese and ducks have been replaced by large integrated operations with greater emphasis on preventive health and bio-security programmes. However, avian disease problems have no respect for international borders. There is world wide movement of poultry breeding stock, poultry products and biologics. Petbirds, free-flying migratory birds and waterfowls are a very potent source of introduction of avian disease agents from one country to another. Influenza viruses, Newcastle disease virus and other paramyxoviruses are good examples of

important pathogens that have been spread in this fashion. Several newly emerging diseases and infections are being reported recently. It seems that new emerging problems keep ahead of our ability to control and eliminate existing diseases.

Isolation of viral agents from migratory waterfowls have been reported from all parts of the world and these migratory birds play an important role in the transmission of infectious diseases to our domestic poultry. Surveillance studies revealed the wide spread distribution of influenza viruses in wild birds, particularly ducks. Studies have shown that virtually all known antigenic subtypes of type A influenza viruses and combinations of various H and N surface antigens existed in the avian wildlife reservoirs (Suss *et al.*, 1994). A carrier state of duck plague virus was suspected in wild ducks (Burgess *et al.*, 1979). Leibovitz (1991) considered the mallard ducks as possible natural reservoirs of duck plague virus.

From the beginning of the 1970's, a large number of orthomyxoviruses and paramyxoviruses have been isolated from feral ducks throughout the world. Other than the orthomyxo and paramyxoviruses, Duck plague virus, Reovirus, Adenovirus, Duck hepatitis B virus etc. were isolated from a variety of migratory waterfowls. Duck plague virus was isolated from

free-flying waterfowls in Lake Andes (Procter *et al.*, 1975). Duck hepatitis B virus was first recorded in 1976 in Netherlands from three species of ducks (*Aix galericulata*, *Anas penelope*, *Cairina moschata*) (Smit *et al.*, 1980). A haemagglutinating duck adeno virus was isolated from the faecal samples from 12 species of waterfowls and other aquatic birds by Gulka *et al.* (1984). Viruses such as influenza virus and Newcastle disease virus have been isolated from domestic as well as migratory waterfowls (Sulochana and Nair, 1979; Sulochana *et al.*, 1987). The present practice of rearing chicken and ducks in Kerala and other parts of the country is quite conducive for easy spread of diseases if these migratory water fowls are reservoirs for these agents. In South India; Kerala with its unlimited backwaters and innumerable lakes is a strong source of attraction to these birds because of its richness in food. In addition, these ecosystems provide nesting, resting and feeding sites protected from ground predators.

The major infectious diseases with considerable economic losses in ducks in India are duck plague and duck pasteurellosis and in chicken, Newcastle disease, Avian influenza and Infectious bursal disease. While the bacterial diseases can be controlled by treatment, viral diseases like duck plague are to be tackled in a different way. Several outbreaks of duck plague have resulted in heavy mortality in

ducks, despite regular vaccinations. Reasons for these outbreaks despite vaccinations could probably be due to the primary introduction of the virus by migratory water fowls.

In Kerala, heavy losses were incurred regularly by the duck farmers in waterlogged areas of Alleppey, Kottayam, Pathanamthitta and Thrissur districts. The incidence was very high during March to May (Kulkarni, 1993). This period coincides with the arrival of migratory water birds to Kerala. The most commonly seen waterfowls in Kerala are the Gargany followed by Pintails and Lesser Whistling Teals (Nameer, 1995).

It is also quite interesting to note that from outbreaks of Epizootic ulcerative syndrome which took a heavy toll of fresh water fishes in Kerala during 1991-92, a virus which can survive in birds, particularly in ducks, was isolated, indicating the possible role of migratory waterfowls in the introduction and dissemination of this disease in our state (Sulochana *et al.*, 1992). Moreover these waterfowls are also to be suspected for the introduction of Japanese encephalitis in Kerala.

In the above circumstances it was felt worthwhile to screen the migratory waterfowls to establish their reservoir status to some of the above mentioned infectious agents.

Objectives of the study include,

1. Screening of waterfowl, to isolate viruses.

2. Physico-chemical and biological characterization of the viruses isolated.

3. Elucidation of the role of waterfowls in the spread of viral diseases to domestic birds.

Review of Literature

2. REVIEW OF LITERATURE

Pet birds, free-flying migratory birds and waterfowls are very potent source of introduction of avian disease agents from one country to another. Newcastle disease virus, other paramyxoviruses and influenza virus are good examples for this type of infection. Isolation of viral agents from migratory waterfowls has been reported from all parts of the world and these have been reported to play an important role in the transmission of infectious diseases to domestic poultry. Studies revealed the wide spread distribution of influenza viruses and paramyxoviruses in wild birds, particularly ducks. Studies have shown that virtually all known antigenic subtypes of type A influenza viruses with various combinations of H and N surface antigens existed in the avian wild life reservoirs (Suss *et al.*, 1994). A carrier state of duck plague virus was suspected in wild ducks (Burgess *et al.*, 1979 and Leibovitz, 1991) considered mallard ducks as possible natural reservoirs of duck plague virus.

From the beginning of the 1970's, a large number of orthomyxo viruses and paramyxoviruses have been isolated from feral ducks through out the world. In addition to orthomyxo and paramyxoviruses, duck plague virus was isolated from free-flying waterfowls in Lake Andes (Procter *et al.*, 1975). Duck hepatitis B virus was first recorded in 1976 in

Netherlands from three species of ducks namely *Aix galericulata*, *Anas penelope* and *Cairina moschata* (Smit *et al.*, 1980). A haemagglutinating duck adenovirus was isolated from the faecal samples from 12 species of waterfowls and other aquatic birds (Gulka *et al.*, 1984). The viral agents and the common waterfowls from which these viral agents have been isolated are listed below:

a. Influenza virus : Northern pintail (*Anas acuta*), Common teal (*Anas crecca*), mallard (*Anas platyrhynchos*), European wigeon (*Anas penelope*), Tufted duck (*Anas fulvigula*), Northern shoveler (*Anas clypeata*), Common shelduck (*Tadorna tadorna*), Whistling swans (*Cygnus columbianus*), Black headed gull (*Larus ridibundus*), Muscovy ducks (*Cairina moschata*)

b. Newcastle disease virus: Common teal (*Anas crecca*), Muscovy ducks (*Cairina moschata*), Mallard (*Anas platyrhynchos*), Lesser whistling teal (*Dendrocygna javanica*), Coot (*Fulica atra*), American blackduck (*Anas rubripes*), Wood duck (*Aix sponsa*), Blue winged teal (*Anas discors*)

c. Duck viral enteritis/duck plague: Mallard (*Anas platyrhynchos*), American black duck (*Anas rubripes*), Common teal (*Anas crecca*), Canada goose (*Branta canadensis*), Northern pintail (*Anas acuta*)

d. Reo virus : Mallard (*Anas platyrhynchos*), Muscovy ducks (*Cairina moschata*)

e. EDS-76 virus : Mallard (*Anas platyrhynchos*), Muscovy ducks (*Cairina moschata*)

f. Duck hepatitis B virus : Mallard (*Anas platyrhynchos*), Mandarin ducks (*Aix galericulata*), European wigeon (*Anas penelope*), Muscovy ducks (*Cairina moschata*)

Domestic poultry especially chicken, ducks and turkeys are under a constant threat to innumerable diseases spread by the migratory waterbirds. Some of these agents cause illness in the wild birds themselves, for others, the birds act as mechanical carriers. Sulochana (1991) isolated a velogenic strain of Newcastle disease virus from the cloacal swabs collected at weekly intervals from apparently healthy Lesser whistling teal (*Dendrocygna javanica*) for a period of eight weeks. This clearly indicates the role of this species as silent carrier of the virus.

2.1 Isolation

2.1.1 Orthomyxo virus

In many outbreaks of influenza in domestic poultry, spread from wild birds have been considered as the most likely mechanism of primary infections (Higgins, 1971; Bahl *et al.*,

1979). Hwang *et al.* (1970) reported an outbreak of respiratory disease in domesticated muscovy ducks in which ten per cent 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 were 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 epornitic of influenza A outbreak.

Cloacal and tracheal swabbings from 159 migratory waterfowls of different species shot by hunters in Delaware and Maryland, United States revealed influenza A virus in four cases (Rosenberger *et al.*, 1974). 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) antigen of influenza A virus. 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 (H_{10}, H_4, H_5, H_6 and H_3) in combination with seven different neuraminidase subtypes (N_1, N_5, N_6, N_8, N_2, N_3 and N_9) were isolated from ducks (6 nos.) and a chicken. Haemagglutinin and neuraminidase combinations of seven of the isolates ($H_{10}N_{15}$, H_1N_5, H_4N_2, H_3N_3, H_3N_9) were not reported earlier. All these new combinations were from ducks except H_3N_9 which was from chicken. Twenty-seven isolations of influenza virus A reported by Bahl *et al.* (1977) were from the tracheal swabbings of apparently healthy mallard ducks. None of the 60 giant Franklin gulls (*Larus pipixcan*) and 65 giant geese (*Branta canadensis*) gave any positive isolations.

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 this was found in high concentration in the faeces. 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 (H_1N_2) (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.

Rao *et al.* (1979) isolated influenza virus from ducks in Tirunelveli area of Tamilnadu. The isolate was designated as duck/India/78/1114 (Hav_4N_2). Weisser (1979) isolated 15 haemagglutinating agents from 1027 cloacal swabs from 959 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 occurrence of various subtypes in wild ducks were due to genetic recombination. In a study involving cloacal and tracheal swabs from 235 resident and 396 migratory ducks in Miyagi prefecture, 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 N_2 and N_3. It was suggested that avian influenza A viruses among feral ducks might be isolated in various combinations of haemagglutinin and neuraminidase.

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 deep water collected three meters 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, most frequently (26 per cent) 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 predominant subtype were the ones which were also isolated from the water and faeces and included Hsw_1N_1, Hav_7Neq_2 and Hav_4Nav_1. All the major H and N subtypes of human strains were represented among these duck isolates. Antigenic counterparts of these duck viruses (Hav_1Nav_2; Hav_7Nav_1; Hav_4Nav_2 and Hav_8Nav_4) have been associated with 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 suplies with faeces of infected yet healthy feral ducks offered a mechanism for maintaining a wide variety of influenza viruses, within the duck population and for introducing these viruses into other species.

Alexander *et al.* (1980) isolated an influenza virus from 39 fresh faecal samples collected at a reservoir frequented by ducks, gulls and other birds and the isolate was designated as A/avian faeces/England/CR1/80 (Hav_3Neq_2).

Boudreault *et al.* (1980) in July-August, 1977 isolated 145 influenza A viruses from ducks, geese and passerine birds. The majority of the viruses were Hsw_1N_2, antigenically related to influenza viruses in swine and man. They opined that the large reservoir of influenza A viruses circulating in ducks might well be involved in the appearance of new viruses in other species. 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. Most of these isolates were influenza viruses belonging to four haemagglutinin (Hav_1, Hav_6, Hav_7 and Hsw_1) types and five neuraminidase (N_2, Nav_2, Nav_4, Nav_5 and Neq_2) types (Hannoun and Devaux, 1980).

Kocan *et al.* (1980) isolated nine type A influenza viruses from migratory and wintering ducks in Oklohama during 1976-77. These isolations were grouped into three (Hav_1Nav_2, Hsw_1N_1 and Hav_6N_2) by subtyping.

Eleven influenza strains of antigenic subtype A/USSR 90/71 (H_1N_1) were isolated from six species of wild birds (Grey heron, *Ardea cinera*; great cormorant, *Phalacrocorax carbo*;

artic loon, *Garia arctica*; squacco heron, *Ardeola valloides*; Gargany, *Anas querquedula* and glossyibis, *Plegadis falcinellus*) (Iftimovici *et al.*, 1980).

Hinshaw *et al.* (1980a), during their survey of feral ducks for orthomyxovirus and paramyxovirus from 1976 to 1978 in the Vermillion area of Alberta, Canada have shown that influenza A viruses were 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 Tennesse. The isolates belonged to 27 different combinations of H and N subtypes. Hinshaw *et al.* (1980b) observed that seven per cent of the cloacal samples collected from Canadian feral ducks contained two or more antigenically distinguishable influenza viruses, indicating mixed infection.

Eighteen strains of avian influenza type A virus were isolated from cloacal swabs from waterbirds and songbirds 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 A/duck England/56 and two A/duck/England/56 in haemagglutinin

and to chicken/Brescia/1902 in neuraminidase. Two others corresponded to duck/England/62 in HA and Quail/Italy/65 in NA (Stunzer *et al.*, 1980).

Influenza A virus antigenically related to swine influenza (Hsw_1N_1) was reported to have been isolated from an adult mallard in Southern Germany. (Ottis and Bachmann, 1980), which was serologically related to A/duck/Alberta/35/76. Thorsen *et al.* (1980) isolated influenza viruses from the samples collected from 720 ducks, 100 terns and 50 gulls.

Boudreault and Lecomte (1981) isolated 357 influenza viruses from 2,293 cloacal samples collected from ducks and other wild birds. Seven H and six N subtyes 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 might be involved in the appearance of new subtypes in other species including man. 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 H_1N_1 subtypes and the remaining 17 were Hav_2Nav_5 subtype.

Manjunath and Mallick (1981) screened the cloacal swabs and faecal samples from 1188 domesticated, semi-domesticated, wild and migratory birds, mostly from the Himalayan region and isolated two influenza A viruses from the samples collected. 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 Hav_2Nav_5. Smitka and Maassab (1981) reported isolation of an avian influenza A virus A/duck/Michigan/77 (Hsw_1Nav_2) from 100 cloacal samples collected from migratory waterfowls during 1977 hunting season in Michigan.

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 Hav_1Neq_1 (11), and Hav_6Neq_3 (16) were isolated from 90 faecal samples from whistling swans. They had also isolated 13 similar viruses, 11 of them with surface antigens Hav_1Neq_1; and one Hav_6Nav_3, from 245 faecal samples of black tailed gulls. Two viruses of Hav_1Neq_1 were from 40 tufted ducks.

Abenes *et al.* (1982) isolated eight influenza viruses from 411 feral birds of 16 species. Influenzaviruses isolated from 40 teals consisted of three H_3N_8, one H_4N_6, one H_6N_6, one H_8N_1 and one designated as $H_8N_?$, because the neuraminidase was

not inhibited by any antiserum to N_1-N_8 neuraminidases. One H_4N_6 strain was also isolated from five spot billed ducks. The H_8N_1 isolate represented the first isolation in nature of an influenza virus possessing this combination of haemagglutinin and neuraminidase.

From 14 of the 278 migrating wild ducks in Hokkaido between 1978-1980, 11 influenza A viruses were isolated (Mikami *et al.*, 1982). Seven of the 11 isolates had the surface antigen $H_{10}N_3$ and three of their isolates had no antigenic relationship to the 26 previously known strains of avian, swine, equine and human influenza viruses. Ottis and Bachmann (1983) during the period of 1977-1980 screened cloacal and tracheal swab samples as well as droppings from 3392 feral birds in the Federal Republic of Germany, the Netherlands and Kenya. Almost half of the birds were migratory ducks, while the remainder consisted of other waterfowls, snipe, rails, geese, song birds and imported psittacines. Sixty nine influenza A virus strains of the haemagglutinin subtypes H_1 (Hsw_1), H_2 (H_2), H_3 (Hav_7, H_4 (Hav_4), H_6 (Hav_6, H_7 (Hav_1), H_{11} (Hav_3) and neuraminidase subtypes N_1 (N_1), N_2 (N_2), N_3 ($Nav_{2/3}$), N_5 (Nav_5), N_6 (Nav_1), N_8 (Neq_2) and N_9 (Nav_6) were isolated. They occurred in various combinations including H_1N_1 (Hsw_1N_1) and H_2N_2 (H_2N_2) strains. H_3N_8 (Hav_7Neq_2) and H_6N_5 (Hav_6Nav_5) influenza A virus strains were isolated from the droppings of migratory mallard ducks.

Sinnecker *et al.* (1983) isolated 351 influenza viruses from the trachea/cloaca of 3344 apparently healthy ducks, gulls, swans, terns and geese. They were of 14 subtypes. The isolation rates were 10.7 per cent in feral ducks, one per cent in other feral birds and 38 per cent in pekin ducks. The isolation ratio was a little higher from cloacal (75 per cent) than from tracheal swabs (70 per cent). It was also reported that 8.2 per cent of the pekins had dual infections.

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 H_4N_6. Repeated isolation of this subtype during their five year period of study suggested its permanent circulation in wildlife.

Cloacal swabs were collected from 3736 birds of 67 species over three years (July 1977 - December 1979) in Western Australia. Twenty four influenza A viruses were isolated from ducks, shearwaters, noddies, terns and a coot and were typed as H_1N_9, H_3N_8, H_4N_4, H_6N_4, H_7N_2, H_7N_6 and H_7N_9. The H_7 haemagglutinin did not react in tests with reference antisera. Whether they represented a new haemagglutinin subtype or a typical member of an established subtype was not known, although preliminary results indicated that they might be atypical members of the H_7 subtype. The H_1N_9 isolate was

the first reported isolate of this particular antigenic combination. This study indicated that influenza viruses were present in wild birds in Australia (Mackenzie *et al.*, 1984).

Otsuki *et al.* (1984) isolated three strains of influenza A virus belonging to H_5N_3 subtype from the faeces of whistling swans (*Cygnus columbianus*) in San-in district, Western Japan in November 1983.

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 virus A subtype H_4N_6.

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 the antigenic subtypes H_4N_6, H_1N_3 and $H_{11}N_3$ were isolated from mallards. They considered importation of poultry and game birds as the source of infection in Newzealand.

Deibel *et al.* (1985) collected 1598 cloacal samples from 1430 migratory waterfowl of 12 species in Newyork state during 1977-1983. Influenza virus was isolated from 42 per cent of 270 *Anas platyrhynchos*, 30 per cent of 40 *Anas rubripes*, 11 per cent of 19 *Anas discors* and 2.3 per cent of 639 *Aix*

sponsa. All positive ducks appeared clinically healthy. 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 June 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, cooler environmental temperature and prolonged reservoir through ground water contamination. They have also opined that ducks were not only a natural reservoir of influenza virus but also had a seasonal infection that appeared to be related to the seasonal outbreak in domestic turkey, in migratory waterfowls and in other species including man.

Lu *et al*. (1985) reported isolation of influenza A virus, A/duck/Taiwan/72 (Hav_6N_1) 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 gathered, these birds were suspected as the source of infection for domestic ducks.

Hinshaw *et al.* (1986) isolated influenza A virus H_5N_2 from wild birds, primarily ducks, in the same geographical area where there was an earlier lethal H_5N_2 avian influenza outbreak. There were 13 antigenic combinations of which only one belonged to H_5N_2 subtype. Though this isolate was antigenically related to the lethal H_5N_2, genetic and antigenic analysis revealed that it was quite distinct from the virulent H_5N_2.

Alexander and Gough (1986) analysed the conditions under which 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 H_4 subtype which was indicative of maintenance of virus on commercial duck farms. The difference in the neuraminidase subtype in combination with the same H type (H_4) 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 Hokkadido 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 H_3N_8, H_4N_6 and H_5N_2, while the remaining four were paramyxoviruses. Otsuki *et al.* (1987a) reported isolation of eight influenza A viruses from 354 faecal samples of whistling swan from 1982 to March 1983. None of the faecal samples from 261 black tailed gulls, 13 pintails and 10 mallards were positive for virus isolation. Of the eight isolates from whistling swans five belonged to human pandemic subtype H_2N_2, two isolates to fowl plague subtype H_7N_7 and the remaining one to subtype H_4N_6. 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 black tailed gulls. These birds were winter migratory birds flying from the USSR or Northern China and staying in Shimane and Tottori prefectures in San in district of Western Japan. A total of 40 influenza A viruses were isolated, 11 from whistling swan, 28 from pintails and one from black tailed gull. The isolates from whistling swan belonged to subtype H_5N_3 and $H_{10}N_4$, from pintails H_2N_2 and $H_{10}N_4$ and that of black tailed gulls $H_{13}N_6$. From November 1980 to April 1981 only two strains $H_{13}N_1$ and $H_{11}N_6$ 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 H_4N_3, 2 H_1N_6 and 2 H_3N_8). Two viruses of $H_{13}N_3$ and $H_{13}N_6$ subtypes were isolated from 425 faecal samples from black tailed gulls. A strain belonging to H_1N_3 subtype was isolated from 30 faecal samples from mallards but all the 242 samples from pintails were negative (Otsuki *et al.*, 1987c).

Sulochana *et al.* (1987) isolated influenza A virus from two to six week old ducklings during an outbreak of respiratory disease in Government duck farm, Niranam, Kerala. The symptoms were droopiness, malaise, occulonasal discharge, swollen eyelids and oedema of the head. Some birds also had paralysis. The mortality rate ranged between 15 to 20 per cent though in one hatch it mounted to 80 per cent. Of the 31 isolates during early 1985 outbreak 10 were subtyped and found to belong to subtypes H_9N_2(9) and H_9N_3 (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 H_3N_2 and H_9N_7 (Sulochana, 1988).

From 1986 to 1987, six influenza strains were isolated from the 276 cloacal swabs obtained from wild birds trapped in the Naples area. Four of the strains, from black cap, gull, vatican duck and house sparrow, were of the H_7N_2 subtype, while

two H_6N_2 subtypes were isolated from a house sparrow and black bird (Fioretti *et al.*, 1988).

Luini *et al.* (1988) isolated influenza virus subtype H_6 from random sample tests carried out on 45 batches of ducks imported from Brittany. The ducks involved had shown no clinical signs of the disease.

Isolation of avian influenza virus has been reported from 12 orders and 88 species of freeliving birds. Most isolations were reported from species in the order Anseriformes and Charadriformes and it was recognised that the species in Anseriformes represented important reservoirs of avian influenza virus (Stallknecht and Shane, 1988).

Of the 927 serum samples taken from 84 flocks of poultry, 33 per cent of the serum samples and 63 per cent of the flocks were positive to ELISA for antibody against influenza A virus and almost all the flocks were situated within areas where there were waterfowl. Of the 231 wild birds tested, belonging to 18 species of 9 families, 40 per cent were seropositive to ELISA. High infection rates were found among Anatidae (43 per cent), flamingoes and sparrows (31 per cent). Sparrows played an important role in carrying the disease from its natural reservoirs to domestic farms. Antibody titres found in wild birds were considerably higher than those found in poultry (Arenas *et al.*, 1990). Cloacal and tracheal swabs were

collected from 1389 hunter killed ducks in Cameron Parish, Louisiana, during the 1986 and 1987 waterfowl seasons. Twenty-eight avian influenza viruses were isolated from 605 blue-winged teal (*Anas discors*), 75 mottled ducks (*Anas fulvigula*), 375 gadwalls (*Anas strepera*) and 334 green-winged teal (*Anas crecca*). Two isolations from resident mottled ducks provided evidence of transmission of avian influenza virus from these wintering areas. Nine of the 13 haemagglutinin and nine of nine neuraminidase subtypes were typical of avian influenza viruses commonly associated with water fowls (Stallkencht *et al.*, 1990). Graves (1992) reported the isolation of type 4 influenza virus from the faeces of 5013 birds of 16 species.

Astorga *et al.* (1994) screened 712 samples from wild water fowls and shore birds in Donana National Park, 44 (6.2 per cent) were seropositive for antibodies to influenza A virus. Birds belonging to 10 of the 13 species studied were positive. Infection rates varied widely. Spoonbill (*Platalea leucorodia*) 32.2 per cent, mallard (*Anas platyrhynchos*) 9.9 per cent, Gadwall (*Anas strepera*) 8.6 per cent, red-crested pochard (*Netta rufina*) 8.1 per cent, pochard (*Aythya ferina*) 6.4 per cent, Shoveler (*Anas clypeata*) 5 per cent, great crested grebe (*Podiceps cristatus*), 4.3 per cent, avocet (*Recurvirostra avosetta*) 3.1 per cent, greyheron (*Ardea cinerea*) 3.1 per cent and coot (*Fulica atra*) 0.8 per cent.

Although infection rates were not high, the wide range of avian species susceptible to influenza virus A suggested circulation of the virus amongst wild fowl at Donana National park and constituted an epidemiological risk.

In a 12-year surveillance (1977-1989) in Eastern Germany, avian influenza A viruses were isolated from 236 feral ducks, 89 other wild birds, 735 domestic ducks from a single farm and from 193 pekin ducks used as sentinels for populations of wild aquatic birds. The efficiency of virus isolation was 9.9 per cent overall, 8.7 per cent in wild ducks, 0.9 per cent in other wild birds, and 38 per cent in Pekin ducks. Among the 40 different combinations of haemagglutinin and neuraminidase subtypes H_6N_1 (23.6 per cent) was the most common for all species, followed by H_4N_6 (11 per cent) (Suss *et al.*, 1994). Alfonso *et al.* (1995) conducted a survey at two wildlife management areas of Pennsylvania (USA) for the detection of avian influenza viruses in the cloacal swabs from water fowl and to determine the influenza A virus subtypes and the distribution of these viruses among waterfowl. Three hundred and thirty cloacal swabs were collected from hunter-killed waterfowl during 1990 and from cage-captured waterfowl in the summer of 1991. Twenty seven influenza A viruses were isolated from the cloacal swabs by chicken embryo inoculation.

To provide information on the mechanism of perpetuation of influenza viruses among waterfowl reservoirs in nature, virological surveillance was carried out in Alaska during their breeding season in summer from 1991 to 1994. Influenza viruses were isolated mainly from the faecal samples of dabbling ducks in their nesting places in Central Alaska. The number of subtypes of 108 influenza virus isolates were 1 H_2N_3, 37 H_3N_8, 55 H_4N_6, 1 H_7N_3, 1 H_8N_2, 1 $H_{10}N_2$, 11 $H_{10}N_7$ and 1 $H_{10}N_9$. Influenza viruses were also isolated from water samples of the lakes where they nested (Ito *et al.*, 1995).

2.1.2 Paramyxovirus

Friend and Trainer (1970) detected serological evidence for Newcastle disease virus (NDV) infection in mallards of three commercial game farms in Wisconsin. They reported HI antibody titres of 1:20 and above in adult female, male and ducklings at the rate of 40 per cent, 36 per cent and 5.67 per cent respectively. Thirty one per cent of the 3010 serum samples from migratory and nonmigratory canada geese were also found to have HI antibodies ranging from 1:20 to 1:1280 (Palmer and Trainer, 1970).

An acute outbreak of Newcastle disease was observed among ducks in Hongkong, characterised by rapid spread and high morbidity and mortality. The symptoms included anorexia, diarrhoea, wasting, occulo-nasal discharge, paraplegia and

decrease in egg production and Newcastle disease virus was isolated from six cases (Higgins, 1971).

Friend and Trainer (1972) reported that large intravenous doses of Newcastle disease virus produced clinical disease or death in mallards. Clinical signs of the disease, when present, were suggestive of nervous system involvement. Virus isolations were most often from the brain, with a few isolated from lung or liver and spleen. There was no evidence that infective virus was shed by birds inoculated with NDV. Haemagglutinin antibody response appeared within three to four days after inoculation and persisted at diagnostic levels during the 40 day experimental period. The serum of 168 wild ducks (grey teals) which were trapped in Gippsland and Northern region of Victoria in June and July 1972 were examined for Newcastle disease virus infection by haemagglutination inhibition. Sixteen per cent of the ducks from Gippsland and ten per cent of the ducks examined in Northern Victoria were positive (Hore *et al.*, 1973).

Rosenberger *et al.* (1974) reported isolation of nine lentogenic NDV strains from the cloacal and tracheal swabs from several species of migratory waterfowl shot by hunters in Delaware and Maryland.

Pearson and McCann (1975) made a study on the role of free-flying wild birds, captive and free ranging semi-domestic

birds and exotic birds in the epizootiology of NDV during an epornitic of viscerotropic velogenic Newcastle disease (VVND) in Southern California. They concluded that the isolation of domestic Newcastle disease virus strains from freeflying wild ducks and mourning doves suggested the potential for transportation of Newcastle disease virus over long distances by migratory birds.

The four isolates of NDV obtained from canada geese in the Atlantic migratory route were all lentogenic by the embryo mean death time being greater than 100 hours and the relative lack of pathogenicity of one-day-old and three-week-old chicken (Rosenberger *et al.*, 1975). Spalatin and Hanson (1975) opined that though antibodies to Newcastle disease virus were detected in migratory canada geese, wild ducks and domestic geese by haemagglutination inhibition and virus neutralization, experimental inoculation of these birds produced only inapparent infection. Hence the possibility of wildfowl acquiring ND infection from domestic poultry and viceversa was thought to be doubtful.

Webster *et al.* (1976) isolated 13 viral agents from the cloaca of 829 feral ducks, which included Newcastle disease virus also. Twenty seven haemagglutinating agents were isolated from 184 tracheal swabs of apparently healthy migratory mallard ducks (*Anas platyrhynchos*) in Mississippi

flyway. Of these twenty four were type A influena virus and three were lentogenic NDV (Bahl *et al.*, 1977). Alexander *et al.* (1979) isolated a strain of NDV from a faecal swab taken from a wild mallard duck and that particular isolate was found to be of low virulence for chicken. Bozorgmehri-Fard and Keyvanfar (1979) inoculated homogenised brain specimens from dead teals into chicken embryos. The allantoic fluid collected from the embryos were inoculated into ten domestic chicken susceptible to NDV and ten chicken immunized against NDV. Eight out of ten (eighty per cent) susceptible chicken died, while the immunized chicken remained healthy.

Sulochana and Nair (1979) isolated NDV from ducks in the southern regions of Kerala. The potential of migratory birds in the dissemination of NDV infection over vast distances, including national and continental boundries was pointed out by Ahmed *et al.* (1980).

Cloud and Rosenberger (1980) isolated nine avian paramyxoviruses from the tracheal and cloacal swabbings of feral ducks and geese in Atlantic flyway. Intracerebral inoculation of these viruses to day-old chicks and turkeys resulted in recovery of the virus from the tracheas of inoculated birds four to ten days after inoculation. The cloacal swabs and faecal samples from 1188 domesticated, semi-domesticated, wild and migratory birds, from the

Himalayan region when inoculated into 9 day-old chicken embryos yielded 65 isolates. Of these, 34 (2.7 per cent) were NDV, two were influenza A virus and the remaining 31 (2.6 per cent) were parainfluenza type 2, Yucaipa and `Bangor' viruses (Manjunath and Mallick, 1981).

During a surveillance of ortho and paramyxo viruses of lower animals and birds to elucidate the natural history and ecology of Newcastle disease virus and influenza viruses, Smitka and Maassab (1981) reported the isolation of three paramyxoviruses from 100 cloacal samples collected from migratory waterfowls during 1977 hunting season in Michigan. Under experimental conditions two of the paramyxoviruses were recovered from the intestinal tract of chicks of which the second one was pathogenic for chicks, and the third paramyxovirus was recovered from both the respiratory and intestinal tracts of chicks.

The pathogenicity of NDV isolated from ducks in Kerala was carried out in chicken (Sulochana *et al.*, 1981). A total of forty two, eight-week-old chicks were used for infection by cloacal, conjunctival, subcutaneous, oral and contact infection. All the birds died with the typical symptoms and lesions of viscerotropic velogenic form of Newcastle disease.

From 1980 to 1981, 411 feral birds comprising 287 waterfowl of 13 species and 124 small birds of 16 species were

surveyed for the presence of Othomyxo and Paramyxoviruses in Hokkaido, Japan (Abenes *et al.*, 1982). A total of 12 paramyxoviruses were isolated from nine of 40 teals, one of the 11 mallards, one of the 78 Eastern dunlins and one of the 72 Japanese buntings tested. Based on the antigenic specificity of their M proteins, five isolates from teal and that from an eastern dunlin were classified as Newcastle disease virus, four isolates from teal and that from a Japanese bunting as Duck/Mississippi/75, and from the mallard as Duck/Hongkong/199/77. Viruses were recovered only from the lower intestinal tissues and cloacal swabs.

Lipkind *et al.* (1982a) isolated two haemagglutinating agents from coot (*Fulica atra*); one from the brain of dead birds and the other from the tracheal and cloacal swabs of healthy birds. Both agents were identified as Yucaipa like avian paramyxoviruses by means of haemagglutination inhibition and neuraminidase inhibition tests.

Yucaipa like avian paramyxovirus was also isolated from a wild mallard (*Anas platyrhynchos*) wintering in Israel in 1979 (Lipkind *et al.*, 1982b). Mikami *et al.* (1982) isolated a total of 18 haemagglutinating agents from 14 of 278 migrating water ducks in Hokkaido between 1978 to 1981. Of these seven were paramyxoviruses and five isolates of

paramyxovirus reacted specifically with antiserum to duck/HK/199/77.

Vickers (1982) reported isolation of Newcastle disease virus from migratory birds and turkeys. Newcastle disease virus was isolated from the cloaca of one to five per cent of waterfowl in the autumn and antibody to NDV was detected in eight per cent of the birds tested. Isolates from waterfowl obtained in 1974-1980 were avirulent for poultry, based on mean death time in chicken embryos. Those obtained from turkey flocks with respiratory disease were also found to be avirulent. Some of the turkey isolates were similar to waterfowl strains. Isolates from waterfowl and infected turkeys were transmitted to contacts without producing overt disease.

Vickers and Hanson (1982) isolated Newcastle disease virus from the cloaca of one to five per cent of 1162 live-trapped waterfowl (canadagoose, mallard, wood duck and coot) in Wisconsin in the autumn from 1978-1980. Antibody to Newcastle disease virus was detected in eight per cent of the 862 birds tested, with no apparent difference between sex and age classes. Experimental infection of mallards resulted in persistence of virus shedding for months after exposure.

Yamane *et al.* (1982) reported the isolation of seventy-eight strains of avian paramyxoviruses from cloacal and/or

tracheal swabs from 5.5 per cent of 1342 wild ducks of seven species between 1976 and 1979 in Northern Japan. The isolates were separated into three `serotypes - a virulent NDV (28 per cent), viruses related to PMV-4, duck/Hongkong (58 per cent), the remaining 14 per cent were serologically related to PMV-3, but structurally different and therefore a new avian PMV serotype PMV-8 was proposed.

In 1977-1980 cloacal and/or tracheal swab samples, as well as droppings from 3392 feral birds were collected in the Federal republic of Germany, the Netherlands and Kenya and were screened by Ottis and Bachmann (1983) for the presence of Orthomyxo and Paramyxoviruses. Almost half of the birds were migratory ducks, while the remainder consisted of other waterfowls, snipe, rails, geese, songbirds and psittacines. The assay for orthomyxo and paramyxoviruses yielded 150 haemagglutinating virus strains representing an overall isolation rate of 4.5 per cent. Of these, 69 were identified as influenza A virus strains and 81 avian PMV strains. The 81 avian PMV-strains isolated were characterized as belonging to subtypes PMV-1 (NDV), PMV-3, PMV-4 and PMV-6, while PMV-2 (Yucaipa like viruses) were isolated from wag tails in Kenya.

Tracheal and cloacal swabs from apparently healthy mallards, gulls, shearwaters and terns in Newzealand were screened by Austin and Hinshaw (1984) for the presence of NDV.

Nine paramyxoviruses of two antigenic subtypes (PMV-1, PMV-4) were isolated from mallards. They considered importation of poultry and game birds as the source of infection in Newzealand.

Gough and Alexander (1984) isolated avian paramyxovirus type 4 from a ringed teal (*Calonetta leucophrys*). The isolate was apathogenic for chicks and ducklings. This was the first report of PMV 4 from United Kingdom. Tumova *et al.* (1984) isolated 41 paramyxovirus strains (25 PMV-1, 10 PMV-4 and 6 PMV-6 serotypes) from the cloacal swabs of 910 freeliving birds trapped in West Slovakia from 1978 to 1982. Paramyxovirus strains were found in nine species of aquatic birds. Twenty-five belonging to the PMV-1 serotype (NDV) were isolated yearly, indicating its wide distribution and circulation in nature. The strains of PMV-4 and PMV-6 serotypes found only in 1978-1980 represented the first isolations in Europe. The surface antigens of the newly isolated PMV strains were related to those of PMV-4/Duck/ Hongkong D_3/75 and PMV-6/Duck/Hongkong 311/80 strains.

Turek *et al.* (1984) investigated the possible circulation of Orthomyxo and Paramyxoviruses in sentinel domestic ducks during October 1981 to May 1982 during which they isolated three strains of Newcastle disease virus and one strain of PMV-4 from trachea and cloacal swabs.

During 1977-1983, 1598 cloacal samples were collected from 1430 migrating waterfowl of twelve species in Newyork state. Virus was detected in 257 (15.9 per cent) of the samples. Of these seventy-one were serotype 1 (NDV). Other serotypes included PMV-2 (1 isolate), PMV-4 (6 isolates), PMV-6 (7 isolates) and PMV uncharacterised (4 isolates) (Deibel *et al.*, 1985). Hinshaw *et al.* (1985) investigated the possible circulation of influenza viruses and paramyxo viruses in waterfowls originating from two different areas of North America. Two marshalling areas of different migratory flyways of waterfowl in North America were compared over 6-8 years. Isolations of paramyxoviruses were characterized mainly as avian PMV-1. Viruses were isolated from ducks immediately before they appeared in other species. These studies indicated that ducks in different areas represented a continuous source of paramyxoviruses which might infect other species. A four year study (1980-1983) involving the use of sentinel ducks that intermingled with wild ducks was carried out in Minnesota by Kelleher *et al.* (1985). A total of ninety-eight paramyxovirus (PMV) isolates were obtained (84 NDV, 14 PMV-6) between June and mid November each year. These findings indicated that wild waterfowl were the natural reservoirs of PMV.

Mbugua and Karstad (1985) isolated avian paramyxoviruses (Yucaipa-like) from the cloacal swab of 57 wild birds in Kenya

between February 1980 and August 1982. All the birds from which the avian paramyxoviruses were isolated were apparently healthy.

Telbis (1986) screened various species of wild birds to detect the presence of any virus and reported the isolation of viruses with cytopathic and haemagglutinating properties from black-headed gull, *Larus ridibundus*, tree sparrow, *Passer montanus* and muteswan, *Cygnus olor*. On the basis of haemagglutination inhibition tests the isolates were placed in the paramyxovirus serogroup 1. Of the five isolates reported, on the basis of the mean death time and the intracerebral pathogenicity index, two were found to be avirulent (lentogenic), while the other three were virulent (velogenic). Three Newcastle disease viruses (NDV) isolated from wild ducks in Japan were examined for their pathogenicity and immunogenicity in one-day-old chickens. One isolate was of mesogenic type and the other two were of lentogenic type. The lentogenic isolates immunized chicken against challenge with virulent strain of NDV (Kawamura *et al.*, 1987).

Cloacal swabs collected from 166 wild free flying ducks shot by hunters on the Pacific flyway on the Ishikari river in Central Hokkaido revealed ten haemagglutinating agents (Mikami *et al.*, 1987). Four of these agents were

paramyxoviruses isolated from mallard and teal species and were Paramyxo virus subtypes PMV-1 and PMV-4 respectively.

Paramyxovirus serotype 1 isolated from waterfowl during a surveillance programme in Northern Germany were inoculated into embryonated eggs and chicken to assess their pathogenicity. On the basis of the mean death time and intracerebral pathogenicity index, two of the isolates were found to be virulent (velogenic) (Telbis *et al.*, 1989).

Sulochana (1991) isolated a velogenic strain of NDV from the cloacal swabs collected at weekly intervals for a period of eight weeks from lesser whistling teal (*Dendrocygna javanica*). Continuous excretion of the virus was indicated by virus isolation at weekly intervals for a period of two months without manifesting any clinical symptoms, clearly indicating that this species could act as silent carrier of the virus. The NDV isolated from teal were inoculated to a total of sixteen, eight-week-old chicks subcutaneously and intranasaly to assess the pathogenicity of the isolate. The infected birds died after five days with symptoms and lesions typical for Viscerotropic velogenic newcastle disease (VVND). The virus was also isolated from the liver and spleen of these birds.

Serological survey of the serum samples of wild waterfowl in Southern Spain were carried out between 1988 and 1990

(Maldonado *et al.*, 1992) for the presence of avian paramyxoviruses. Two hundred and twenty two birds of eleven species (including 104 mallards) were tested for antibodies to avian paramyxovirus of eight types. Two per cent were positive to PMV-1, 8 per cent to PMV-2, 6 per cent to PMV-6, 8 per cent to PMV-7, 40 per cent to PMV-8 and 10 per cent to PMV-9.

Wobeser *et al.* (1993) reported the investigation on the mortality of wild water birds in Western Canada during the late 1990's. Newcastle disease virus was isolated from the birds and most of the viruses isolated were classed as velogenic and had a similar monoclonal antibody profile to viruses from the 1970 to 1974 panzootic. Ziedler and Hlinak (1993) screened 262 samples from wild birds of 26 species in Germany for the presence of antibodies against NDV. Twenty two serum samples showed positive antibody titres to NDV by HI test.

During a study carried out to detect the prevalence of antibodies to avian paramyxovirus serotypes in wild and domestic birds in Southern Spain, Maldonado *et al.* (1994) examined 361 serum samples collected from 24 wild species of birds. The incidence was highest for PMV 2 (69 per cent), followed by PMV-3 (six per cent) and PMV-1 (one per cent).

Selleck *et al.* (1994) reported the isolation of seven haemagglutinating agents from the cloacal swabs collected from a disease outbreak in three week old muscovy ducks in July 1992 from Victoria. The clinical symptoms recorded were high mortality rate and severe respiratory distress. Of the seven haemagglutinating agents, six were identified as NDV by HI test and the seventh agent as an influenza A virus.

A survey was conducted at two wildlife management areas of Pennsylvania for the isolation of orthomyxo and paramyxo viruses. Three hundred and thirty cloacal swabs were collected from waterfowl killed by hunters in the autumn of 1990 and from cage-captured waterfowl in the summer of 1991. Thirty-one haemagglutinating agents were isolated by chicken embryo inoculation, of which 27 were influenza A virus and four were NDV (Alfonso *et al.*, 1995).

Maldonado *et al.* (1995) carried out a serological survey of avian paramyxoviruses among wild fowls in Southern Spain during the period 1990 to 1992, using the haemagglutination inhibition technique. Five hundred and seventy-nine serum samples from 24 avian families were collected. Antibodies were detected to all paramyxoviruses in waterfowls, with a notable prevalence of antibodies to PMV-8 (43 per cent) and to a lesser extent to PMV-6 (21 per cent). In non-aquatic species high antibody prevalence was detected only to PMV-2

(60 per cent) while antibody prevalence to other PMVs were moderate or less.

Cloacal and tracheal swabs from 36 wild mallards shot in Israel were propagated through embryonated chicken eggs. A total of four haemagglutinating agents were isolated, one from the tracheal swab and three from the cloacal swabs. One of the isolate was confirmed to be a NDV by HI test by the reference antiserum. The other three isolates were found to be avian PMV-4 (Shihmanter *et al.*, 1995).

Graves (1996) screened the faecal sample of nearly fifteen avian species in the Atlantic flyway over a three year period (1977-1979) for NDV. Newcastle disease virus were isolated from adult canada geese, nesting royal terns, European mute swan and Tundra swan on the Eastern flyway. The prevalence of elution inhibition antibodies in swans and geese ranged from 3 to 41 per cent and that of haemagglutination inhibition antibodies ranged from 4 to 62 per cent.

2.1.3 Duck viral hepatitis

Ulbrich (1971) conducted a study on the significance of wild ducks in the transmission of duck viral hepatitis. In his study neutralizing antibodies to duck viral hepatitis were not detected in thirty-six wild ducks (*Anas platyrhynchos*, *Anas strepera*, *Anas querquedula*, *Nyroca ferina*) from ponds

where the disease had occurred in domestic ducks. At the same time, wild duck embryonated eggs from an infected area were all susceptible to experimental infection.

Friend and Trainer (1972) in their studies on experimental duck virus hepatitis in mallard ducks have reported that 60 per cent of the infected and 30 per cent of contact ducklings died of the disease with clinical symptoms and lesions of duck heptitis.

A study conducted in 1977 showed that three species of waterfowls (*Aix galericulata*, *Anas penelope*, *Cairina moschata*) were also susceptible to Duck hepatitis virus (DHV) in addition to domestic ducks (Smit *et al.*, 1980).

A virus closely related to duck hepatitis B virus (DHBV) was isolated from the serum and liver samples of migratory ducks (mallards) caught in two wild life reserve parks in France (Cova *et al.*, 1986). Duck hepatitis B virus was isolated from 12 per cent of mallards in the Dombes region and three percent of mallards on the River Somme. The various replicative forms of DHBV were also detected in the liver of wild viraemic mallards. The DNA restriction enzyme pattern of the wild mallard strain was also found to be different from that of American and French DHBV strains. White pekin and mallard ducklings were experimentally infected with the DHBV isolate, which induced chronic viraemia in both the species.

Gough and Wallis (1986) on conducting postmortem examination of ducks which died of respiratory disease observed infraorbital caseous sinusitis and enlarged pale liver with petechiae and echymosis. They isolated duck hepatitis type I virus from the liver suspensions of the dead birds. The isolations were made in developing chick embryos by allantoic inoculation.

Song and Guo (1988) examined the serum samples from ten ducks and two goose species and also mandarin ducks by spot hybridization with DNA and RNA probes. Duck hepatitis B virus DNA was detected in samples from five species.

2.1.4 Adeno virus

During an investigation on respiratory disease in migratory birds in Barathpur Sanctuary, Rajasthan, Mathur *et al.* (1972) detected antibodies to chicken embryo lethal orphan virus (CELO virus) in three of seven *Anas clypeata* (Shoveler) and four of eight *Anas strepera* (Gadwalls). McFerran *et al.* (1976) isolated Adenoviruses from pigeons, mallard ducks, budgerigars and bantam showing a variety of clinical conditions. Primary identification of these viruses was by electronmicroscopy. The four adenoviruses isolated were related to recognized fowl serotypes.

Schloer (1980) screened the serum samples of domestic ducks and wild waterfowl for the presence of antibodies to adenovirus 127. Nearly 90 per cent of the serum samples from breeder ducks and 50-70 per cent of ducklings possessed HI antibodies to "adenovirus 127" (associated with egg drop syndrome). About 3.7 per cent of serum samples from mallard ducks and 14.7 per cent from canada geese were also positive. The distribution of antibody was sporadic with low titres in wild goose than in mallard ducks. Serum samples containing HI antibodies were also positive by serum neutralization test and it was opined that adenovirus 127 was enzootic in commercial ducks.

In a serological survey for the prevalence of antibodies to egg drop syndrome 1976 virus in domesticated and wild birds in Israel, Malkinson and Weismann (1980) have detected antibodies in all pekin duck flocks tested, and in all muscovy duck flocks that were in direct or indirect contact with pekin ducks. No antibodies could be demonstrated in thirty-three wild palm doves or in wild birds, including waterfowl belonging to the zoological department.

Bouquet *et al.* (1982) isolated a virus from muscovy ducks and was classified as an adenovirus on chemical and morphological grounds. Though this virus shared avian adenovirus group antigen it was distinct from fowl, turkey and

duck prototype adenovirus strain 127. This virus multiplied well in cells of duck and fowl origin, and was considered as a new duck adenovirus serotype.

Seroprevalence of duck adenovirus in twelve species of aquatic birds were studied in detail (Gulka *et al.*, 1984). Duck adeno virus (DAV) was serologically indistingushable from egg drop syndrome - 76 virus. A total of 285 serum samples were tested by the haemagglutination inhibition test (HI test). Forty two per cent of the birds had HI antibodies, the titres ranging from 8 to 256. Wild ducks showed the highest frequency of antibodies viz. 56 per cent. It was only 33 per cent and 26 per cent in coots and grebes respectively. Attempted isolations from 79 faecal samples did not yield any result. The authors were of the opinion that these data supported the hypothesis that DAV was indigenous in wild duck populations and suggested that infection and viremia were limited in time and occurred at a very early age.

Antibodies to egg drop syndrome - 76 was detected in 65 wild ducks of eight species captured in different areas of Buenos Aires Province. Twenty three (35 per cent) were serologically positive by HI test. The reactions were confirmed with the serum neutralization test (Risso *et al.*, 1985).

Riddel *et al.* (1992) isolated adeno virus from goslings between four and eleven days of age. The most common clinical signs exhibited by the bird was respiratory distress. The postmortem lesions were white opaque plug of fibrin and cellular debris in the trachea. The tracheal epithelium was hyperplastic and metaplastic with intranuclear inclusion bodies in the superficial cells. Adeno virus was isolated in both chicken and goose embryo liver cells.

2.1.5 Herpes virus (Duck plague/duck virus enteritis)

Wild ducks, geese and mute swans which are the members of the family Anatidae were reported to be susceptible to duck plague virus (Van Dorssen and Kunst, 1955). They had also reported that Europeanteals, pintails, mallard and grey call ducks were resistant to fatal infection, though the virus could multiply in them as evidenced by antibody titration. Isolation of duck plague virus from wild ducks and swan was also recorded by Jansen (1964).

Leibovitz and Hwang (1968) reported an outbreak of duck plague in a duck farm on Long Island, New York in 1967, but the virus was isolated from wild ducks and swans upto 40 miles away from the original outbreak, indicating the role of wild ducks in the causation of duckplague. Asplin (1970) screened the serum samples from free flying wild waterfowl near Gloucester and Peterborough for antibodies against duck plague

virus. The three mallard serum samples he studied had significant neutralization titres which indicated the exposure of these birds to duckplague.

Outbreaks of duck viral enteritis in various duck species and swan in Great Britain was reported by Hall and Simmons (1972). They observed heavy mortality with symptoms of severe diarrhoea, listlessness, followed by collapse. Postmortem findings were haemorrhagic enteritis, necrotic membranes in the cloaca and oesophagus, petechiation of the subepidcardium, pancreas and liver. Eosinophilic intranuclear inclusion bodies were demonstrated in the liver, pancreas and intestine. The disease was confirmed by virus isolation and identification.

Snyder *et al.* (1973) confirmed duck viral enteritis in an outdoor aviary in San Francisco which resulted in the death of entire resident population of 46 muscovy ducks and other species of waterfowl. Though the clinical signs were not prominent, post-mortem examination revealed ulcerations with pseudomembranes in the small intestine, annular band ulceration in the small intestine, ulceration with plaque like pseudomembranes in the oesophagus and eosinophilic intranuclear inclusions in the liver. The diagnosis was confirmed by virus isolation and identification. A major epizootic of duck plague in freeflying waterfowl at Lake

Andes, South Dakota in January-February 1973 was reported (Procter *et al.*, 1975). Duck plague was diagnosed in black ducks, mallards, pintails, mallard hybrids, American widgeon, wood ducks and canada geese, indicating a general susceptibility to this disease. Clinical signs reported in mallards were droopiness, lethargy, weakness, reluctance to fly, swimming in cricles, bloody diarrhoea, bloody fluid draining from the nares and bills and terminal convulsions. Burgess *et al.* (1979) carried out a surveillance of healthy waterfowls and reported them to be carriers of duck plague. Black ducks (*Anas rubripes*) and canada geese (*Branta canadensis*) surviving a natural outbreak of duck plague at Coloma, Wisconsin, in 1973 yielded the same isolate in the cloacal swabs four years after infection. Experimental infection of previously unexposed mallard ducks (*Anas platyrhynchos*) with the coloma strain of duck plague virus also produced cloacal viral shedding for four years after infection. Another duck plague virus strain, "LA-SD (73)" from South Dakota was detected from cloacal swabs of pintail ducks (*Anas acuta*), gadwall ducks (*Anas strepera*), wood ducks (*Aix sponsa*) and canada geese (*Branta canadensis*) infected experimentally one year earlier. Erosions also occurred at the openings of the sublingual salivary gland ducts in all species tested except canada geese and gadwall ducks.

An outbreak of duck virus enteritis (duck plague) in a captive flock of mixed waterfowl was reported by Montgomery *et al.* (1981). The captive waterfowls were mallards (*Anas platyrhynchos*), black ducks (*Anas rubripes*) and canada geese (*Branta canadensis*). Although, all the three species were housed together, morbidity and mortality were confined to the black ducks and canada geese. Gross lesions of black ducks and canada geese, which died were epicardial haemorrhage, haemorrhage on the surface of heart, haemorrhagic bands of the intestine, pale foci in the liver, necrosis of the cloacal lining and raised plaque-like areas of the oesophagus.

Kaleta *et al.* (1983) isolated duck plague virus from two dead muscovy ducks (*Cairina moschata*) in an extensively maintained reserve of free living waterfowl. The post mortem lesions showed grossly enlarged liver with minute greyish yellow foci, enlarged spleen and acute catarrhal enteritis. Virus was isolated in chicken embryo fibroblast culture from liver homogenate.

An outbreak of duck virus enteritis in domesticated mallards, pekin ducks and geese in Denmark in 1982 was reported by Prip *et al.* (1983), in which the virus was isolated by inoculating ducklings.

Balla (1984) studied the pathogenicity of duck plague virus for ducklings and geese using KLM7 strain of DPV

(isolated from geese). When injected subcutaneously, it produced 100 per cent mortality by 28-45 days, while strain KPV8 (isolated from ducks) was less virulent. However KLM7 and KPV8 strains obtained after seven and eight passages respectively in day-old ducklings killed 74.3 per cent of birds at 48-70 days and 86.7 per cent at 120-155 days, respectively.

Gough (1984) in his studies on duck virus enteritis in the United Kingdom from 1977 to 1982 observed that most of the nineteen outbreaks confirmed in captive waterfowls were associated with contact with migratory waterfowls, particularly male mallards. It was also observed that the most sensitive system for virus isolation was muscovy duck embryo fibroblasts.

Lin *et al.* (1984) isolated a herpes virus from waterfowl dying from duck viral enteritis from California, in April 1983 and was designated as Sheridan-83. It was serologically related to the Holland and Lake Andes strains of duck enteritis virus. Sheridan-83 was nonpathogenic to ducks, but ducks inoculated with this virus developed resistance to challenge with virulent Lake Andes strain.

Gough and Alexander (1987) analysed the conditions under which duck enteritis virus isolations were made in Great Britain during 1977 to 1983. Most of the outbreaks were

recorded in captive waterfowl, including muscovy ducks, in non commercial waterfowls from private collections, municipal parks, zoological gardens, wild fowl reserves and game farms. The arrival of freeflying waterfowl, usually mallard drakes, one to two weeks before losses occurred was commonly observed in many outbreaks. It was suggested that free-range commercial ducks and geese that might come in contact with wild waterfowl between April and June were at considerable risk. The authors opined that the carrier status of mallards about four years after infections and the seasonal incidence of duck viral enteritis outbreaks suggested stress factors such as breeding, sexual activity and over crowding contributing to the epizootiology of this disease. Wobeser (1987) carried out an experimental infection with liver tissue from a natural case of duck plague in a wild mallard (*Anas platyrhynchos*). Ten adult blue winged teal (*Anas discors*) and six canada goslings (*Branta canadensis*) were inoculated with the liver homogenate. Four additional teals were placed in contact with the inoculated teals. Inoculated teals died, 63.5-68 hr after inoculation, contact teals and goslings were killed when moribund 90-133 hr after infection. The clinical course was extremely rapid in both the species. Signs were limited to sudden onset of profound weakness, ataxia, tremors and terminal convulsions. The consistent gross lesion in the teal was a small dark spleen and also had inconspicuous foci of epithelial necrosis in the distal oesophagus and in the

cloaca. Goslings had focal hepatic necrosis and mucosal necrosis over the intestinal lymphoid tissue and intestinal hemorrhage.

Brand and Docherty (1988) carried out a surveillance during 1979-86 in waterfowls surviving duckplague outbreaks. Duckplague virus was found in combined oral and cloacal swabs of birds from three outbreaks, and duckplague neutralizing antibody was detected in some birds from all nine outbreaks. When freeflying waterfowl from within 52 km of four duckplague outbreak sites were also sampled, virus was not found in any birds, but DPV antibody was found in urban waterfowl close to an outbreak in Potterville, Michigan.

Barr *et al.* (1992) isolated duck virus enteritis from muscovy and mallard ducks which died over a three week period. Gross and microscopic changes were compatible with duck virus enteritis and the virus was isolated from the intestines. In addition to intranuclear viral inclusion bodies in several tissues, intracytoplasmic inclusion bodies were present in the oesophageal and cloacal epithelium. By electron microscopy, the membrane bound intracytoplasmic inclusions were found to contain enveloped herpes virus and nuclei contained herpes viral nucleocapsids.

In an investigation of duck plague in wild birds during the period from 1980 to 1988, Ziedler and Hlinak (1992)

reported the incidence of DP in wildbirds of 15 species. The virus was isolated from nine birds and positive antibody was detected in 20 serum samples.

Vanrompay *et al.* (1992) reported isolation of duck plague virus from a disease outbreak in a colony of 120 aquatic birds (wild ducks, geese and swans) in April 1991. The authors isolated DPV from a mute swan, two geese and a duck.

Spieker *et al.* (1996) carried out a study to assess the virulence of Lake Andes strain of duck plague virus to waterfowls. Susceptibility of the waterfowls to this virus was assessed by intramuscular inoculation of adult muscovies (*Cairina moschata*), mallards (*Anas platyrhynchos*), Canada geese (*Branta canadensis*), wood ducks (*Aix sponsa*), red heads (*Aythya americana*), gadwalls (*Anas strepera*), blue-winged teal (*Anas discors*) and pintails (*Anas acuta*). Based on mortality, Lake Andes strain was pathogenic to blue winged teal, wood ducks and red heads; muscovies and gadwalls were moderately susceptible, while mallards and canada geese were less susceptible, and pintails the least susceptible. From the results the authors opined that as in the case of European water fowls the North American waterfowls also had varying susceptibility to DP virus.

2.2 Characterisation

2.2.1 Thermostability

Temperature sensitivity is one of the properties studied in the characterization of viruses. Influenza A viruses are considered to be temperature sensitive. They get inactivated at 56°C for 30 min (Merchant and Packer, 1967), Papparella *et al.*, 1969). Hess and Dardiri (1968) reported complete inactivation of duckplague virus in 10 min at 56°C and in 90-120 min at 50°C.

Rosenberger *et al.* (1975) isolated four Newcastle disease virus from canada geese in the Atlantic migratory route. The haemagglutinin of two of the isolates were stable at 56°C for 15 min, one for 30 min, and the other for atleast two hours.

Nine avian paramyxoviruses isolated from tracheal and cloacal swabs of feral ducks and geese were examined for their thermostability. Eight of the isolates possessed haemagglutinin that was stable for less than 15 min at 56°C, whereas that of the remaining virus was stable between 15 and 30 min (Cloud and Rosenberger, 1980).

2.2.2 pH stability

The pH stability is another criterion used for grouping viruses. Exposure of duck plague virus (DPV) for 6 hr at pH levels of 7, 8 and 9 resulted in no loss of titre, but a measurable titre reduction was noted at pH 5, 6 and 10. At pH 3 and 11 DPV was rapidly inactivated. A marked difference in inactivation rates was noted between pH 10 and 10.5 (Hess and Dardiri, 1968).

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.* (1989). Influenza viruses were found to be labile at pH 3.0 when held at room temperature for a period of one hr (Papparella *et al.*, 1969; 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.

Rinaldi *et al.* (1968) have presented a comprehensive review of stability studies of avian adeno virus. There was an insignificant loss in virus infectivity at pH 3. Newcastle disease virus had a rather broad stability in presence of varying hydrogen ion concentrations. Infectivity of NDV was

lost at pH 3 and was retained at pH 10 (Sulochana *et al.*, 1981).

2.2.3 Chloroform sensitivity

Choloroform sensitivity holds an important position in the characterization of viruses, as it helps in identifying whether the virus is an enveloped virus or a naked one.

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

Duck hepatitis virus resisted treatment with ether or chloroform (Pollard and Starr, 1959).

Benton *et al.* (1967) found that infectious bursal disease virus resisted treatment with ether and chloroform. Herpes viruses containing considerable amount of lipids in their envelope are sensitive to chloroform (Roizman and Roane, 1963). Hess and Dardiri (1968) found duck plague virus to be sensitive to ether and chloroform.

2.2.4 5-iodo-2-deoxyuridine

This drug is used to know whether the nucleic acid is DNA or RNA; if a DNA virus comes in contact with IudR it is inactivated and so no multiplication, or Cytopathic effect (CPE) is seen in the monolayer. The structural base of avian adeno virus was reported to be DNA by Kawamura *et al.* (1964), and confirmed by Burke *et al.* (1968). Kunst (1968) found that the replication of duck plague virus and the development of cytopathic effect (CPE) could be inhibited by IudR. From its sensitivity to deoxyribonuclease and insensitivity to ribonuclease, Breese and Dardiri (1968) considered the duck plague viral nucleic acid as DNA type. Acridine orange staining of infected cell cultures also showed characteristic nuclear fluorescence indicative of DNA (Hess and Dardiri, 1968). Presence of DNA was substantiated by the same authors by staining with acridine orange. Fluorescence of intranuclear inclusion bodies of DPV in cell cultures stained with acridine orange indicated the presence of DNA (Hess and Dardiri, 1968). From studies using labelling with 3H Thymidine and inhibition with iododeoxyuridine, EDS 76 virus was found to contain DNA (Todd and McNulty, 1978, Yamaguchi *et al.*, 1981).

2.2.5 Pathotyping

Paramyxoviruses particularly avian strains are usually grouped into lentogenic, mesogenic or velogenic based on the intracerebral pathogenicity index (ICPI), mean death time in chicken embryos (MDT) and intravenous pathogenicity index (IVPI) commonly known as pathotyping (Poultry Biologics, 1963).

2.2.6 Haemagglutination

It was Hirst in 1941 who first showed that influenza virus could agglutinate chicken red cells. Later on it was found that all strains of influenza virus agglutinated erythrocytes of chicken, guniea pigs, human and many other species.

Winslow *et al.* (1950) reported that NDV agglutinated erythrocytes of all amphibia, reptiles and birds, but some mammalian erythrocytes were inagglutinable. Human, mouse and guniea pig erythrocytes were agglutinated by NDV, while those of cattle, goats, sheep, swine and horses were agglutinated by some but not all strains of NDV.

Safonov *et al.* (1964) have reported that fowl plague virus agglutinated erythrocytes of sheep, horse, cattle, man, fowl, pigeon, guniea pig, rat and mouse.

2.2.7 Propagation in cell cultures

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 hr. Niven *et al.* (1962) reported that the cytopathic effects of influenza virus in cellculture consisted of rounding and eventual detachment of cells.

Rosenberger *et al.* (1975) studied the pathogenicity of four isolates of NDV obtained from canada geese in chicken embryo fibroblast (CEF) cell cultures. All the four isolates were found to produce plaque in CEF culture.

Paramyxovirus isolates from wild birds were tested for their pathogenicity in chicken embryo fibroblast monolayer (Telbis *et al.*, 1989). The isolates were found to produce different plaque morphology in chicken embryo fibroblast monolayer.

2.2.8 Electron microscopy

The electronmicroscopic morphology of the viruses were as per the details given in the fourth report of the International Committee on Taxonomy of Viruses (Mattews, 1982).

Paramyxovirions are pleomorphic, usually roughly spherical or filamentous, with a diameter of 150 nm or more. They consist of a nucleocapsid of helical symmertry, 13-18 nm in diameter, which is surrounded by an envelope derived from the cell surface membranes. The envelope possesses two types of glycoproteins, 8-12 nm in length, exposed as projections at the viral surface, and one or two unglycosylated proteins.

Typical virions of influenza A virus are spherical and about 100 nm in diameter, but larger, more pleomorphic forms are commonly seen. It is an enveloped virus.

The capsid of adenovirus is an icosahedron 70-90 nm in diameter, composed of 252 capsomers

Herpes virus virions are enveloped and about 150 nm in diameter. The capsid is an icosahedron, 100 nm in diameter, composed of 162 hollow capsomeres. The envelope of the virion is some what pleomorphic with a diameter of 120 to 200 nm.

The picorna virus virion is a non enveloped icosahedron 25-30 nm in diameter.

Materials and Methods

3. MATERIALS AND METHODS

MATERIALS

3.1 Reagents

All the chemicals used in this study were of analytical grade.

3.1.1 Tryptose phosphate broth (TPB-A)

Readymade media purchased from Hi-media, Bombay were reconstituted as per the manufacturer's instructions and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 min. Penicillin (500 IU/ml) and Streptomycin (500 µg/ml) were added to the medium before it was used for collection/dilution of specimens for virus isolation.

3.1.2 Phosphate buffered saline (PBS)

PBS was prepared as per the procedure described by Bishai *et al.* (1974).

3.1.3 Calcium magnesium free phosphate buffered saline (CMF-PBS)

Readymade media purchased from Hi-media, Bombay were reconstituted as per the manufacturer's instructions and sterilized by autoclaving at 15 lbs at 121°C for 15 min.

3.1.4 Lactalbumin Hydrolysate (DIFCO)

Lactalbumin hydrolysate was added to tissue culture medium at 0.5 per cent level before sterilization.

3.1.5 Yeast extract (DIFCO)

Yeast extract, 0.2 per cent, was prepared in tissue culture medium before sterilization.

3.1.6 Calf serum

Blood was collected from calves, below two months of age, maintained at the University Livestock Farm, Mannuthy and the serum was separated. This serum was clarified, sterilized by filtration, inactivated at 56°C for 30 min, distributed into small aliquots and was stored at -20°C.

3.1.7 Hanks balanced salt solution (HBSS)

Readymade media purchased from Hi-media, Bombay were reconstituted as per the manufacturer's instructions. This was autoclaved at 10 lb pressure for 45 min or was sterilized by filtration. The pH of this solution was adjusted to 7.4 with 7.5 per cent sodium bicarbonate solution.

3.1.8 Cell culture growth medium

The growth medium was prepared by adding five per cent calf serum to HBSS containing 0.5 per cent Lactalbumin hydrolysate and 0.2 per cent yeast extract. Antibiotics at the rate of 250 IU of penicillin and 250 μg of streptomycin per ml were also added.

3.1.9 Maintenance medium

Same as 3.1.8 except that the serum concentration was reduced to two per cent.

3.1.10 Trypsin

A 0.2 per cent trypsin (1:250 Difco) was prepared in HBSS and sterilized by filtration using seitz filter pads.

3.1.11 7.5 per cent sodium bicarbonate solution

Prepared as per the method given by Bishai *et al*. (1974).

3.1.12 Antibiotic solution

Streptomycin	-	1 g
Penicillin	-	1,000,000 units
Sterile distilled water/HBSS	-	40 ml

One ml of this solution was added to 100 ml of tissue culture medium to give a final concentration of 250 IU of penicillin and 250 μg of streptomycin per ml.

3.1.13 Chloroform

Obtained from Sisco Research Lab (SRL) was used.

3.1.14 Normal saline

Prepared by adding 0.9 g of sodium chloride (SRL) in 100 ml of double glass distilled water and sterilized by autoclaving.

3.1.15 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.

3.1.16 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 solutions. Solution A 87.7 ml 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 min.

3.1.17 5-iodo-2-deoxyuridine (IudR)

5-iodo-2-deoxyuridine from Koch-Light laboratories, England, was used for the study.

3.1.18 May-Grunwald giemsa stain

May-Grunwald stain 2.5 gm was dissolved in 100 ml of absolute methanol and allowed to age for one month. Giemsa stain 1 gm was dissolved in 66 ml of glycerol at 55°C overnight and added 66 ml of absolute methanol.

3.2 Collection of specimens

The specimens such as cloacal swab, throat swab, trachea, intestine, liver, spleen, lung and brain collected from 52 teals caught from Kumarakom and Nedupuzha areas were used in the present study. TPB containing 500 μg of streptomycin and 500 IU of penicillin per ml was added to all the specimens and stored at -20°C until used. Blood smears and impression smear from the cut surface of livers were also taken for direct

examination for the presence of bacteria and for intranuclear inclusion bodies.

3.3 Hen eggs

Hatching eggs from White Leghorn/Austrawhite breeds received from the University Poultry Farm, Mannuthy and All India Co-ordinated Research Project for poultry improvement (A.I.C.R.P), Mannuthy were utilized for this work.

3.4 Chicks

Day old unvaccinated male chicks received from A.I.C.R.P., Mannuthy were used for assessing intracerebral pathogenicity index. Day old unvaccinated male chicks were also maintained till they attained the appropriate age for pathogenicity study.

3.5 Duck eggs

Duck eggs for this study were received from the University Poultry Farm, Mannuthy, Government Duck Farm, Niranam or from the local market.

3.6 Ducklings

Day old ducklings (Desi) used for this study were purchased from the local market and were maintained till they attained the required age for pathogenicity study.

3.7 Red blood cells for haemagglutination

3.7.1 Chicken RBC

Blood from the wing vein of cockerels was collected in Alsever's solution. It was centrifuged at 1000 rpm for 10 min. The suspended RBC's were washed thrice in sterile normal saline and resuspended in fresh normal saline to get a 0.5 per cent/one per cent concentration for haemagglutination test.

3.7.2 Red cells from other species

Blood from the jugular vein of cattle and sheep, the ear vein of rabbits and pigs, the wing vein of ducks and the cubital vein of man was collected in Alsever's solution and processed (3.7.1).

METHODS

3.8 Processing of the specimens

3.8.1 Cloacal swab/throat swab

Swabs soaked in Tryptose phosphate broth containing antibiotics (TPB-A) stored at -20°C were allowed to thaw at room temperature. These 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 was centrifuged at 2000 rpm for 10 min. The clear supernatant fluid was inoculated into 11-12 day old duck embryos by CAM route of inoculation and 9-10 day embryonated chicken eggs by allantoic route.

3.8.2 Trachea/intestine

Portions of the trachea and intestine were cut open and the contents scrapped with a clean slide, which was then triturated with a pestle and mortar, centrifuged at 2000 rpm for 10 min and was inoculated by the CAM route to 11-12 day old duck eggs and by allantoic route of 9-10 day old chicken eggs.

3.8.3 Tissues

At the time of use the tissues were emulsified in TPB-A with the help of a pestle and mortar and sterile silica gel to obtain a 15-20 per cent w/v suspension. This homogenate was then clarified, incubated for one hour after adding antibiotics and was used for inoculation into embryonated eggs.

Liver and spleen were processed and inoculated into 11-12 day old duck embryos by the CAM route and the processed brain and lungs were inoculated into 9-10 day old chicken eggs by the allantoic route.

3.9 Inoculation of embryonated chicken/duck eggs

In all the cases three embryos were inoculated per sample.

3.9.1 Allantoic cavity inoculation

The procedure described by Bishai *et al.* (1974) was adopted. The position of the air cell and the embryo of 9-10 day embryonated chicken eggs, was marked. After disinfecting the air cell region with tincture of iodine, a hole was drilled at 0.5 cm towards the centre from the base of the air cell, and 0.2 ml of the inoculum was introduced into the

allantoic cavity, with the help of a tuberculin syringe and a 22 gauge needle. The hole was then sealed with melted paraffin, and the eggs were incubated at 36-37°C in an upright position. Control eggs were also similarly treated with 0.2 ml of sterile normal saline. All the eggs were examined daily. The embryos that died after 24 hr, and those alive after 6 days, were transferred to the refrigerator.

3.9.2 Chorioallantoic membrane method

The procedure described by Betts (1967) was followed. Eleven to twelve day old embryonated duck eggs were candled, and the air cell, the embryo, and an area on the side, free from large blood vessels, were located. The air cell region and the spot marked on the side, were sterilized with tincture of iodine. With the help of a dental drill, a hole was made at the spot on the side of the shell, taking care not to puncture the shell membrane. Another hole was made at the centre of the air cell, to pierce both the shell and shell membrane. A drop of sterile saline was placed on the hole on the side of the egg and the fibres of the shell membranes were gently separated apart. Just below this hole, an artificial air cell was created. After checking the false air cell by candling, 0.2 ml of inoculum was dropped on to the CAM. Both the openings were sealed with melted paraffin. The eggs were gently rotated for a couple of times, to spread the inoculum

over the entire CAM. Control eggs were similarly treated except that 0.2 ml of sterile saline was used in place of the inoculum. All the eggs were incubated at 36-37°C in a horizontal position with the false air cell upwards. The eggs were candled every morning and evening. The embryos that died after 24 hr and those alive after six days, were chilled at 4°C before harvesting.

3.10 Harvesting of the inoculated eggs

The air cell of the embryonated eggs were disinfected with 70 per cent alcohol. The air cell region was cut and removed with sterile scissors. The shell membrane and chorio allantoic membrane were peeled off and allantoamniotic fluid was collected aseptically with sterile pipettes. The fluid from each embryo was tested by rapid plate agglutination using one per cent of chicken, duck, pig, rabbit, cattle, horse and human `O' RBC. The embryos and CAM were further examined for any lesions.

All the negative samples were passaged thrice in the same system before they were declared as negative.

3.11 Non-haemagglutinating agents

In cases where there was no haemagglutination but lesions were seen on the embryo, the following tests were done to detect the presence of the virus.

3.11.1 Impression smears from the cut surface of the embryo liver were stained with May-Grunwald Giemsa for inclusion bodies.

3.11.2 The embryo fluid, CAM and whole embryo were processed and inoculated into chicken embryo fibroblast/duck embryo fibroblast cultures and kept under observation for a period of six days for the development of any cytopathic effect.

3.11.3 The above processed sample was inoculated into one week and six week old ducklings/chick and kept under observation for three weeks for any clinical symptoms.

3.12 Characterization of haemagglutinating isolates

3.12.1 Haemagglutination

The method adopted was that given in Poultry Biologics (1963). Two fold dilutions of the viruses were made in normal saline in perspex haemagglutination plates. Equal quantity of 0.5 per cent washed chicken RBC was added to each dilution. RBC control was prepared simultaneously. The plates were incubated at room temperature for 30 min. The readings were recorded after the red cells in the control wells had settled.

3.12.2 Embryo infective dose 50 (EID50)

Serial ten fold dilutions of the isolates were made in TPB-A. Each dilution was then inoculated at the rate of 0.2 ml/embryo into nine day old embryonated chicken eggs using four 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).

3.12.3 Thermostability

The isolates were distributed in one ml volumes into screw capped vials and immersed in a water bath at 56°C for 30 min. The treated and untreated samples were titrated as described above. The isolates exposed to 56°C for 30 min were tested for its haemagglutinating activity and compared to that of the untreated sample.

3.12.4 pH sensitivity

One in ten dilutions of the isolates were made in citrate phosphate buffer (pH 3.2) and phosphate buffer pH 7.2 and pH 9.0 and kept at room temperature for one hr. After this period virus titrations were made in 9 day old embryonated chicken eggs, (0.2 ml per embryo), using three eggs per

dilution. The untreated samples were also titrated simultaneously. Post inoculation incubation, candling and harvesting were done as described earlier.

The isolates treated with citrate phosphate buffer (pH 3.2) and phosphate buffer (pH 7.2 and 9.0) were also tested for haemagglutination and they were compared to that of the untreated sample.

3.12.5 Chloroform sensitivity

Chloroform sensitivity was done as per the method of Feldman and Wang (1961). Two ml each of the infected allantoic fluid was mixed with 0.1 ml of chloroform and kept at room temperature for 10 min with intermittent shaking and centrifuged at 2000 rpm for 10 min. The chloroform then appeared at the bottom of the tube and the supernatant clear fluid was separated with a pasteur pipette and used for titration of haemagglutination activity and infectivity as described above.

3.12.6 Propagation in chicken embryo fibroblasts/duck embryo fibroblasts

3.12.6.1 Chicken embryo fibroblast cultures

The procedure followed was that of Cunningham (1966). Eleven day embryonated chicken eggs were selected. After

cleaning the shell at the air cell region it was cut with sterile scissors, the shell membrane and the chorio allantoic membrane were torn and the embryo was lifted with sterile forceps and transferred into a sterile petridish containing HBSS-A. The head and limbs 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 trypsinisation flask containing 0.2 per cent trypsin in HBSS-A at the rate of 5 ml/embryo. Incubated for 10-16 min with intermittent shaking at 37°C. The supernatant was poured off, fresh trypsin was added and then kept for 20-30 min at 37°C with intermittent shaking. The supernatant was removed and kept at 4°C after adding foetal calf serum to a final concentration of five per cent. The procedure was repeated, the supernatant was collected and pooled and kept at 4°C for 30 min. The resulting cell suspension was filtered through a double layered muslin cloth. The filtrate was centrifuged at 800 rpm for 8 to 10 min, discarded the supernatant, resuspended the cells in growth medium and recentrifuged. Like wise 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 $1x10^6$ cells/ml.

The above cell suspension was seeded into test tubes containing coverslips at the rate of one ml per tube and incubated at 37°C in slanting position. When a satisfactory

monolayer was obtained it was used for studying the cytopathic effect (CPE).

3.12.6.2 Duck embryo fibroblasts

The procedure followed was the same as above except that 12-13 day old duck embryos were used for preparing the duck embryo fifbroblast cultures.

3.12.6.3 Inoculation of the cell culture

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 isolate/ sample was added at the rate of 0.2 ml per tube and incubated at 37°C for one hr to facilitate adsorption. At the end of this period, the inoculum was poured off, washed with maintenance medium and added maintenance medium at the rate of 1.5 ml per tube. Control tubes were similarly treated except that instead of the inoculum, 0.2 ml of the maintenance medium was added. At twenty-four hr intervals the tubes were examined for CPE under an inverted microscope. When the CPE was evident, the coverslip cultures were collected at intervals of 24 hr, 36 hr and 72 hr, fixed in formol saline for 24 hr and stained with May-Grunwald's Giemsa for 40 min to demonstrate CPE/inclusion bodies.

Negative samples were subjected to three blind passages before they were declared as negative.

3.12.6.4 Staining of coverslip cultures

The coverslip cultures were fixed overnight in formol saline. After fixing it was stained for 10 min in May-Grunwald stain and for 20 min in dilute Giemsa solution (1:10 in distilled water). The coverslip cultures were then rinsed rapidly in two changes of acetone and again in two parts acetone and one part xylol for five sec. The coverslips were then placed in one part acetone and two parts xylol for one min and cleared in two changes of xylol for two min each, mounted on a clean slide and examined for CPE/inclusion bodies.

3.13 Sensitivity to 5-iodo-2 deoxy uridine (IudR)

The method described by Nath *et al.* (1971) was followed in this case. The growth medium was poured off, the monolayer was washed and replaced with maintenance medium containing 100 micrograms per ml of IudR. Control tubes were also kept with the same medium but without the drug. All the tubes were incubated at 37°C for four hr and then inoculated with 0.2 ml of the isolates. Again incubated for one hr at 37°C for adsorption of the isolates onto the cells. The inoculum was poured off and the maintenance medium was added as above and

incubated. Absence of cytopathic effect in the presence of the drug, compared to that of the control tubes, was taken as an indication of inhibition of viral multiplication. Newcastle disease virus, a known RNA virus was used simultaneously for comparison.

3.14 Pathotyping

Pathotyping of isolates was carried out as per the procedure given in Poultry Biologics (1963).

3.14.1 Mean death time at terminal dilution (MDT)

Serial ten fold dilutions, upto 10^{-9} of the isolates were prepared in TPB-A and inoculated into nine day embryonated chicken 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 hr intervals and the death time of the embryos was noted. The mean death time was then calculated.

3.14.2 Intra cerebral pathogenicity index (ICPI)

Ten, day old chicks were inoculated intracerebrally with 0.05 ml each of 1:10 dilution of isolates 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.

3.14.3 Intravenous pathogenicity index (IVPI)

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

3.15 Pathogenicity

Pathogenicity of the haemagglutinating isolates were carried out in one week-old chicks/one week-old ducklings (nine numbers each) and six week-old chicks/six week-old ducklings (sixteen numbers each).

3.15.1 Experiment I

Nine, one week-old chicks were used for this study. They were divided into three groups of three each. Group A received 10^7 EID50 of T_{18} and Group B 10^6 EID50 of T_{22} isolate,

both by oral (1 ml) and subcutaneous route (0.5 ml). Chicks in Group C served as uninfected controls. Following infection different groups were kept separately to avoid cross infection. All the birds were observed twice daily for the development of any clinical symptoms or death for a period of 3 weeks.

3.15.2 Experiment II

Nine, day-old ducklings were used for this study. Three of the ducklings received 10^7 EID50 of T_{18} and another three 10^6 EID50 of T_{22} both by oral (1 ml) and subcutaneous route (0.5 ml) and another three were kept as uninfected controls. After infection different groups were kept separately and extreme care was taken to prevent cross-contamination. The birds were observed daily for a period of three weeks for the development of any clinical signs or death.

3.15.3 Experiment III

Of the total sixteen six-week old chicks, six numbers each were inoculated with the undiluted embryo propagated isolates (T_{18} and T_{22}) at the rate of one ml per bird subcutaneously. The remaining four chicks were kept as controls and were given sterile saline inoculation. Cloacal and throat swabs were collected at 3, 5, 7, 19, 14, 21 and 28 days postinfection (P.I) for virus isolation as described

previously. Serum from the birds were collected and subjected to haemagglutination inhibition test of the end of the observation period for detection of antibodies. All the groups were kept in separate cages and extreme precautions were taken to prevent cross-contamination.

3.15.4 Experiment IV

Out of the sixteen ducklings, six number of six-week old ducklings were given 10^7 EID50 of T_{18} and the other six 10^6 EID50 of T_{22} both by oral (1 ml) and subcutaneous routes (0.5 ml). The remaining four birds were treated as controls and were inoculated with sterile saline. All the birds were observed for a period of six weeks. The different groups were kept separately and extreme care was taken to avoid cross contamination.

3.15.5 Antibody titration

The immune response to the above isolates was detected by Haemagglutination-inhibition test (HI) on the 28th day post infection using the corresponding isolate 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 in normal saline were mixed with 0.2 ml of four HA units of the virus and incubated at room

temperature for 20 min. After this time 0.4 ml of 0.5 per cent 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 serum in which there was complete inhibition of HA.

3.15.6 Antigenic relationship of the isolates with other viruses

Haemagglutination inhibition tests of the isolates were carried out with NDV-antiserum and EDS antiserum.

The antiserum against T_{18} and T_{22} were also tested against NDV, EDS-76 virus and Epizootic ulcerative syndrom virus (EUS) of fishes (FV and F6) by HI.

3.16 Electron microscopy

The embryo processed samples showing lesions and the allantoic fluid containing the isolates T_{18} and T_{22} were clarified twice by low speed centrifugation at 3000 rpm for 30 min at 4°C. The supernatant was subjected to ultracentrifugation at 28,000 rpm in a Beckman Type 28 rotor for 3 hr 15 min at 4°C. The pellet was resuspended in 0.2 ml of distilled water, centrifuged at 5000 rpm for 15 min and the supernatant was used for electron microscopic examination. One drop of this virus suspension was placed on a formavar

coated grid. After 45 sec, the excess fluid was absorbed in a Whatman No.1 filter paper. The grid was then stained with potassium phosphotungstate (PTA-one per cent aqueous solution, pH 6.5) for 35 sec. After blotting the excess PTA, the grid was dried at room temperature for 10 min and examined in a Hitachi 500 A electron microscope at 75 KV.

Results

4. RESULTS

4.1 Identification of waterfowls

There are different types of waterfowls that are seen in Kerala. They are Lesser whistling teals, Comb duck, Cotton teal, Common teal, Spot billed duck, Pintail and Gargany. The numbers of birds that visit Kerala vary between the years.

Migratory water birds from wetlands of Kerala were captured with permission from Chief conservator of forest, Government of Kerala. During this investigation a total of 52 waterfowls were screened for the presence of viruses.

Teals migrate to wetlands during December to April. Two seasons were included in this study i.e. from December 1995 to April 1996, twenty-two birds were captured. Among these 22, 15 were from Kumarakom bird sanctuary area and the remaining seven from Palghat area. The birds, particularly in the Kumarakom lake arrive in swarms. They roost during day time and are dawn and dusk feeders. They take off when they are disturbed and fly around in swarms (Fig.1).

The birds captured from this area were having the following characteristics, the forehead and crown brown, head-neck reddish-brown changing into brown on the scapulars

Fig. 1 Birds flying in swarms

Fig. 2 Lesser whistling teal

Fig. 3 Lesser whistling teal

Fig. 4 Lesser whistling teal

and back, where the feathers are broadly margined with golden rufous, rump black, tail brown; lesser and medium wing-coverts chestnut; greater wing-coverts darkashy; quills black; upper-breast yellowish-grey or yellowish-fulvous, this changing to chestnut on the breast and abdomen, bill slaty-grey to almost black, legs and feet plumbeous-grey or plumbeous-blue, the webs and claws blackish and were identified as Lesser whistling teals or locally callted "Cheolan eranda" (Fig.2,3,4).

Another type of waterfowl captured was identified as Garganey, locally known as "vari eranda". They were captured from Kuzhalmannam area of Palghat district. A total of seven numbers were procured from this area.

The characteristics of these birds were crow and nape deep brown; back, rump, upper tail coverts and tail brown, inner scapulars black, glossed with green and with broad white central streaks and narrow white edges; outer scapulars the same but with the outerwebs broadly blue-grey; wing-coverts bright pale french grey, the greater broadly edged with white, forming a wing-bar; outer secondaries brown-grey, glossed with green and tipped with white; breast brown with black or dark brown markings, concentric on the upper breast, in the form of bars on the lower breast; abdomen white, more or less speckeled and brown towards the vent; bill-brownish black, the

Fig. 5 Gargany

Fig. 6 Gargany

nail quite black; the legs and feet dark grey and were identified as Gargany (Fig.5 and 6).

During the second season attempts were made to capture the birds from Thrissur Kole lands. A total of 30 birds were captured from different localities of this wetland. All of them had the same characteristics as that of the birds caught from Palghat area and were identified as Gargany.

The number of birds captured from different areas are shown in the Table 1.

Table 1. Number of birds captured from different areas of Kerala

Area	Lesser whistling teal	Gargany
Kumarakom	15	-
Kuzhalmannam	-	7
Nedupuzha	-	30

3.2 **Bacteriological investigation**

The blood smears prepared from these birds were negative for any bacterial infections (Fig.7).

Fig. 7 Blood smear of teal

Fig. 8 Post-mortem examination of teal revealing the internal organs

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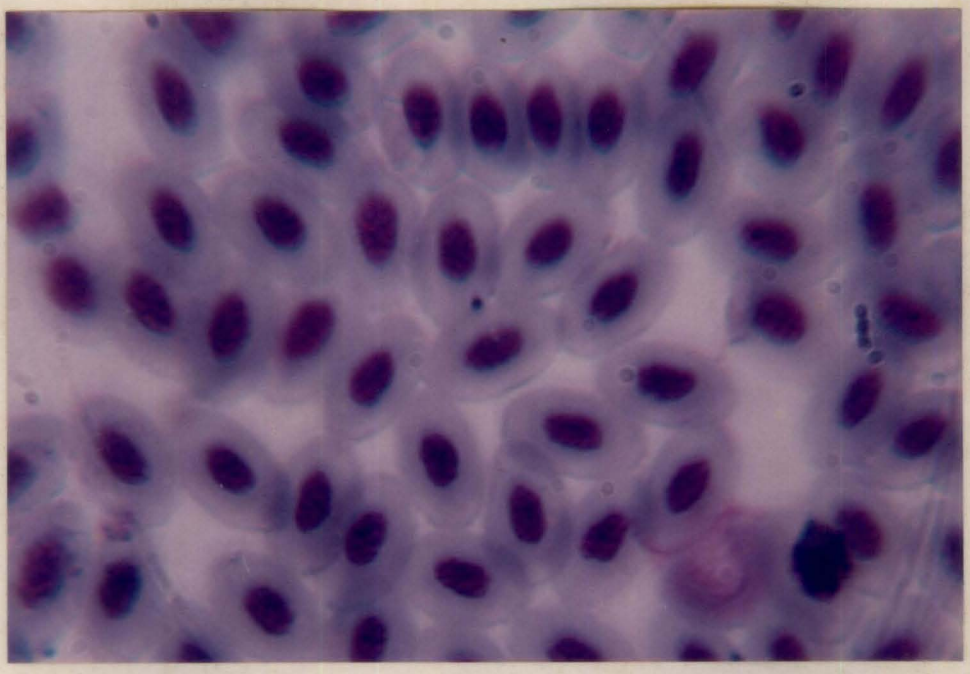
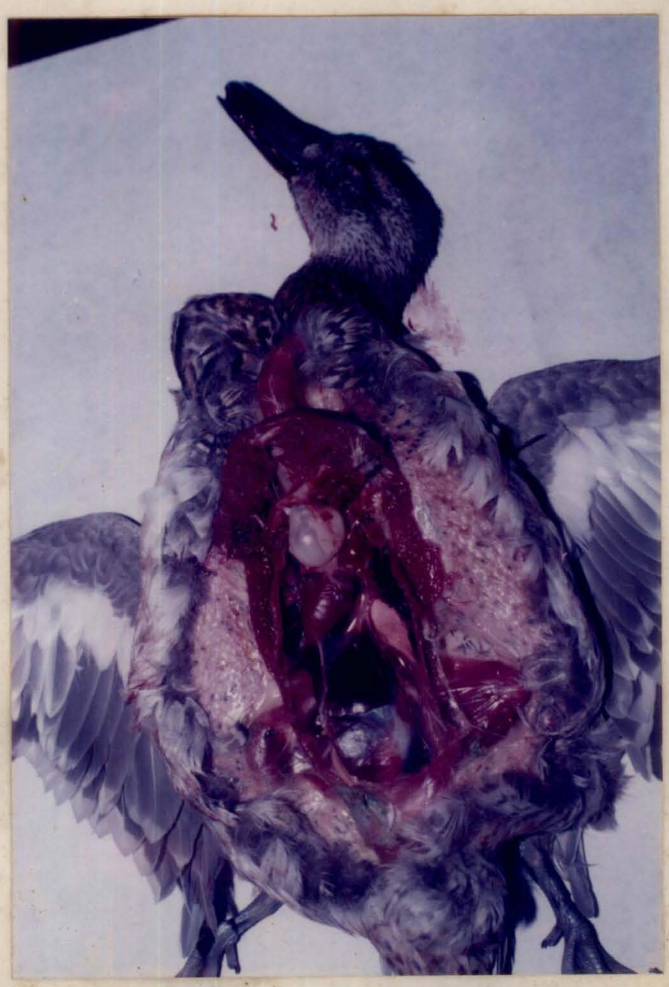




4.3 Parasitological examination

The intestinal contents were examined for the presence of any parasites. Most of them were negative except a few which showed the presence of trematodes of Genus Echinostoma and Cestodes of Genus Hymenolepis.

4.4 Virological investigation

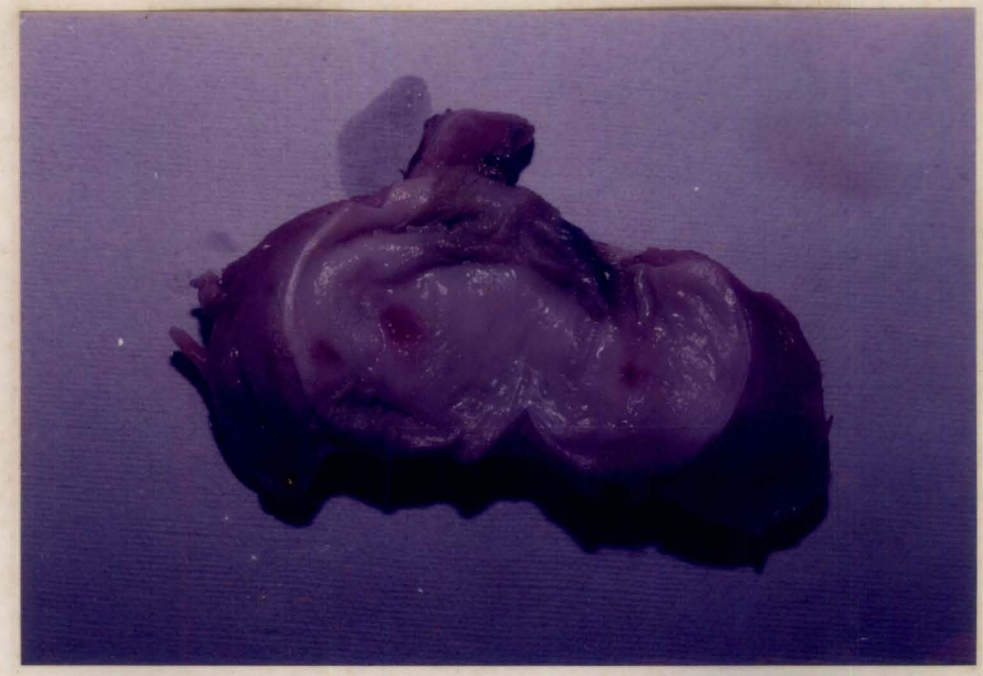
All the 52 birds were used for collection of specimens for virological studies. Postmortem examination of all these 52 birds were conducted and tissues collected in TPB containing 200 µg of streptomycin and 200 IU of penicillin/ml. The anatomical features of these birds were similar to that of ducks (Fig.8).

Of the 52 birds 10 had macroscopic lesions indicating some infection. The details of macroscopic lesions seen in these birds are given in Table 2. The common lesions noticed in the liver were congestion/enlargement/necrotic patches. Similarly enlargement and congestion of spleen, necrosis and ulcers of the gizzard musculature (Fig.9) and in two cases haemorrhagic enteritis were also noticed. In one case mucosa was found oedematous and haemorrhagic.

In most cases impression smears prepared from the cut surfaces of the liver did not show any indication of inclusion

Fig. 9 Ulceration and necrosis of the gizzard musculature

Fig. 10 Impression smear from the cut surface of the liver (Bird No.17) showing intranuclear inclusion bodies (large arrow)



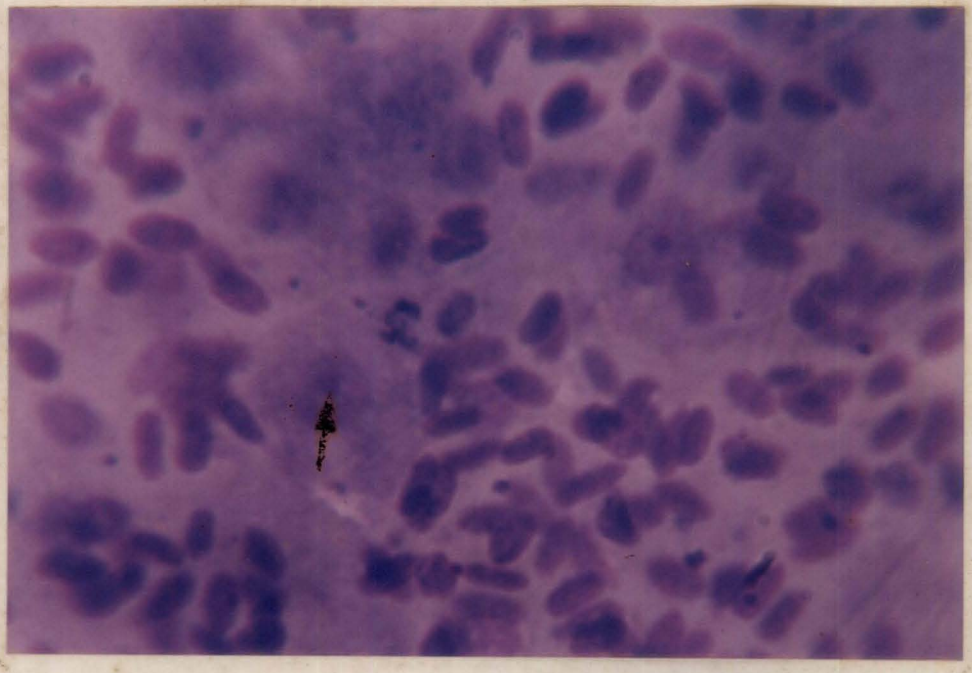
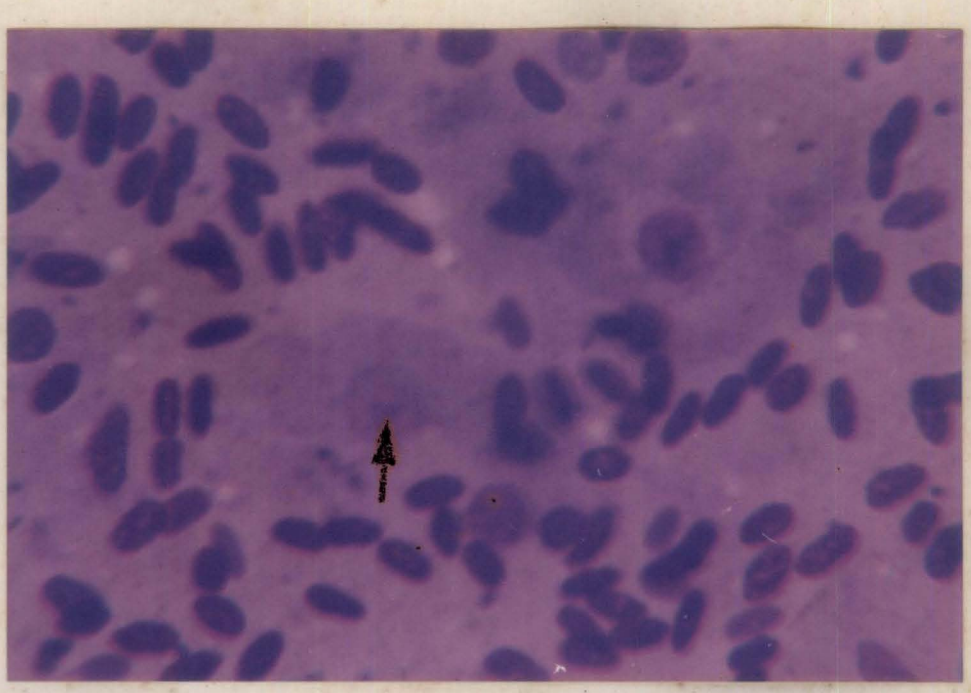


Fig. 11 Impression smear from the cut surface of the liver (Bird No.23) showing intranuclear inclusion bodies (large arrow)

Fig. 12 Impression smear from the cut surface of the liver (Bird No.27) showing intranuclear inclusion bodies (large arrow)



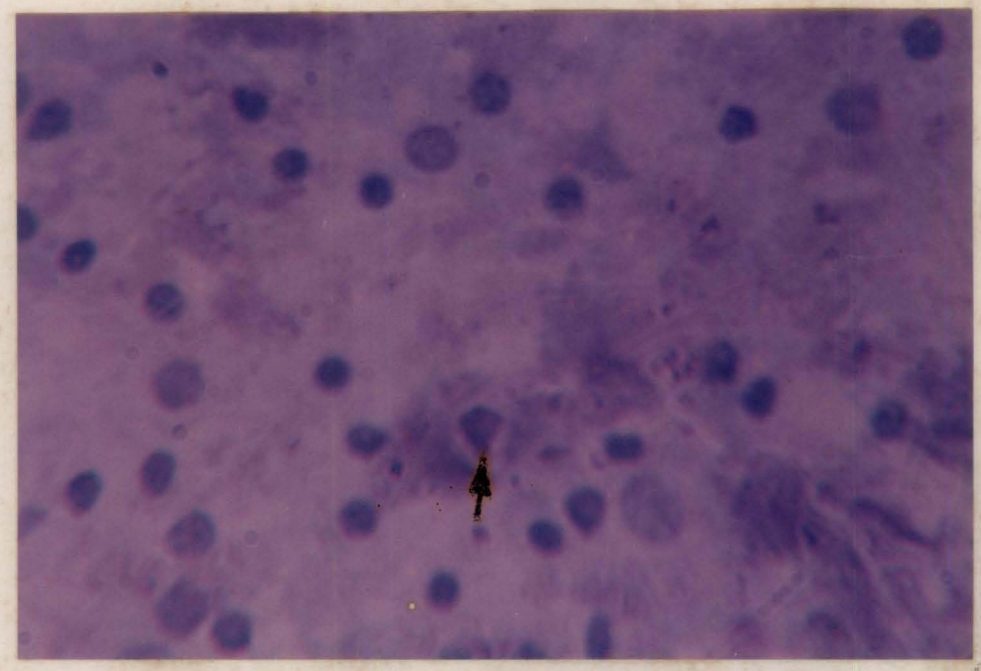


Table 2. Gross lesions observed during the post-mortem examination of teal

Bird No.	Lesions observed
Teal 11	Liver - white necrotic patches, spleen congested, gizzard musculature showed necrosis, haemorrhagic enteritis
Teal 13	Liver - Highly congested, gizzard musculature - showed necrosis
Teal 16	Liver - Congested, white necrotic patches, gizzard musculature - showed necrosis, haemorrhagic enteritis
Teal 17	Liver - pale, showed streaks of congestion at the borders. Gizzard musculature - showed necrosis
Teal 18	Liver - Dark red, spleen - small, highly congested, Gizzard musculature - necrotic patches
Teal 20	Heart - Enlarged, liver, spleen - enlarged, bursa - haemorrhagic enlarged
Teal 24	Liver - necrotic lesions
Teal 27	Liver - Streaks of hemorrhage, spleen - slightly enlarged
Teal 28	Liver - Focal areas of haemorrhage throughout the liver
Teal 33	Liver - haemorrhagic streaks

bodies either in the nucleus or in the cytoplasm. In cases viz. nos. 17, 23, 27 and 49 the hepatic cells had intranuclear inclusion bodies, but virus isolations could not be made from them (Fig.10,11,12,13).

4.4.1 Virus isolation

The specimens collected and processed from these birds were - liver, spleen, lungs, brain, cloacal swab, intestinal scrappings, throat swab and tracheal scrappings. Of these cloacal swab, intestinal scrappings, throat swab, tracheal scrappings and brain were processed and inoculated into 12 day old embryonated duck eggs by the CAM route and liver, spleen and lungs into 10 day old chicken eggs by the allantoic route. All the samples except those from numbers 18 and 22 were found to be negative for any viral isolates even after three blind passages. In the case of numbers 18 and 22 haemagglutinating agents were isolated from the cloacal swabs only in second and third passages. The isolates T_{18} and T_{22} were obtained from Lesser whistling teals caught from Kumarakoom during 1995 December-January 1996. The bird number 18 had revealed gross lesions during necropsy while bird number 22 did not exhibit any changes.

The results after three blind passages in chicken and duck embryos are given in Table 3.

Table 3. Result after three passages in duck/chicken embryo

Bird No.	Throat swab/ tracheal scrappings	Cloacal swab/ intestinal scrappings	Liver and spleen	Lung	Brain
1	-ve	-ve	-ve	-ve	-ve
2	-ve	-ve	-ve	-ve	-ve
3	-ve	-ve	-ve	-ve	-ve
4	-ve	-ve	-ve	-ve	-ve
5	-ve	-ve	-ve	-ve	-ve
6	-ve	-ve	-ve	-ve	-ve
7	-ve	-ve	-ve	-ve	-ve
8	-ve	-ve	-ve	-ve	-ve
9	-ve	-ve	-ve	-ve	-ve
10	-ve	-ve	-ve	-ve	-ve
11	-ve	-ve	-ve	-ve	-ve
12	-ve	-ve	-ve	-ve	-ve
13	-ve	-ve	-ve	-ve	-ve
14	-ve	-ve	-ve	-ve	-ve
15	-ve	-ve	-ve	-ve	-ve
16	-ve	-ve	-ve	-ve	-ve
17	-ve	-ve	Intra nuclear inclusions	-ve	-ve
18	-ve	+ve	-ve	-ve	-ve
19	-ve	-ve	-ve	-ve	-ve
20	-ve	-ve	-ve	-ve	-ve
21	-ve	-ve	-ve	-ve	-ve
22	-ve	+ve	-ve	-ve	-ve
23	-ve	-ve	Intra nuclear inclusions	-ve	-ve
24	-ve	-ve	-ve	-ve	-ve
25	-ve	-ve	-ve	-ve	-ve
26	-ve	-ve	-ve	-ve	-ve

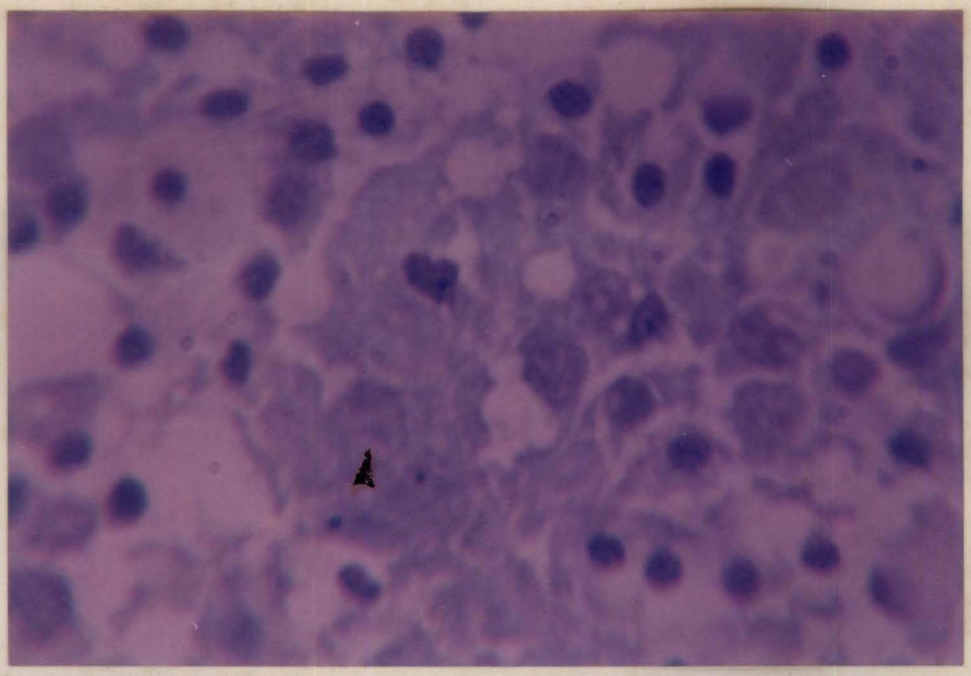
Contd...

Table 3. (Contd.)

Bird No.	Throat swab/ tracheal scrappings	Cloacal swab/ intestinal scrappings	Liver and spleen	Lung	Brain
27	-ve	-ve	Intra nuclear inclusions	-ve	-ve
28	-ve	-ve	-ve	-ve	-ve
29	-ve	-ve	-ve	-ve	-ve
30	-ve	-ve	-ve	-ve	-ve
31	-ve	-ve	-ve	-ve	-ve
32	-ve	-ve	-ve	-ve	-ve
33	-ve	-ve	-ve	-ve	-ve
34	-ve	-ve	-ve	-ve	-ve
35	-ve	-ve	-ve	-ve	-ve
36	-ve	-ve	-ve	-ve	-ve
37	-ve	-ve	-ve	-ve	-ve
38	-ve	-ve	-ve	-ve	-ve
39	-ve	-ve	-ve	-ve	-ve
40	-ve	-ve	-ve	-ve	-ve
41	-ve	-ve	-ve	-ve	-ve
42	-ve	-ve	-ve	-ve	-ve
43	-ve	-ve	-ve	-ve	-ve
44	-ve	-ve	-ve	-ve	-ve
45	-ve	-ve	-ve	-ve	-ve
46	-ve	-ve	-ve	-ve	-ve
47	-ve	-ve	-ve	-ve	-ve
48	-ve	-ve	-ve	-ve	-ve
49	-ve	-ve	Intra nuclear inclusions	-ve	-ve
50	-ve	-ve	-ve	-ve	-ve
51	-ve	-ve	-ve	-ve	-ve
52	-ve	-ve	-ve	-ve	-ve

Fig. 13 Impression smear from the cut surface of the liver (Bird No.49) showing intranuclear inclusion bodies (large arrow)

Fig. 14 Severely congested embryo and CAM (infected with T_{18})



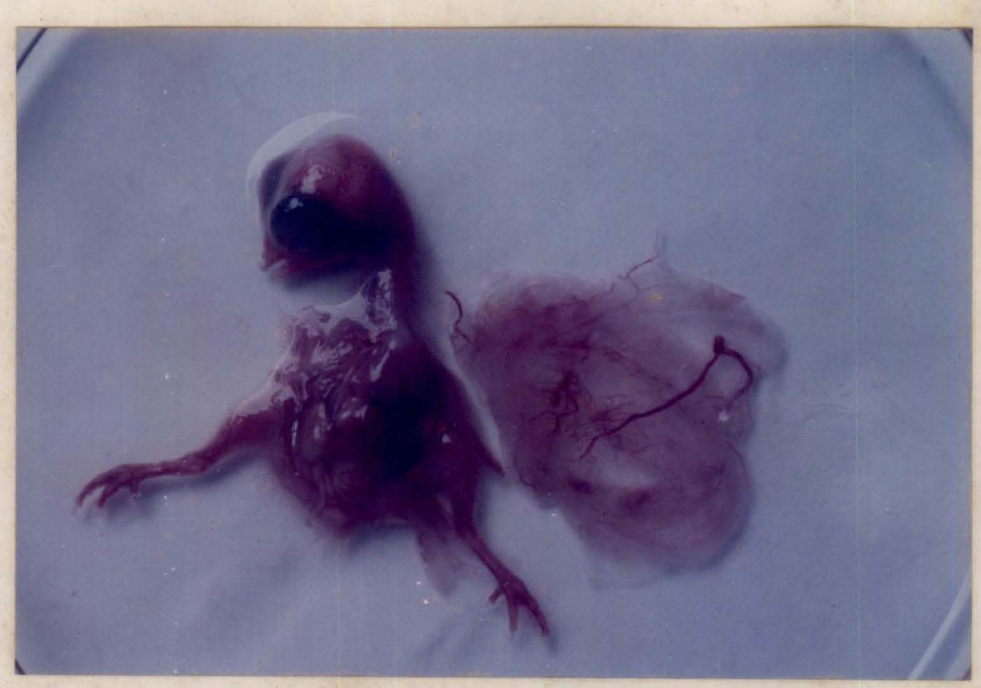
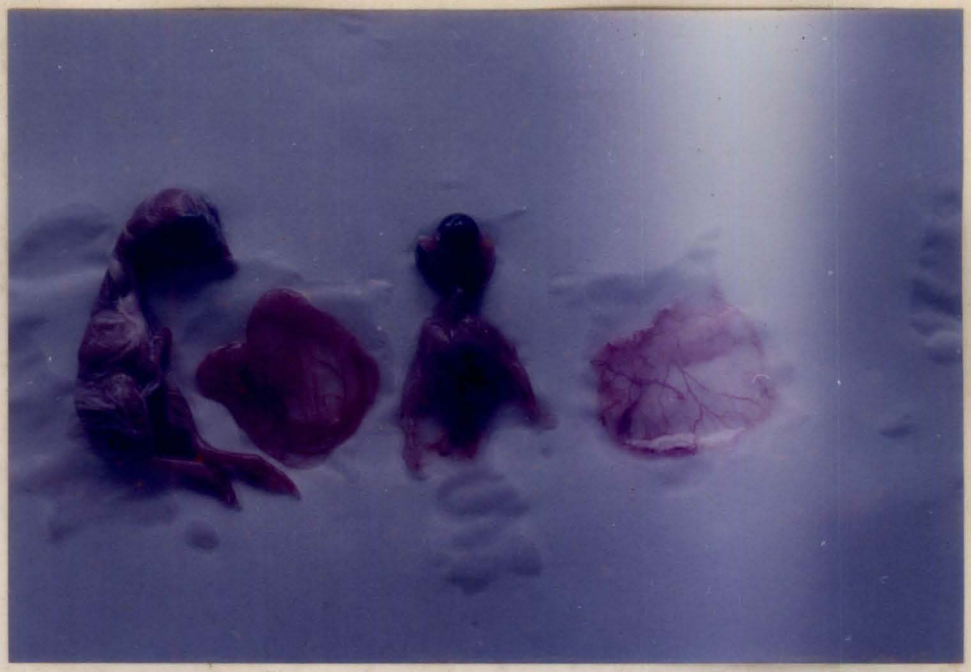


Fig. 15 Highly congested CAM and stunted embryo (infected with T_{22})

Fig. 16 Unstained, normal chicken embryo fibroblast monolayer (low power)



4.5 Characterisation of haemagglutinating isolates

4.5.1 Chicken embryo propagation and lesions in the infected embryo

T_{18} - The isolate killed the embryo in 3 to 5 days time. The infected embryo had lesions such as congestion of the CAM and embryo (Fig.14). No specific lesions could be seen in the internal organs. The allantoamniotic fluid showed agglutination of red cells from chicken and teal.

T_{22} - In this case also mortality was detected between 3 to 5 days time. Unlike in T_{18} the embryos were highly stunted and curled, at the same time congested. The chorioallantoic membranes were also congested (Fig.15). The liver of the embryo had yellowish brown patches. Impression smears from the cutsurface of the liver did not show any inclusion bodies. The allantoaminotic fluid showed agglutination of red cells from chicken and teal.

4.5.2 Duck embryo propagation

All the samples were processed and propagated in 11 to 12 day old duck embryos by the CAM route of inoculation. Both T_{18} and T_{22}, though exhibited changes in chicken embryo, changes were not prominent in duck embryos.

4.6 Haemagglutination

With chicken RBC haemagglutination titre of T_{18} and T_{22} were only 1:8 during the early passages which rose to 512 and 128 respectively by the sixth passage in chicken embryos (Table 4).

Table 4. Rise of HA titre of the isolates with increase in passage

Passage number in chicken embryo	HA titre	
	T_{18}	T_{22}
1	-	-
2	8	-
3	32	8
4	128	32
5	256	64
6	512	128

The results of the HA activity of the isolates T_{18} and T_{22} with red cells from various species are given in the Table 5.

Table 5. Results of HA activity of the isolates with the red cells of various species

Red cells	T_{18}	T_{22}
Chicken	512	128
Teal	128	128
Duck	-	-
Cattle	-	-
Rabbit	-	-
Pig	-	-
Horse	-	-
Human `O'	-	-

4.7 Embryo lethal dose 50 (ELD_{50})

The ELD_{50} of T_{18} and T_{22} were $5x10^7$ and $3.2x10^6$ respectively. ELD_{50} of both the isolates were calculated as per the method of Reed and Muench (1938). The ELD_{50} of T_{18} and T_{22} were given in Table 6 and Table 7 respectively.

4.8 Chloroform sensitivity

Both the isolates were sensitive to chloroform when treated at five per cent level for 10 min at room temperature, indicating that these isolates were enveloped. The chloroform sensitivity of the isolates are given in Table 8.

Table 6. ELD_{50} of T_{18} (6th passage material in chicken embryo)

Dose/ embryo	Virus dilution	No. of eggs inocu-ted	No. of infected/ dead	No. of unin-fected alive	Accumulated values		Ratio of +ve	Percent-age of +ve
					No +ve	No -ve		
0.1 ml	Undiluted	4	4	0	29	0	29/29	100
	10^{-1}	4	4	0	25	0	25/25	100
	10^{-2}	4	4	0	21	0	21/21	100
	10^{-3}	4	4	0	17	0	17/17	100
	10^{-4}	4	4	0	13	0	13/13	100
	10^{-5}	4	4	0	9	0	9/9	100
	10^{-6}	4	4	0	5	0	5/5	100
	10^{-7}	4	1	3	15	3	1/4	25
	10^{-8}	4	0	4	05	7	0/7	00

$$\text{Proportionate distance} = \frac{\text{Per cent of infectivity just above 50 per cent} - 50}{\text{Per cent of infectivity just above 50 per cent} - \text{Per cent of infectivity just below 50 per cent}}$$

$$= \frac{100-50}{100-25} = \frac{50}{75} = 0.66 = 10^{-6+0.66}$$

$$= 10^{-6.66}/0.1 \text{ ml}$$

$$\text{For 1 ml} = \frac{1}{0.1} \times 10^{-6.66}$$

$$= 10 \times 10^{6} \times \text{antilog of } 0.66$$

$$= 10 \times 10^{6} \times 5 = 5 \times 10^{7} \text{ } ELD_{50}/\text{ml}$$

Table 7. ELD_{50} of T_{22} (6th passage material in chicken embryo)

Dose/ embryo	Virus dilution	No. of eggs inocu-ted	No. of infected/ dead	No. of unin-fected alive	Accumulated values		Ratio of +ve	Percent-age of +ve
					No +ve	No -ve		
0.1 ml	Undiluted	4	4	0	26	0	26/26	100
	10^{-1}	4	4	0	22	0	22/22	100
	10^{-2}	4	4	0	18	0	18/18	100
	10^{-3}	4	4	0	14	0	14/14	100
	10^{-4}	4	4	0	10	0	10/10	100
	10^{-5}	4	4	0	6	0	6/6	100
	10^{-6}	4	2	2	2	2	2/4	50
	10^{-7}	4	0	4	0	6	0/6	0

$$\text{Proportionate distance} = \frac{\text{Per cent of infectivity just above 50 per cent} - 50}{\text{Per cent of infectivity just above 50 per cent} - \text{Per cent of infectivity just below 50 per cent}}$$

$$= \frac{100-50}{100-25} = 0.5$$

$$= 10^{-5+0.5}/0.1 \text{ ml}$$

$$\text{For 1 ml} = \frac{1}{0.1} \times 10^{-5.5}$$

$$= 10 \times 10^{5} \times 3.2$$

$$= 3.2 \times 10^{6} \text{ } ELD_{50}/\text{ml}$$

Table 8. Chloroform sensitivity of the isolates T_{18} and T_{22}

Sample No.	Infectivity titre in chicken embryo		HA titre	
	Control	Chloroform treated	Control	Chloroform treated
T_{18}	10^7	0	512	16
T_{23}	10^6	0	128	8

Table 9. Effect of temperature at 56°C for 30 min

Sample No.	HA titre		Infectivity titre in chicken embryo	
	Control	Treated	Control	Treated
T_{18}	512	0	10^7	0
T_{22}	128	0	10^6	0

4.9 Thermostability

Thermoinactivation of both the isolates were presented in the Table 9. It was observed that infectivity of both the isolates T_{18} and T_{22} were completely destroyed by heating at 56°C for 30 min. Haemagglutination activity of both the isolates were lost within 30 min.

4.10 pH stability

Results of stability of the isolates at various pH levels are shown in the Table 10. It was seen from this table that the infectivity and haemagglutinins were unaffected at pH 7.2 for both the isolates. At pH 3.2 and 9.0 the infectivity and haemagglutinating activities were completely lost in both the isolates.

Table 10. Stability of the isolate T_{18} and T_{22}

Sample No.	pH 3.2		pH 7.2		pH 9.0	
	HA	Infecti-vity	HA	Infecti-vity	HA	Infecti-vity
T_{18}	no HA	0	512	10^7	no HA	0
T_{22}	no HA	0	128	10^6	no HA	0

Fig. 17 May-Grunwald Giemsa stained CEF monolayer (high power)

Fig. 18 Unstained CEF monolayer infected with isolate T_{18}

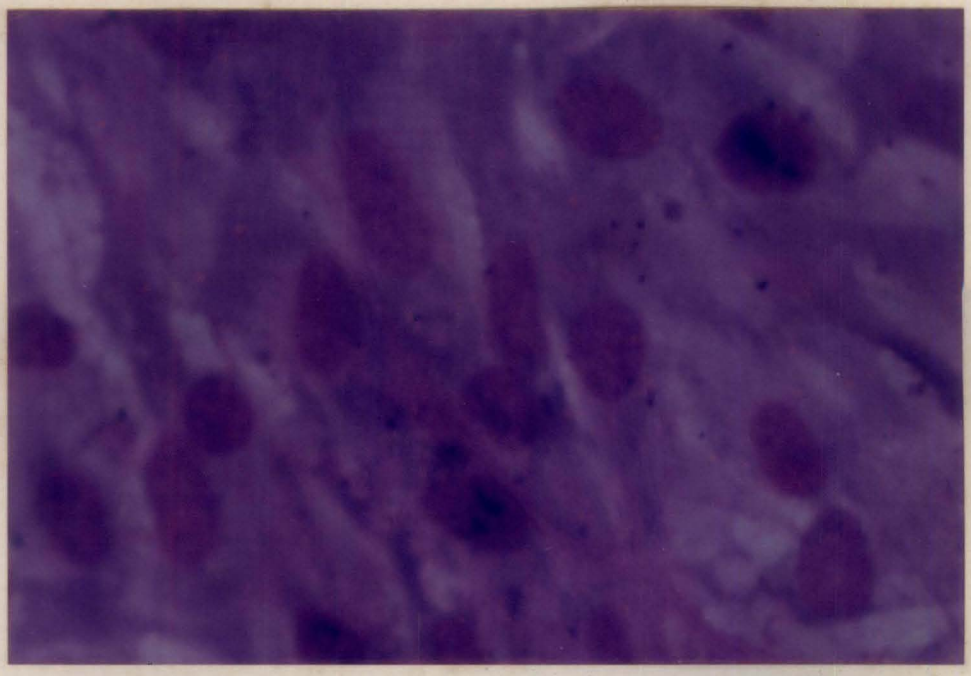
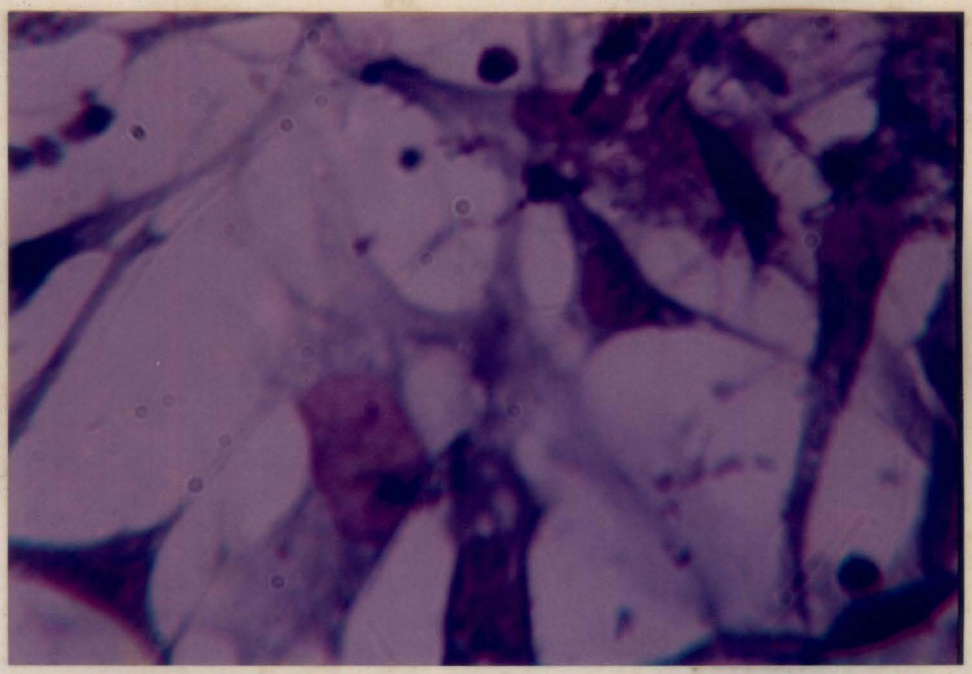
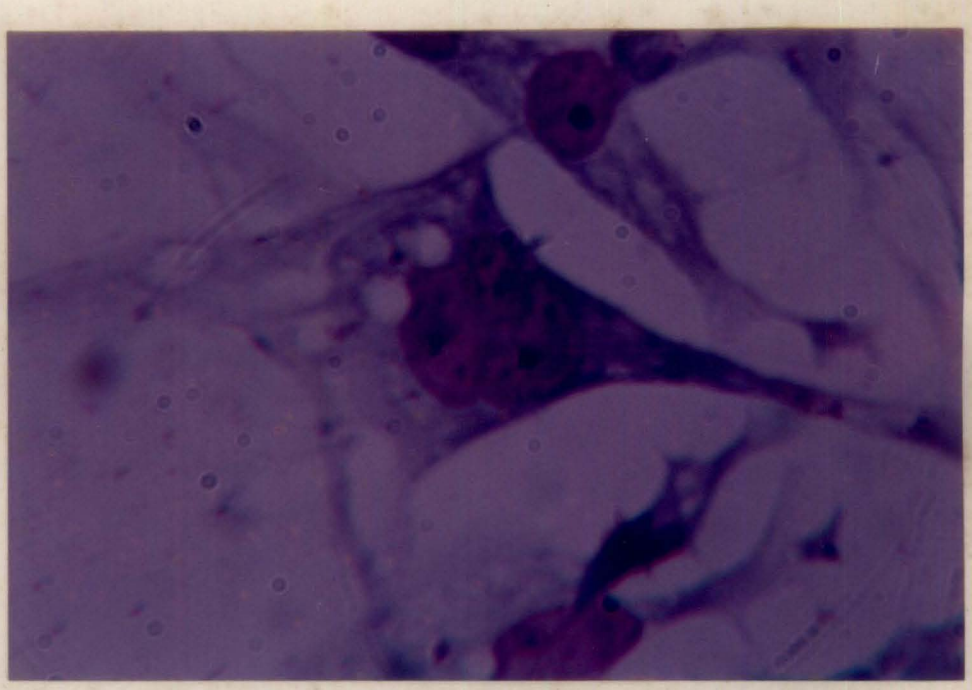


Fig. 19 Enlarged and rounded cells with long cytoplasmic fibrils

Fig. 20 Syncytium





4.11 Propagation in chicken embryo fibroblasts

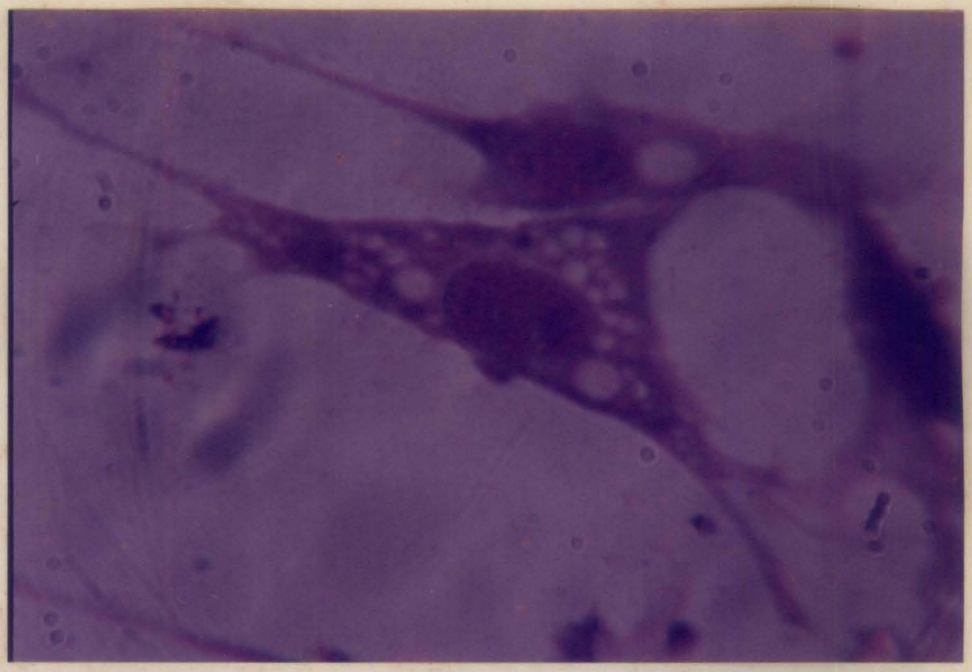
Primary chicken embryo fibroblasts were grown in Hanksbalanced salt solution supplemented with 0.5 per cent lactalbumin hydrolysate, 0.15 per cent yeast extract and five to seven per cent calf serum. Satisfactory monolayers were formed in 24 hr. Morphologically the cells were more or less spindle shaped and in areas of high density were oriented with their long axis parallel to one another (Fig.16,17). The monolayers were inoculated with isolates T_{18} or T_{22}. For T_{18} and T_{22} the monolayer started showing CPE 48 hr after inoculation and the CPE was complete within 96 hrs. In unstained preparations, the initial changes were noticed from the periphery of the monolayer. Subsequent changes were uniformly distributed and finally the whole monolayer was affected and the cells got detached from the glass surface (Fig.18).

4.12 Examination of coverslip cultures

The coverslips showing CPE and the controls were fixed in formol saline overnight and were stained with May-Grunwald Giemsa. In the case of T_{18} the stained monolayer exhibited CPE which was characterised by rounding and clumping of the cells. The infected cells showed a tendency to get separated from the neighbouring cells leaving long cytoplasmic fibrils (Fig.19). There were syncytium with four or five nuclei without any

Fig. 21 Infected cells with cytoplasmic vacuoles

Fig. 22 Unstained monolayer infected with isolate T_{22}



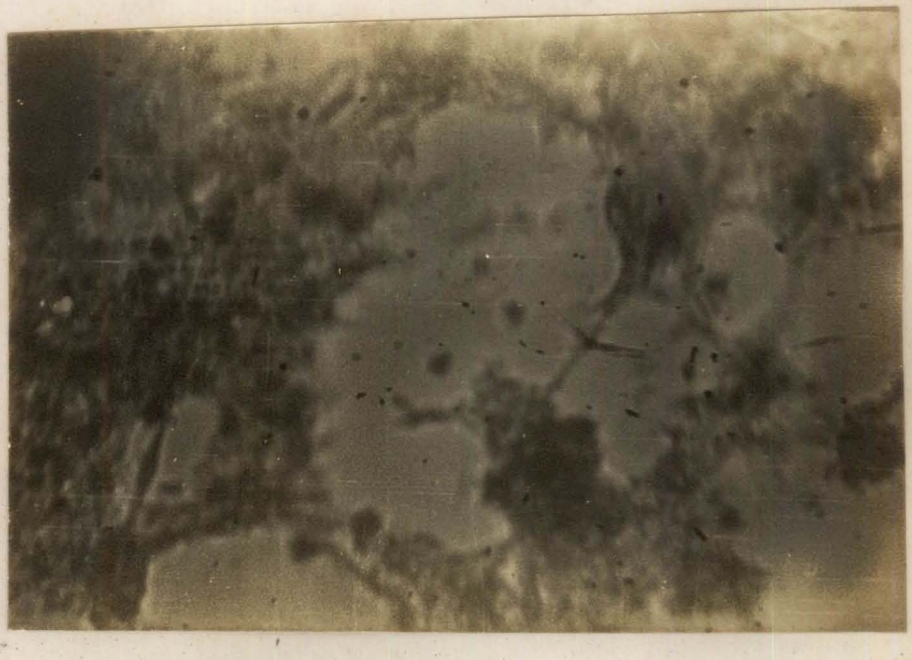
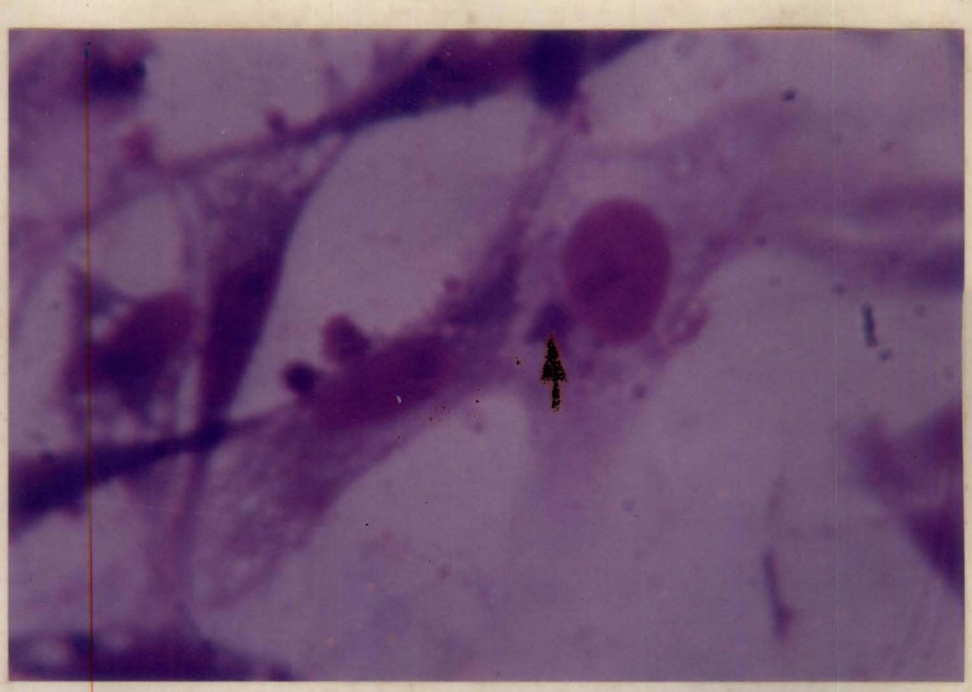


Fig. 23 Rounding of cells with cytoplasmic vacuolation

Fig. 24 Cytoplasmic vacuoles with intranuclear (small arrow) and intracytoplasmic (large arrow) inclusion



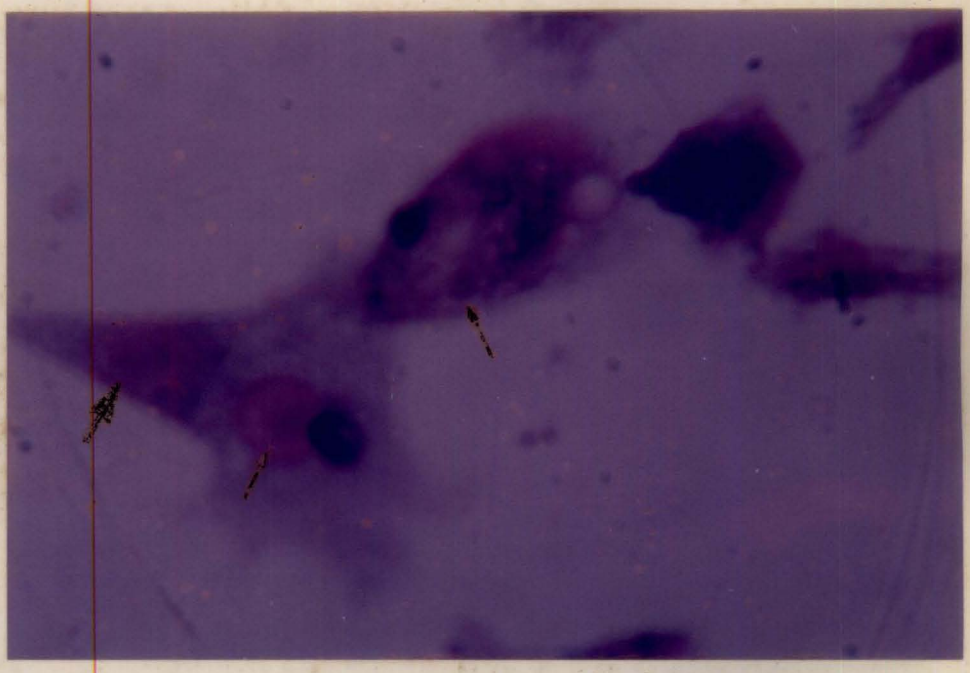
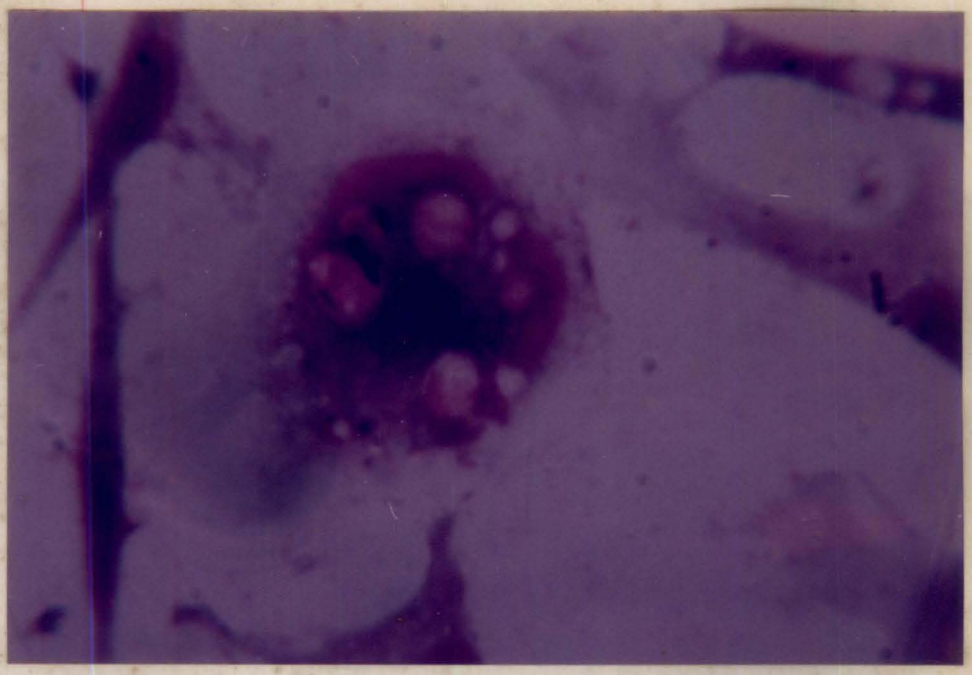
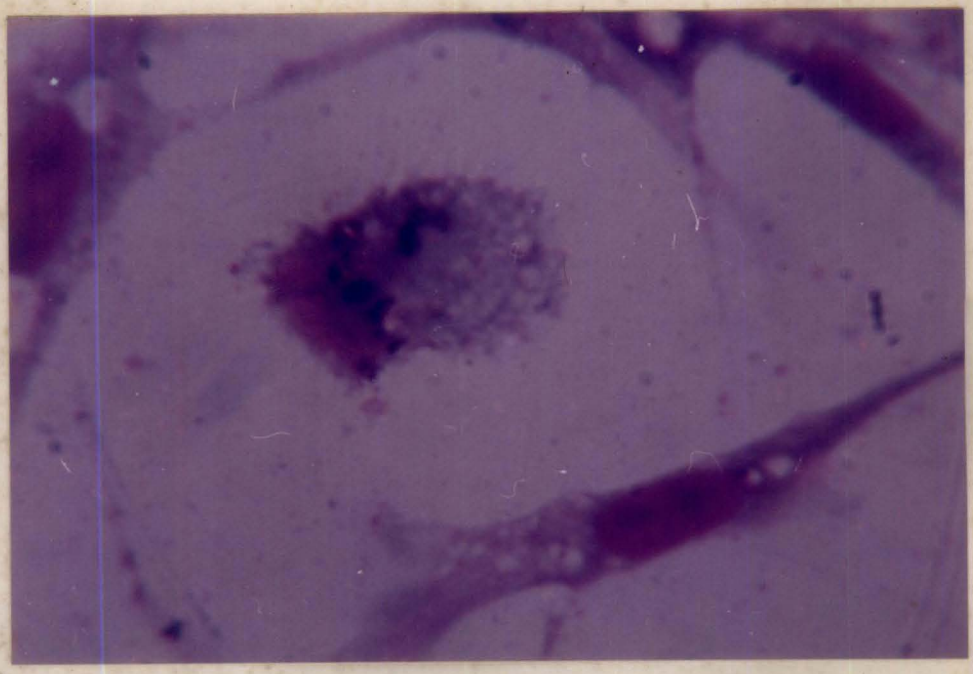


Fig. 25 Large syncytium

Fig. 26 Total disintegration of cells





cytoplasmic demarcation (Fig.20). Severe cytoplasmic vacuolation was another constant feature of most of the infected cells (Fig.21). Inclusion bodies were not detected either in the nucleus or in the cytoplasm.

In T_{22} the CPE was characterised by cytopathic changes though developed later, the cellular changes were more marked compared to T_{18}. The clumps of infected cells were larger (Fig.22) and due to detachment and separation from neighbouring cells the monolayer showed a honey comb appearance by naked eye examination. The early changes detected in stained monolayers were again rounding of cells with cytoplamic vacuolation. Vacuolation of the cytoplasm slowly extended to cover the whole cell when it also showed perinuclear inclusion bodies (Fig.c). A basophilic mass seen in the nucleus was quite distinct from the normal chromatin deposition. This could be early stages of inclusion body formation (Fig.23). As infection progressed there was extensive degeneration of the affected cells with well marked cytoplasmic and intranuclear inclusion bodies (Fig.24). Large syncytia and total disintegration of cells were observed as the infection progressed to 96 hrs (Fig.25, 26).

4.13 Nucleic acid type

Pretreatment of chicken embryo fibroblast cell cultures with 200 micrograms of 5-iodo-2 deoxyuridine (IUdR) inhibited

the replication of T_{22} isolate. Such an inhibitory effect was not observed when they were grown in untreated control wells. On the other hand the T_{18} isolate showed characteristic CPE both in the drug treated as well as control tubes. A known RNA virus - Newcastle disease virus was not inhibited by treatment of the monolayers with IUdR as CPE was evident in both drug treated and untreated cell cultures. The same results were obtained even when the concentration of the drug used was reduced to 100 μg/ml. At both the levels the drug was non-toxic to the cells as no morphological or structural changes were observed in cell cultures. The results indicate that the isolate T_{18} is an RNA virus and T_{22} a DNA virus. IUdR sensitivity of the isolates T_{18} and T_{22} are given in the Table 11.

Table 11. IUdR sensitivity of the isolates

Sample No.	IUdR treated (200 μg/ml)	IUdR untreated
T_{18}	CPE in chicken embryo fibroblast	CPE in C.E.F.
T_{22}	No CPE in C.E.F.	CPE in C.E.F.
NDV	CPE in C.E.F.	CPE in C.E.F.

4.14 Pathotyping

4.14.1 Intracerebral pathogenicity index

Intracerebral pathogenicity index was studied by inoculating 12, day old chicks with 0.05 ml of 1 in 10 dilution of the isolates T_{18} and T_{22} respectively.

Day old chicks inoculated with T_{18} were normal on the day of inoculation. On the second day two chicks died and four showed clinical symptoms. On the 3rd day three more died and one was showing clinical symptoms. The chick showing the clinical signs died on the 7th day, after which no more mortality was recorded. The intracerebral pathogenicity index of T_{18} is given in the Table 12.

Day old chicks inoculated with T_{22} were normal during the day of inoculation. On the second day after inoculation, two chicks started showing clinical symptoms, one of which died on the 4th day and the other one on the 5th day. Another chick started showing clinical symptoms on the 8th day and died on the 9th day, after which none of the chicks died. The intracerebral pathogenicity index of T_{22} is given in the Table 13.

4.14.2 Mean death time (MDT) at terminal dilution

The mean death time at terminal dilutions for both the isolates T_{18} and T_{22} was 120 hr. MDT of T_{18} and T_{22} were given in the Tables 14 and 15 respectively.

Table 12. Intracerebral pathogenicity index of T_{18} (ICPI)

Status	Days										Sum	Factor	Total
	1	2	3	4	5	6	7	8	9	10			
Dead	-	2	5	5	5	5	6	6	6	6	46	2	92
Sick	-	4	1	1	1	1	0	0	0	0	8	1	8
Live	12	6	6	6	6	6	6	6	6	6	66	0	0
Total											120		100

$$\text{Neuropathic Index} = \frac{100}{120} = 0.83$$

Table 13. Intracerebral pathogenicity index of T_{22} (ICPI)

Status	Days										Sum	Factor	Total
	1	2	3	4	5	6	7	8	9	10			
Dead	-	-	-	1	2	2	2	2	3	3	15	2	30
Sick	-	2	2	1	0	0	0	1	0	0	6	1	6
Live	12	10	10	10	10	10	10	9	9	9	99	0	0
Total											120		36

$$\text{Neuropathic Index} = \frac{36}{120} = 0.30$$

Table 14. Mean death time (MDT) of the minimum lethal dose (MLD) of T_{18}

Virus dilution log	Hours post inoculation														D/L	Percentage mortality	MDT
	24	32	40	48	56	64	72	80	88	96	104	112	120	128			
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5	0	
8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1/5	20	MLD=7
7	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5/5	100	MLD= $\frac{5}{5}$x120
6	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5/5	100	=120
5	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5/5	100	
1	0	0	0	0	0	0	0	0	0	5	0	0	0	0	5/5	100	

Table 15. Mean death time (MDT) of the minimum lethal dose (MLD) of T_{22}

Virus dilution log	Hours post inoculation														D/L	Percentage mortality	MDT
	24	32	40	48	56	64	72	80	88	96	104	112	120	128			
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5	0	
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5	0	MLD=6
7	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2/5	40	MLD= $\frac{5x120}{5}$
6	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5/5	100	=120
5	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5/5	100	
1	0	0	0	0	0	4	1	0	0	0	0	0	0	0	5/5	100	

4.14.3 Intravenous pathogenicity index (IVPI)

This was studied in six week old chicks. None of the chicks inoculated with one ml of the isolates T_{18} and T_{22} 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 both the isolates indicating virus infection. The IVPI was calculated to be zero. The IVPI of T_{18} and T_{22} are given in Tables 16 and 17 respectively.

Comparison of the characteristics of isolate T_{18} and T_{22} are given in Table 18.

4.15 Pathogenicity

4.15.1 Experiment I

Three, one week old chicks each were given 0.5 ml each of the isolates T_{18} and T_{22} subcutaneously and 1 ml of each of the isolates orally. The controls were given 0.5 ml of sterile normal saline subcutaneously and 1 ml normal saline orally and kept separately. The birds were monitored daily for any signs of disease or mortality for a period of four weeks. None of the birds exhibited clinical signs or mortality during the period of observation. The postmortem examination of the birds at the end of the observation period did not revealed any lesions. The results of experimental infection of one

Table 16. Intravenous pathogenicity index of T_{18} (ICPI)

Status	Days of observation											Sum	Factor	Total
	1	2	3	4	5	6	7	8	9	10	11			
Death	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Signs	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Normal	6	6	6	6	6	6	6	6	6	6	6	66	0	0
									Total			66		0

$$\text{IVPI} = \frac{\text{Coded number}}{\text{Total observations}} = \frac{0}{66} = 0$$

Table 17. Intravenous pathogenicity index of T_{22} (ICPI)

Status	Days of observation											Sum	Factor	Total
	1	2	3	4	5	6	7	8	9	10	11			
Death	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Signs	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Normal	6	6	6	6	6	6	6	6	6	6	6	66	0	0
									Total			66		0

$$\text{IVPI} = \frac{\text{Coded number}}{\text{Total observations}} = \frac{0}{66} = 0$$

Table 18. Comparison of the characteristics of the isolates T_{18} and T_{22}

Isolates	ELD_{50}	Haemagglutination								pH stability	Thermostability 56°C and 30 min		Chloroform		Nucleic ACID type	IVPI	ICPI	MDT
		Cattle	Horse	Pig	Human 'O'	Rabbit	Chicken	Teal	Duck		Infectivity	HA	Infectivity	HA				
T_{18}	$5x10^7$ ELD_{50}/ml	-ve	-ve	-ve	-ve	-ve	512	128	-ve	Stable at pH 7.2	0	0	0	16	RNA	0	0.83	120 hrs
T_{22}	$3.2x10^6$ ELD_{50}/ml	-ve	-ve	-ve	-ve	-ve	128	128	-ve	Stable at pH 7.2	0	0	0	8	DNA	0	0.3	120 hrs

week old chicks with the isolates T_{18} and T_{22} are given in the Table 19.

Table 19. Experimental infection of one week old chicks with isolate T_{18} and T_{22}

Isolate No.	No. of birds infected	Clinical symptoms	Mortality
T_{18}	3	-	-
T_{22}	3	-	-
Sterile normal saline	3	-	-

4.15.2 Experiment II

Three numbers of one week old ducklings each were given 0.5 ml each of the isolates T_{18} and T_{22} subcutaneously and also 1 ml each orally. The controls were injected with 0.5 ml of sterile normal saline subcutaneously and also 1 ml of saline orally. All the birds were closely watched daily for a period of four weeks for any clinical symptoms or death. The birds infected with T_{18} and also those birds infected with T_{22} did not show any clinical symptoms or mortality. Postmortem examination of the birds at the end of four weeks did not reveal any macroscopic changes. The details of the experimental infection are given in Table 20.

Table 20. Experimental infection of one week old ducklings with isolate T_{18} and T_{22}

Isolate No.	No. of birds infected	Clinical symptoms	Mortality
T_{18}	3	-	-
T_{22}	3	-	-
Sterile normal saline	3	-	-

4.15.3 Experiment III

Six week old chicken infected either with the isolate T_{18} and T_{22} 1 ml subcutaneously did not show any clinical symptoms or death till the 28th day after inoculation. Virus isolation from the tracheal and cloacal swabs collected from these birds are given in Tables 21 and 22.

With isolate T_{18} all the six infected birds were excreting the virus on days 3, 5 and 8 post infection, as the virus could be isolated from the cloacal swabs of all of them. The number of isolations decreased thereafter to 50 per cent of the cloacal swabs by day 14. Cloacal shedding of the virus thus continued to the 14th day and all the birds were negative by the 21st day. Throat swabs collected at various time intervals along with the cloacal swabs were negative. The infected birds showed an antibody response with an HI titre of 16.

Table 21. Experimental infection of six week old chicks with isolate T_{18}

Time of collection/ observation in days	No. of first infected	Virus isolation		Clinical symptoms	Mortality	Percentage of isolation	
		CS* No +ve/ No screened	TS** No +ve/ No screened			CS*	TS**
3	6	6/6	0/6	-	-	100	0
5	6	6/6	0/6	-	-	100	0
7	6	6/6	0/6	-	-	100	0
14	6	4/6	0/6	-	-	66	0
21	6	0/6	0/6	-	-	0	0
28	6	0/6	0/6	-	-	0	0

* CS - Cloacal swab
** TS - Throat swab

Table 22. Experimental infection of six week old chicks with isolate T_{22}

Time of collection/ observation in days	No. of first infected	Virus isolation		Clinical symptoms	Mortality	Percentage of isolation	
		CS* No +ve/ No screened	TS** No +ve/ No screened			CS*	TS**
3	6	6/6	0/6	-	-	100	0
5	6	6/6	0/6	-	-	100	0
7	6	6/6	0/6	-	-	100	0
14	6	3/6	0/6	-	-	50	0
21	6	0/6	0/6	-	-	0	0
28	6	0/6	0/6	-	-	0	0

* CS - Cloacal swab
** TS - Throat swab

In the case of T_{22}, the cloacal swabs collected at 3rd, 5th and 7th day post infection were positive for virus isolation from all the six infected birds, while no isolation was made from the tracheal swabs collected on the above days. Virus could be isolated between 3rd and 14th day. The date of isolation was 100 per cent upto the 7th day which was reduced to 66 per cent by the 14th day and nil on the 21st day. HI antibodies could be demonstrated in the serum of infected birds with a titre of 8.

4.15.4 Experiment IV

Six numbers of six week old ducklings each were given the isolates T_{18} and T_{22} 0.5 ml each by the oral and subcutaneous routes. Four birds were kept as control and were given 0.5 ml of sterile normal saline subcutaneously and 0.5 ml sterile normal saline orally. The birds were examined daily for any clinical symptoms or death for a period of six weeks. None of the birds exhibited any clinical signs or mortality. Experimental infection of the birds with the isolates are given in Table 23.

Table 23. Experimental infection of six week old ducklings with isolate T_{18} and T_{22}

Isolate No.	No. of birds infected	Clinical symptoms	Mortality
T_{18}	6	-	-
T_{22}	6	-	-
Sterile normal saline	4	-	-

4.16 Antigenic relationship between other haemagglutinating avian viruses

Both isolates were antigenically distinct from NDV, EDS. Similarly isolate T_{18} was distinct from T_{22}. Isolate T_{18} showed antigenic similarity with Fish viruses isolated from spizootic ulcerative syndrome of fishes (FV and F_6) but isolate T_{22} did not show any antigenic relationship with fish viruses. The details of the antigenic relationship between different viruses are shown in the Table 24.

Fig.27 & 28. **T_{18} under electron microscope**

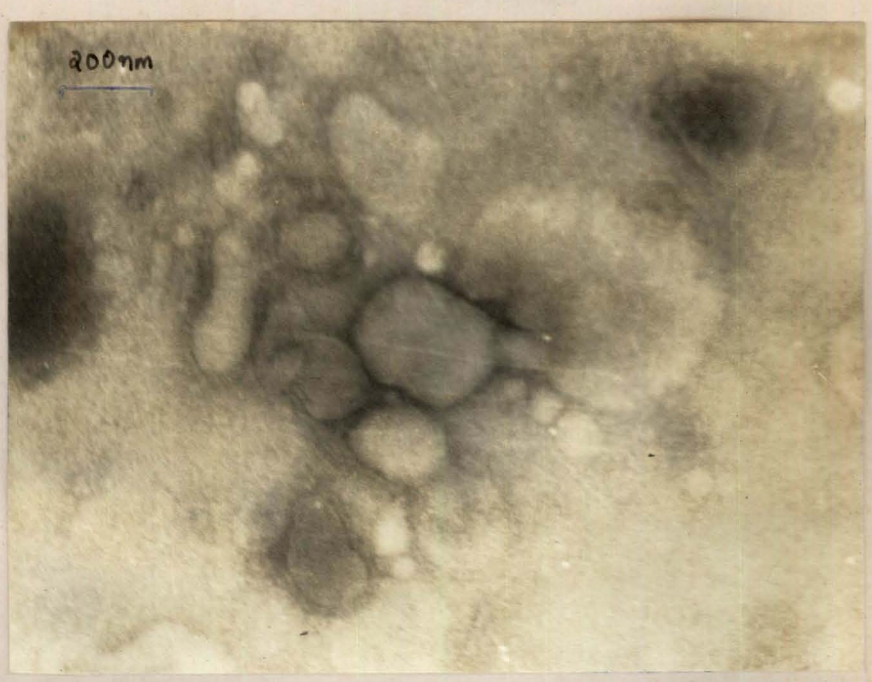

200nm

Table 24. Antigenic relationship between different viruses

Antigen	Antiserum		HI titre	
	T_{18}	T_{22}	NDV	EDS
T_{18}	16	-	-	-
T_{22}	-	8	-	-
NDV	-	-	32	-
EDS	-	-	-	128
FV*	16	-	-	-
F6**	8	-	-	-

* Fish virus isolated from epizootic ulcerative syndroms (EUS) in fishes

** Fish virus isolated from EUS in fishes

4.17 Electron microscopy

T_{18} - the virus had the morphological features of a paramyxovirus. It was an enveloped virus with a size of 150-185 nm and had pleomorphic forms. The peplomers were 18-20 nm in length (Fig.27, 28).

Discussion

5. DISCUSSION

5.1 Common waterfowls seen in Kerala

The different types of waterfowls seen in Kerala are Lesser whistling teals, Comb duck, Cotton teal, Common teal, Spot billed duck, Pintail and Gargany. Few unidentified waterfowls which do not belong to any of the above seven groups are also being reported. The most commonly seen in Kerala is gargany followed by pintail, lesser whistling teal, cotton teal, common teal, spot billed duck and comb duck. These birds are mainly seen in the backwaters of Southern Kerala, particularly in Kuttanad area and in the Kole lands of Trichur, Peechi and wet lands of Malappuram district. Ninety per cent of the population is seen in the first two places (Nameer, 1995). The migratory types come to Kerala during December to April. During day time they roost on the water surface and by evening when the sun sets they fly in swarms to the paddy fields either during sowing season or at the time of harvesting.

Of the total of 52 birds captured for the study, 15 were identified as lesser whistling teals and the remaining 37 were identified as gargany by the characteristics described by Backer (1929). Though the second most common reported in Kerala is the pintails (Nameer, 1995); none of the birds captured for the present study belonged to this group. It is

possible that pintails may be localizing in areas other than Kumarakom, Palghat and Thrissur Kole lands from where the birds were captured for this study. The failure to capture more number of birds from each area could also be a reason as different species are reported to be seen in each flock.

5.2 Virus isolation

A total of 52 waterfowls including 37 gargany and 15 lesser whistling teals were utilized for virus isolations. The specimens used from each bird were brain, liver, spleen, lung, throat swab, tracheal scrapping, cloacal swab and intestinal scrappings.

Impression smears from the cut surface of liver from all the birds were subjected to microscopical examination for inclusion bodies in the hepatic cells. Impression smears from four birds (bird nos. 17, 23, 27 and 49) revealed intranuclear inclusions. The viruses that were reported to produce intranuclear inclusions in waterfowls are duck plague virus (herpes virus) and EDS-76 (adeno virus) and waterfowls are reported to act as reservoirs for these two viruses. Clinical manifestation and mortality among waterfowls other than domestic ducks have been reported with DP virus (Procter *et al.*, 1975, Montgomery *et al.*, 1981). In the present study it was impossible to say whether any mortality had occurred in the flock from which these birds were caught as we had visited

the area only once and it was difficult to locate any dead birds in the flock as there were thousands of birds in each flock. Moreover it was very difficult to reach the area as these birds roost on the water bodies and permission was not granted to visit the area frequently.

Virus isolation attempts from the above four birds in developing duck embryos by the CAM route of inoculation were not successful. Hence neither duck plague virus nor EDS-76 could be isolated from these cases. However, Sulochana (1996) had reported isolation of duck plague virus from one of the gargany, caught from Palghat district which had intranuclear inclusion bodies in the liver impression smears. She had also reported mortality of two week old ducklings with symptoms of DP when they were infected with the isolate from waterfowl. The isolate was confirmed as DPV by serum neutralization test.

The failure of virus isolations from the liver of birds showing intranuclear inclusion bodies could be (1) the less number of passages done in developing duck embryos (2) the possible presence of antibodies to duck plague virus in the duck eggs used for propagation as they were all purchased locally since they were not available from the University duck unit. Also attempt was not made to inoculate the above specimens into susceptible ducklings.

Haemagglutinating agents were isolated from the cloacal swabs of bird with numbers 18 (T_{18}) and 22 (T_{22}). These

isolations were initially made in duck embryos. In both cases no HA activity could be detected in the first passages. Haemagglutinating activity was detected by the second passage in T_{18} and only by the third passage in T_{22}. All the other specimens were negative even after three blind passage.

The virus isolations were from apparently healthy birds. Orthomyxo and paramyxo viruses have been reported to be widely distributed among ducks throughout the world. The two haemagglutinating agents from the lesser whistling teals, appear to corroborate the above finding. The migratory and foraging patterns of waterfowl provide ideal conditions for transmission of viruses from migratory waterfowl to domestic poultry or even man and other animals. Although the haemagglutinating agents were isolated from apparently healthy birds, this does not indicate that the isolates lack pathogenic potential, since a serotype may be virulent for one species and avirulent for another (Deibel *et al.*, 1985).

The recovery of viruses from the cloacal swabs rather than the tracheal swabs indicate the faecal shedding of virus, an important factor in the survival and transmission of the agent. The faecal and oral route of shedding of virus would provide an excellent method of transmitting the virus from migratory ducks to domestic ducks or poultry population or even man.

Abenes *et al.* (1982) reported isolation of orthomyxo and paramyxo viruses from the cloacal swabs/lower intestinal tract of 9.7 per cent of the teals, they screened from Hokkaido, Japan. The percentage of isolations of influenza virus by Deibel *et al.* (1985) in various species of waterfowls was also higher. It was 42 per cent (*Anas platyrhynchos*); 30 per cent (*Anas rubripes*); 11 per cent (*Anas discors*) and 2.3 per cent (*Aix sponsa*). All positive ducks appeared clinically healthy. Higher percentage of isolations were also reported by Sinnecker *et al.* (1983), Mikami *et al.* (1987) and Shortridge *et al.* (1977). The percentage of isolation in the present study was 3.8 per cent. Though this was lower than the reports of Abenes *et al.* (1982) and Deibel *et al.* (1985) it was higher when compared to the reports of Stallkencht *et al.* (1990) in various species of waterfowls.

The percentage of isolation of viruses from waterfowls depends on various factors. They are (i) the chances of the birds to pick up infection, (ii) mode of virus excretion, (iii) mode of spread and transmission, (iv) susceptibility of the species and (v) how long the virus can persist in them. However to get an exact picture of the flock a good number of birds are to be screened. In the present study we were unable to satisfy these criteria as these birds come under wildlife protection act 1972 and it was very difficult to capture these birds. Hence the percentage of isolation reported in this study may not be a true picture of the situation. However, the

present study indicates the possibility of waterfowls acting as a source of infection to the domestic ducks and poultry, particularly in the waterlogged areas of Kerala where these birds visit every year. It is also interesting to note that it is in these areas the ducks are concentrated and often outbreaks of duck plague and duck pasturellosis occur resulting in heavy mortality. In addition during 1995 in the districts of Alleppey and Kottayam there was an outbreak of JE which could possibly be introduced by the waterfowls. All these indicate that the migratory waterfowls can be considered as a source of infection not only to birds but also to the human population.

5.2 Propagation in embryonated chicken and duck eggs

Both the isolates T_{18} and T_{22} killed the chicken embryo in three to five days time. The HA titres of the allantoic fluids were 1:512 and 1:128 respectively. In the case of T_{18} the lesions noticed in the embryos were congestion of the CAM and embryo. The embryo infected with isolate T_{22} was highly stunted and curled, at the same time congested. The liver of the embryo had yellowish brown patches.

Moses *et al.* (1948) observed death of embryos with congestive and haemorrhagic lesions, particularly in the skin and musculature of the embryo infected in the case of influenza virus. Lang *et al.* (1968) observed dark red skin of

the embryos, peticheal haemorrhages on the head, congestion of the muscles and punctiform haemorrhages on the sternum with influenza virus.

Inoculation of both the isolates into embryonated duck eggs did not show any changes.

5.3 Thermostability

The isolates T18 and T22 lost their infectivity and haemagglutination property by heating at 56°C for 30 min, suggesting that these two properties are thermolabile. Similar findings of inactivation of influenza A virus was also reported by Merchant and Packer (1967), Papparella *et al.* (1969). Eight of the nine paramyxo viruses isolated from tracheal and cloacal swabbings of feral ducks and geese in the Atlantic flyway possessed haemagglutinin that was stable for less than 15 min at 56°C, whereas the haemagglutinin of the remaining viruses was stable between 15 and 30 min (Cloud and Rosenberger, 1980).

Moses *et al.* (1948) reported that infectivity of fowl plague virus was destroyed by exposure to 60°C for a period of five min or less. Infectivity and haemagglutination activity of the velogenic NDV isolated from lesser whistling teal was lost when the virus sample was treated at 56°C for 30 min (Sulochana, 1991). The haemagglutinin of NDV isolated from the cloacal swab of a wild mallard was resistant to 56°C

(Alexander *et al.*, 1979). Rosenberger *et al.* (1975) reported the isolation of four strains of NDV from canada geese in the Atlantic migratory route. The haemagglutinin of two of the isolates were stable at 56°C for 15 min, one for 30 min, and the other for at least 2 hr.

All the avian paramyxovirus isolated from tracheal and cloacal swabbings of feral ducks and geese in the Atlantic flyway possessed haemagglutinin that was destroyed at 56°C for 30 min (Smitka and Maassab, 1981). Yadav *et al.* (1974) reported that all 11 isolates of avian CELO virus were resistant to 56°C for 30 min.

5.4 pH stability

Sensitivity to pH is one of the criteria that could be employed for grouping a newly isolated virus into a particular group. The observations made during this study indicated that the infectivity of both the isolates were lost when exposed to pH 3.2 for a period of one hr at room temperature. There was complete absence of HA activity at pH 3.2. Same was the case when both the isolates were exposed to a pH of 9. At pH 7.2 both infectivity and HA of both the isolates were retained. Both the isolates were labile at pH 3.2 and pH 9.0 and the isolates could survive only at a narrow range of pH, unlike most of the orthomyxo and paramyxovirus, which were stable at alkaline pH 8-10.9 (Mini, 1988; Merchant and Packer, 1967).

5.5 Haemagglutinating property

Red cells of a variety of species such as cattle, pig, horse, rabbit, Human `O', chicken, duck and teal were used for examining the haemagglutinating property of the isolates T_{18} and T_{22}. Both the isolates agglutinated only chicken and teal erythrocytes. This characteristic is different from avian paramyxovirus and influenza virus which agglutinated erythrocytes of various other species (Safonov *et al.*, 1964; Mini, 1988).

5.6 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 greatly reduced. Both the isolates, T_{18} and T_{22} showed complete inactivation of the strains when treated with five per cent chloroform indicating that both the isolates were enveloped. Chloroform was preferred to ether because of its polarity as a lipid solvent. Chloroform is heavier than ether and can be easily separated by centrifugation. Moreover, chloroform is as effective as ether (Feldman and Wang, 1961).

5.7 Propagation in chicken embryo fibroblast

Of the two isolates propagated in monolayer cultures of chicken embryo fibroblasts, both the isolates produced marked CPE. For the isolate T_{18} CPE was characterized by rounding and clumping of cells which extended to the whole of the monolayer by 96 hr. For isolate T_{22}, CPE was characterized by cytoplasmic vacuolation, karryorhexis and syncytium formation which extended to the whole of the monolayer. The CPE manifested by isolate T_{18} was similar to that described for orthomyxovirus (Narayan *et al.*, 1969) while that of T_{22} to paramyxovirus (Telbis *et al.*, 1989), but there was no cytoplasmic inclusions. Multiplication of the isolates in cell cultures was evidenced from the HA activity and infectivity of the cell culture fluid after repeated freezing and thawing.

5.8 Nucleic acid type

Certain halogenated deoxyuridines such as 5-iodo-2-deoxyuridine, could inhibit the replication of DNA viruses, due to their effect on DNA synthesis (Prusoff, 1972). But they do not have any effect on the multiplication of RNA viruses.

Kunst (1968) found that the replication of duck plague virus and the development of CPE could be inhibited by IUdR. The isolates T_{18} and T_{22} were infected into CEF monolayer

cultures which were pretreated with IUdR at the level of 100 μg and 200 μg/ml. In the case of T_{18} both the treated and the untreated cell cultures exhibited CPE indicating that it was an RNA virus. Unlike in untreated control cells, T_{22} failed to produce any CPE in cells treated with IudR. However, no effect on the multiplication of a known RNA virus, Newcastle disease virus, was observed when it was grown in drug treated cells. From these observations, it was concluded that T_{18} was an RNA virus and T_{22} a DNA virus.

5.9 Pathotyping

For assessing the virulence of the isolates T_{18} and T_{22}, pathotyping was carried out as in the case of NDV. Based on the criteria like mean death time, intravenous pathogenicity index, intracerebral pathogenicity index, NDV is grouped into lentogenic, mesogenic and velogenic strains (Poultry Biologics, 1963). Lentogenic strains rarely kill day old chicks, mesogenic strain kills all the chicks within a period of 8 days and velogenic strain kill all the chicks at a fast rate. The ICPI for the above types of NDV are 0.1 to 0.2, 0.8 to 1.5 and <1.5 respectively. The ICPI of T_{18} and T_{22} were 0.83 and 0.3 respectively. If these values were analysed based on the criteria given for NDV, it is seen that the isolates are less virulent and of the two, T_{18} seems to be more virulent.

Mean death time, another criterion 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 hrs, 60 to 90 hrs and over 100 hrs respectively. On this basis, the isolates T_{18} and T_{22} are to be grouped with the least virulent type (120 hrs) as against the evaluation based on ICPI. Intravenous pathogencity index (IVPI), another criterion used for pathotyping NDV was also used in this study. In the case of NDV, the values for velogenic, mesogenic and lentogenic strains of NDV are 1.5, 0.05 to 0.31 and `O' respectively. On this basis, the isolates T_{18} and T_{22} studied were grouped under lentogenic as the IVPI was zero.

5.10 Pathogenicity

To study the pathogenicity of the isolates T_{18} and T_{22}, different age groups of chicken and ducklings were used and different routes of inoculations were tried.

Both the isolates T_{18} and T_{22} were found to be nonpathogenic to one week old duckling and chicken. The isolates were given by the subcutaneous route and also the oral route. The birds did not show any clinical symptoms or mortality during the period of observation, i.e., four weeks.

Six week old ducklings were resistant to the isolates T_{18} and T_{22}, when the isolates were given by the oral and

subcutaneous routes. The birds did not exhibit any clinical signs or mortality for a period of six weeks.

Six week old chicken infected with the isolate T_{18} and T_{22} respectively did not exhibit any clinical symptoms or mortality even after 28 days of inoculation. However, the virus could be isolated from the cloacal swabs from the third day of infection upto the 14th day in both cases and no virus isolations were obtained from the tracheal swabs indicating that the virus multiplies well in the cells lining the intestinal tract. Gough and Alexander (1984) isolated PMV-4 from a ringed teal which was non-pathogenic for chicken and ducklings. The four isolates of NDV obtained from canada geese in the Atlantic migratory route were lentogenic as the embryo mean death time was greater than 100 hr and relative lack of pathogenicity of one-day old and three week old chicken (Rosenberger *et al.*, 1975).

The persistance of virus in the system indicate the possibility of its transmission through contaminated water. The infected birds showed an antibody response with an HI titre of 16 in the case of isolate T_{18} and HI titre of 8 in the case of isolate T_{22}. The results obtained during the study indicate that the virulence and immunogenicity of both the isolates were very poor. In orthomyxovirus and paramyxovirus, host adaptation is considered to be one of the factors that determine the virulence/pathogenicity (Russel and Edington,

1985). But it is possible that when these avirulent isolates get a chance to infect a different species - chicken/ducks these may acquire virulence. The poor immunogenicity of the isolate should be considered as the antibody titres were low in chicken. None of the control birds picked up infection as neither virus nor antibody could be detected in them. This shows poor transmissibility of both the isolates T_{18} and T_{22}. Though transmissibility could not be established experimentally, in natural habitat this may not be the case when the environment, especially if the water is heavily contaminated and the birds are under stress due to various factors.

5.11 Antigenic relationship of different viruses with isolates T_{18} and T_{22}

The antigenic relationship between NDV, EDS and the fish viruses isolated from epizootic ulcerative syndrome (EUS) in fishes (FV and F6) were carried out with isolates T_{18} and T_{22}. No antigenic relationship was shown by both the isolates against NDV and EDS. Isolate T_{22} did not exhibit any similarity in antigenicity with both the fish viruses FV and F6. But the fish viruses FV and F6 showed antigenic relationship with the isolate T_{18}, indicating that this virus might be an antigenic varient of fish virus. The antigenic similarity of T_{18} isolated from waterfowls with fish viruses

support the view of Sulochana *et al.* (1992) that EUS in Kerala was disseminated by waterfowls.

5.12 Electron microscopy

Electron microscopy revealed the isolate T_{18} as an enveloped virus with a size of 150-185 nm and pleomorphic. The peplomers were 18-20 nm in length. The virus had the morphological features of a paramyxovirus except peplomers (Mattews, 1982). Avian paramyxo viruses that have been isolated from waterfowls were PMV-1 (NDV) (Palmer and Trainer, 1970; Rosenberger *et al.*, 1974 and Sulochana, 1991). Parainfluenza type 2 (Manjunath and Mallick, 1981). PMV-2, PMV-3, PMV-4 and PMV-6 were also isolated from waterfowls (Ottis and Bachmann, 1983; Manjunath and Mallick, 1981; Sulochana, 1987; Austin and Hinshaw, 1984; Tumova *et al.*, 1984 and Shihmanter *et al.*, 1995). Antibodies to PMV-1, PMV-2, PMV-6, PMV-7 and PMV-8 were also reported in wild water fowls (Maldonado *et al.*, 1994).

In the present study it was not possible to say whether T_{18} belongs to any of the PMV group other than PMV-1 (NDV group) as antisera to these serotypes were not available. However, this isolate has been forwarded to avian virology, Central Veterinary Laboratory, Weybridge, England for typing. From the characteristics studied for T_{22} the following conclusions were made. It is a DNA virus, enveloped, produces

haemagglutination of duck and teal red cells and are inactivated at pH 3 and pH 9. They could be propagated in chicken embryo fibroblasts producing CPE characterized by rounding of cells with cytoplasmic vacuolation and syncytium formation with intranuclear and intracytoplasmic inclusions.

The DNA virus reported from waterfowl are duck plague virus (herpes virus), EDS-76 virus (adenovirus) and duck hepatitis virus. T_{22} does not belong to any of these as DPV is a nonhaemagglutinating virus. EDS-76 though produces HA it is a naked virus. Duck hepatitis virus is a naked and non-haemagglutinating virus. Hence, it is concluded that this could be a DNA virus that has not been reported from waterfowls so far. The morphology of the virus could not be studied due to some technical difficulty. This isolate has also been forwarded to Avian Virology, Central Veterinary Laboratory, Weybridge, U.K. for identification.

In conclusion, two haemagglutinating viruses were isolated from the cloacal swabs of a total of 15 lesser whistling teals and 37 gargany screened. Intranuclear inclusions could be demonstrated in the hepatic cells of four of the 52 birds screened, but no isolations could be made from them. Of the two isolates obtained T_{18} was found to be an RNA virus having the morphological features somewhat similar to paramyxovirus and the isolate T_{22} a DNA virus, but the morphological features were not studied. The antigenic

similarity shown by the isolate T_{18} with that of the fish viruses FV and F6 isolated from epizootic ulcerative syndrome of fishes in Kerala leads to the conclusion that the waterfowls can act as symptomless carriers and can disseminate the fish viruses over long distances leading to the spread of the disease.

Summary

SUMMARY

The role of waterfowls in the dissemination and transmission of virus infection of domestic birds was explored during the present study. A total of 52 birds were screened for this purpose (15 lesser whistling teals and 37 gargany). Postmortem examination of these birds were carried out and the specimens including cloacal swabs, throat swabs, intestinal scrappings, tracheal scrappings, liver, spleen, lungs and brain were collected for virus isolation. Blood smears of all the birds were examined and were found to be negative for any bacterial infection. The impression smears from the cut surface of the liver revealed intranuclear inclusion bodies in the hepatic cells in four cases (lesser whistling teal No.17; gargany No.23, 27 and 49), but no virus isolations could be made from them. Two haemagglutinating agents were isolated from the cloacal swabs of two lesser whistling teals (Bird No.18 and 22) and these were characterized for further identification by morphological, biological and physicochemical characteristics.

Preliminary isolations of both the agents T_{18} and T_{22} were made in duck embryos and haemagglutinating activity was observed only after the second passage in the case of T_{18} and third passage in the case of T_{22}. But changes exhibited by the

duck embryos were not prominent as in the case of chicken embryos.

Nine-day old embryonated chicken eggs were used to increase the virulence of both the isolates. The allantoamniotic fluid of the chicken embryos inoculated with isolates T_{18} and T_{22} exhibited marked haemagglutination activity with chicken and teal red blood cells. The HA titre of T_{18} and T_{22} rose to 512 and 128 respectively after the 6th passages. Both T_{18} and T_{22} inoculated chicken embryos died in 3 to 5 days time. With T_{18} the embryo had lesions such as congestion of the CAM and embryo. No specific lesions could be seen in the internal organs. The chicken embryos inoculated with T_{22} showed highly stunted and curled embryos which were also congested. The chorioallantoic membrane were also congested. the liver of the embryo had yellowish brown patches.

Both the isolates T_{18} and T_{22} were examined for their HA activity against red blood cells of different species like duck, cattle, human `O', horse, pig and rabbit. In all these cases no agglutination was noticed. The infectivity tires in chicken embryos were 10^7 and 10^6 respectively for T_{18} and T_{22}. Both the isolates were thermolabile and got inactivated at 56°C for 30 min. The infectivity and haemagglutinating property were unaffected at pH 7.2, but at pH 3.2 and pH 9, complete loss of infectivity and HA activity were noticed.

The isolates T_{18} and T_{22} were sensitive to chloroform at five per cent level, indicating the enveloped nature of these agents.

In chicken embryo fibroblasts both the isolates exhibited marked CPE and the CPE was complete within 96 hr. For T_{18} the CPE in the stained monolayer revealed syncytium formation with four or five nuclei without any cytoplasmic demarcation and severe cytoplasmic vacuolation. Inclusion bodies were not detected in the nucleus or in the cytoplasm. In T_{22} the CPE was characterized by cytoplasmic vacuolation and rounding of cells. Large syncytium and total disintegration of cells were observed. Intranuclear and intracytoplasmic inclusion bodies could be demonstrated.

The nucleic acid type of the isolates were detected by pretreating the chicken embryo fibroblast cultures with 5-iodo-2 deoxyuridine which led to the conclusion that T_{18} is an RNA virus and T_{22} a DNA virus. The intracerebral pathogenicity indices were 0.83 and 0.30 respectively for T_{18} and T_{22}. The mean death time was 120 hr for both the isolates, while the intravenous pathogenicity indices were 0.00 for both, leading to the conclusion that both the isolates are non-pathogenic.

Pathogenicity was carried out in one-week old and six-week old chicken and ducklings. Both the isolates were

found to be non-pathogenic to chicken and ducklings when given by the oral route, subcutaneous route or by both. In all these cases neither clinical symptoms nor death was observed. However, infection was established by virus isolation and antibody titration from the cloacal swabs, upto the 14th day of infection, after which no virus could be isolated in the case of six-week old chicks. The haemagglutination-inhibition titres (HI) of the sera collected from the corresponding isolates T_{18} and T_{22} were 16 and 8 respectively.

Antigenic relationship of the haemagglutinating viruses T_{18} and T_{22} were analysed. It was found that both the isolates did not have any antigenic similarity with NDV and EDS-76. T_{22} was also distinct from the fish viruses F6 and FV isolated from cases of epizootic ulcerative syndrome (EUS) in fishes. On the contrary isolate T_{18} showed antigenic similarity with both the fish viruses leading to a conclusion that the waterfowls may be responsible for the bouts of disease outbreaks in fishes.

By electronmicroscopy, morphological characteristics of virus isolate T_{18} was studied which revealed it as an enveloped virus with a size of 150-185 nm, pleomorphic with peplomers of 18-20 nm in length. The virus similated paramyxovirus excepting the peplomers. The morphological features of T_{22} could not be studied due to some technical faults.

It was concluded from the study that T_{18} is an RNA virus similating paramyxovirus but entirely different from NDV and T_{22} is a DNA virus which is entirely different from DPV and EDS-76 and it could be a new isolate which has not been reported from waterfowls so far.

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* Originals not seen

ISOLATION AND IDENTIFICATION OF VIRUSES FROM WATERFOWLS SEEN IN KERALA

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ABSTRACT OF A THESIS

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ABSTRACT

In the recent years, frequent outbreaks of poultry diseases have caused significant economic losses and among the various sources of infection, freeflying migratory birds and waterfowls are reported to be an important source for the introduction of disease to domestic flocks. These freeflying birds and waterfowls abound in the waterlogged areas of Kerala especially Alleppey, Kottayam, Thrissur and Malappuram districts. Heavy losses were reported each year due to severe outbreaks of duck plague and duck pasturellosis resulting in the wiping out of the duck population in the state. This may be due to the introduction of the disease agents by migratory waterfowls. Hence a study was undertaken to elucidate the role of waterfowls in the spread of diseases to domestic poultry and ducks.

A total of 52 waterfowls were caught from different parts of Kerala of which 15 were lesser whistling teals and 37 were gargany. Postmortem examination of these birds were carried out and the required materials were collected. Impression smears from the cut surface of the liver revealed intranuclear inclusion bodies in the hepatic cells in four cases (Bird Nos. 17, 23, 27 and 49) and two haemagglutinating agents were isolated from the cloacal swabs of the bird numbers 18 and 22.

The haemagglutinating agents developed lesions in the chicken embryos which died 3 to 5 days after inoculation. the allantoamniotic fluid of the affected embryos agglutinated teal and chicken erythrocytes. The lesions exhibited by the embryos infected with T_{18} were congestion of the CAM and embryos while in T_{22} the embryos were stunted, curled and at the same time congested. The liver of the embryo had yellowish brown patches. The haemagglutination activity of both the viral isolates were tested with red cells of various species like cattle, horse, human `O', duck, rabbit and pig but was negative in all cases.

Both the isolates lost their infectivity and haemagglutination property at 56°C for 30 min. the infectivity and HA activity of the viral isolates T_{18} and T_{22} were retained at pH 7.2 and were completely destroyed at pH 3.2 and pH 9. Both the isolates were sensitive to treatment with chloroform, revealing both as enveloped viruses, wherein the infectivity was completely lost and HA activity was considerably reduced. In the case of T_{18} HA activity was reduced from 512 to 16 and for T_{22} from 128 to 8. The nucleic acid types of the viral isolates were confirmed by inoculating the isolates to chicken embryo fibroblast cultures pretreated with 100 μg and 200 μg per ml of IudR which led to the conclusion that T_{18} was a RNA virus and T_{22} a DNA virus. The ELD_{50} of T_{18} and T_{22} were 10^7 ELD_{50}/ml and 10^6 ELD_{50}/ml. The ICPI, MDT and IVPI were calculated as for NDV. The ICPI for the isolates were 0.83

and 0.30 for T_{18} and T_{22} respectively, MDT was 120 hr for both and the IVPI was calculated to be zero in both cases, indicating that both the isolates were nonpathogenic.

In cell cultures both the isolates produced CPE which affected the whole monolayer by 96 hrs. In the case of T_{18} the CPE was characterised by rounding and clumping of cells, the infected cells showing a tendency to get separated from the neighbouring cells leaving long cytoplasmic strands, syncytium formation with four or five nuclei and severe cytoplasmic vacuolation. For T_{22} the CPE was characterised by rounding and cytoplasmic vacuolation and karryorhexis. Intranuclear and intracytoplasmic inclusion bodies could be demonstrated.

The pathogenicity studies of both the isolates were carried out in one week old and six week old ducklings and chicken, for which both were nonpathogenic and did not exhibit any clinical signs or mortality. But viral infection was established in six week old chicks by virus isolation till the 14th day of infection from the cloacal swabs for both the isolates. The sera collected from these birds revealed an antibody titre of 16 for T_{18} and 8 for T_{22} indicating the infection. The antigenic relationship of the isolates was examined with NDV, EDS-76 and fish viruses (FV and F6), of which T_{22} did not show any antigenic similarity with any of the viruses. But T_{18} on the contrary exhibited antigenic relationship with the fishviruses FV and F6 but no antigenic

similarity with NDV and EDS-76. The antigenic similarity exhibited by T_{18} with the fish viruses leads to the conclusion that waterfowls may be disseminating the viruses responsible for the outbreak of epizootic ulcerative syndrome in fishes.

The morphological features of T_{18} by electronmicroscopy revealed an enveloped virus with a size of 150-185 nm with pleomorphic forms and peplomers of a length of 18-20 nm. Except the length of the peplomers it similated a paramyxovirus. The morphology of T_{22} was not studied due to technical defects.