CHARACTERISATION OF VIRUS ISOLATES FROM LESSER WHISTLING TEALS *(Dendrocygna javanica)* AND CHANNA SPECIES OF FISH

By

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THESIS

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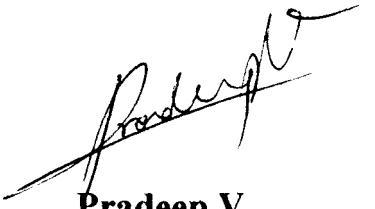
DECLARATION

I hereby declare that the thesis entitled **"Characterisation of virus isolates from lesser whistling teals (*Dendrocygna javanica*) and Channa species of fish"** is a bonafide record of research work done by me during the course of research and that this 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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CERTIFICATE

Certified that the thesis entitled **"Characterisation of virus isolates from lesser whistling teals (*Dendrocygna javanica*) and Channa species of fish"** is a record of research work done independently by **Pradeep V.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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INTRODUCTION

1. INTRODUCTION

India is a country where a large majority of the people depend upon agriculture as an important means of earning their income. The term agriculture in a broad sense includes both the activities involving cultivation of food and cash crops as well as management of livestock and poultry. Nowadays tremendous advances are being made in the field of agriculture, making it possible for the farmers to obtain rapid and profitable returns, thereby allowing them to lead a life of dignity and prosperity.

Poultry farming has in recent years gained much importance as a subsidiary occupation of the farmers. It is believed that poultry farming can play an effective role in improving the socio- economic status of the small farmers. The duck population in India is estimated to constitute about ten percent of the total poultry (Rithombar *et al.*, 1986). Major proportion of ducks are conveniently maintained as a side-line to various domestic operations in villages with wet lands and shallow and stagnant water sources.

Poultry and eggs are items of human food. The annual egg production in India during 1993 had crossed 27570 million eggs. During 1993, 4,06,000 tonnes of poultry meat were produced in the country and about 5000 tonnes of eggs were being exported.

Even as the small scale units of chicken, duck and turkeys with minimum capital investment are being replaced by large integrated operations with investment involving crores of rupees, mixed farming

including rearing of livestock, poultry, pig and ducks along with fish farming is still being followed in many rural areas of our state. Fish farming is another source of income to these farmers where their investment is very less. The excreta of the pigs and poultry are the sole source of food needed by the fishes.

Whatever the type of rearing, all forms of livestock including poultry are under constant threat of innumerable diseases. Large scale transport of animals and birds, finished products and biologics across international borders is leading to introduction of disease agents from one country to another. Other sources of transmission of disease producing agents are the free flying birds and waterfowls, which travel thousands of kilometers during their migratory period. These birds may be carrying a large number of pathogens, including viruses during their travel. Influenza viruses, Newcastle disease viruses and other paramyxoviruses are good examples of important pathogens that have been found spread in this fashion. Also a number of newly emerging diseases are being reported now for which these birds may be acting as silent carriers.

The fish farmers in our state are also facing disease problems with the fresh water fishes being affected with an epizootic ulcerative syndrome leading to formation of ulcers on the surface resulting in mortality or making the fishes unsuitable for consumption.

Isolation of viral agents from migratory waterfowls has been reported from many parts of the world and these migratory birds play an important role in the transmission of infectious diseases to domestic poultry. Since 1968 during surveillance of influenza virus in domestic

and feral birds many new viruses belonging to the group of paramyxoviruses based on their morphology, structure and other properties have been isolated (Tumova *et al.*, 1984). At least nine serogroups of avian paramyxoviruses, designated as PMV-1 to PMV-9 have been recognised. Many of the reported isolations of paramyxoviruses have been made from exotic or feral birds, but several serotypes have been isolated from domestic poultry as well (Alexander, 1982).

Both influenza A viruses and paramyxoviruses have been implicated as causative agents of epidemics in domestic poultry. Therefore the isolation of these viruses from free- flying birds has become an issue of great concern within the poultry industry (Mikami *et al.*, 1987). Migratory waterfowl and shore birds are reported to harbour a wide range of influenza viruses, some of which have been implicated in influenza outbreaks in mammals and domestic birds (Hinshaw *et al.*, 1985). They also found that ducks in different areas represent a continual source of orthomyxo and paramyxo viruses of potential disease producing significance to other species.

Other than orthomyxo and paramyxo viruses, a number of viruses like duck plague virus, adenovirus, duck hepatitis B virus have also been isolated from a variety of migratory waterfowls (Procter *et al.*, 1975; Smit *et al.*, 1980; Gulka *et al.*, 1984).

Viruses such as influenza virus and Newcastle disease virus have been isolated from domestic and feral birds as well as migratory water fowls in Kerala also (Sulochana and Nair, 1979; Sulochana *et al.*, 1982a; Sulochana *et al.*, 1987). Sulochana (1991b) isolated a

velogenic strain of Newcastle disease virus from the cloacal swabs collected at weekly intervals from apparently healthy lesser whistling teals (*Dendrocygna javanica*) for a period of eight weeks which clearly indicates the role of this species as a silent carrier of the virus. In a previous work conducted in the Department of Microbiology, Bindu (1997) had isolated two viruses from lesser whistling teals (*Dendrocygna javanica*) which failed to produce any clinical signs in six week old chicken after experimental inoculation, but virus isolation was possible from 3rd to 14th day from cloacal swabs.

From outbreaks of epizootic ulcerative syndrome (EUS), which took a heavy toll of fresh water fishes in Kerala during 1991-92, viruses which can survive in birds, particularly ducks was isolated indicating the possible role of migratory waterfowls in the introduction and distribution of the disease in our state (Sulochana *et al.,* 1992). Rhabdoviruses have also been reported to be isolated from fishes with epizootic ulcerative syndrome (Ahne *et al.,* 1988; Liley and Frerichs, 1994).

The practice of mixed farming followed in the rural waterlogged areas of Kerala is quite conducive for easy spread of disease agents if these migratory waterfowl are reservoirs of these agents. The waterlogged areas of Kerala are a good migratory place for the waterfowls and are also very rich in food for them, providing protection from other predators. While using these backwaters and lakes as a temporary halting place, these migratory birds can transmit the viral agents harboured within them to both the domestic birds as well as fishes leading to outbreaks of new epidemics in them.

Considering the above facts, an urgent need was felt to conduct a detailed study of viral agents isolated from teals and fishes to find out their relatedness. Hence the present research work was undertaken with the following objectives.

1. Physico- chemical and biological characterisation of isolates from lesser whistling teals and Channa species of fish.
2. Elucidation of the protein profile of the above isolates.
3. Study the antigenic relationship between the isolates.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Domestic poultry, especially chicken, ducks and turkeys are under a constant threat to innumerable diseases spread by the migratory water birds. Some of these agents cause illness in the wild birds themselves and for others, the birds act as mechanical carriers. A number of viruses have been isolated from migratory waterfowls and other wild birds from different parts of the world and they have been reported to play an important role in the transmission of infectious diseases to domestic poultry.

Since 1968, during surveillance of influenza virus in domestic and feral birds, many new viruses belonging to the group of paramyxoviruses have been isolated based on their morphology, structure and other properties (Tumova *et al.*, 1984). Out of the different serogroups of paramyxoviruses, many of the reported isolations of PMV-2 to PMV-7 viruses have been made from exotic and feral birds, but several serotypes have also been isolated from domestic poultry (Alexander *et al.,* 1982). Suss *et al.* (1994) reported that virtually all known antigenic subtypes of type A influenza viruses with various combinations of H and N surface antigens existed in the wild life reservoirs. In addition to orthomyxo and paramyxo viruses, duck plague virus was isolated from free flying waterfowls in Lake Andes (Procter *et al.,* 1975). A haemagglutinationg duck adenovirus could be isolated from the faecal samples of 12 species of waterfowls and other aquatic birds (Gulka *et al.,* 1984).

Sulochana (1991b) isolated a velogenic strain of Newcastle disease virus from the cloacal swabs collected at weekly intervals from

apparently healthy lesser whistling teals (*Dendrocygna javanica*) for a period of eight weeks which clearly indicated the role of this species as silent carriers of virus. Also from outbreaks of epizootic ulcerative syndrome which took a heavy toll of fresh water fishes (Channa species) in Kerala, a virus which can survive in birds, particularly in ducks was isolated, indicating the possible role of migratory birds in the introduction and dissemination of diseases in fishes (Sulochana *et al.*, 1992).

Isolation of rhabdoviruses from Ophicephalus (Channa) species of fishes have been reported by Ahne *et al.* (1988) and Liley and Frerichs (1994).

2.1.CHARACTERISATION OF THE VIRUS ISOLATES

2.1.1. pH sensitivity

Viruses vary in their stability to different pH levels. As a general rule, enteric viruses are stable to low pH since they are able to withstand the acidic conditions of the intestinal tract. Stability to various pH levels is determined by exposing the virus samples to pH 3.0, 7.2 and 9.0 for 60 minutes. A decrease of 2 log or more in infectivity or haemagglutinating activity will be considered indicative of sensitivity to pH. Retention of activity or any decrease less than 2 log will be indicative of stability.

Nerome *et al.* (1978) isolated a haemagglutinating virus from an epizootic among budgerigar flocks in Kunitachi, Tokyo. The virus designated Kunitachi virus was identified to be a member of the paramyxo group whose infectivity was not affected by acid treatment (pH- 2.9).

Sulochana *et al.* (1981) isolated a Newcastle Disease virus form ducks (NDV- D) which was found to be sensitive to pH 3.0 and resistant to pH 10.

The infectivity of a strain of Newcastle disease virus isolated from Japanese quails was observed to be stable at pH 3.0 (Kumanan and Venkatesan, 1991).

Vijayarani *et al.* (1992b) characterised three field isolates of Newcastle disease virus and found that the infectivity of two of the isolates was stable at pH 3.0 whereas that of the other was unstable.

Out of eleven NDV isolates obtained from outbreaks of disease in chicken and Japanese quail in Tamil Nadu, one was reported to be unstable at pH 3.0 while the other 10 were stable. (Kumanan *et al.*, 1992).

Bindu (1997) reported that both the infectivity and haemagglutination properties of two viruses isolated from lesser whistling teals (*Dendrocygna javanica*) were completely lost at pH 3.0 and 9.0 and was normal at pH 7.2.

The infectivity of five isolates of Newcastle disease virus from *desi* birds was observed to be stable at pH 3.0 (Kamaraj *et al.*, 1998).

Lang *et al.* (1968) observed that the infectivity and haemagglutinin of an influenza virus isolated from turkey were labile at low pH (3.0) but were unaffected in the alkaline range of pH 7.0 to 8.0.

Influenza viruses were labile at pH 3.0 when held at room temperature for a period of one hour (Papparella *et al.*, 1969; Buxton and Fraser, 1977; Mini, 1988) but were relatively stable at pH 7.0 to 8.0 (Buxton and Fraser, 1977; Mini, 1988). Mini (1988) observed that at pH 9.0, there was marked reduction in HA activity and slight reduction in infectivity of four strains of influenza virus type A isolated from ducks.

Exposure of duck plague virus (DPV) for six hours at pH levels of 7.0, 8.0 and 9.0 resulted in no loss of titre, but a measurable titre reduction was noted at pH 5.0, 6.0 and 10.0 At pH 3.0 and 11.0 DPV was rapidly inactivated. A marked difference in inactivation rates was noted between pH 10.0 and 10.5 (Hess and Dardiri, 1968). Leibovitz *et al.* (1991) reported that duck plague virus was inactivated at a pH below 3.0 and above 10.0.

Ramkumar *et al* (1991) reported that at an acidic pH of 3.0, a haemagglutinating viral agent resembling EDS-76 virus isolated from poultry flocks was quite stable. A similar observation was made by Swain *et al.* (1992) with a field isolate of EDS- 76 virus isolated from a layer flock.

A rhabdovirus isolated from snake head (*Ophicephalus striatus*) with ulcerative syndrome in Thailand was reported to be inactivated by treatment with acid (pH 3.0) (Ahne *et al.*, 1988). The virus infectivity of a rhabdovirus isolated from penaeid shrimps was found to be sensitive to a low pH (Lu and Loh, 1992).

2.1.2. Thermostability

Viruses vary widely in their response to heat. Some viruses are quickly inactivated at higher temperatures, whereas others are much more heat stable. Knowledge of the thermostability of a given virus can predict if a given procedure will result in destruction or preservation of viral activity.

Most viruses are stable at room temperature, at least for short periods of time, when they are suspended in complex media. Differences occur in the 50- 60°C range where there is protein denaturation. The standard test uses constant exposure to 56°C in a water bath. A given virus is considered heat labile if there is a 2 log (or greater) decrease in infectivity following exposure to 56°C for 30 min.

Hanson *et al.* (1949) conducted detailed thermostability studies on the haemagglutinins from Newcastle Disease Virus (NDV) strains of different virulence and concluded that at 56°C, haemagglutinins of lentogenic strains were heat labile where as those of mesogenic and velogenic strains maintained their activity at least for 30 min. But McFerran and Nelson (1971) reported the isolation of a lentogenic NDV strain whose haemagglutinin showed a relatively high thermostability.

Lomniczi (1975) determined the thermal inactivation rate constant for infectivity of 24 Newcastle Disease virus strains at 50°C and 56°C. A greater part of the NDV strains examined were found to be thermostable irrespective of their virulent properties.

A paramyxovirus, designated Kunitachi virus, isolated from an epizootic which occurred among budgerigar flocks in Kunitachi, Tokyo

was found to be relatively susceptible to heating at 56°C for 30 min. (Nerome *et al.*, 1978).

Hanson and Spalatin (1978) reported the isolation of viscerotropic velogenic strains of NDV having a thermosensitive haemagglutinin and of a lentogenic strain with an unusually thermostable haemagglutinin which confirmed that virulence for chicken was not related to the thermostability.

Kim and Spradbrow (1978) compared some properties of the avirulent Australian V_4 strain of NDV and 12 new isolates of NDV. They found that the infectivity of V_4 and three of the isolates was relatively stable at 56°C while that of eight isolates was labile. Haemagglutinins of all viruses studied were stable at 56°C for longer than 60 min.

Haemagglutination titre of two isolates of avian paramyxovirus belonging to PMV-6 group was reduced to less than two from an initial titre of 2048 in 30 min., and that of another isolate in 45 min at 56°C (Shortridge *et al.*, 1980).

Nine 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).

Alexander *et al.*(1981) found the haemagglutinin activity of two paramyxoviruses isolated from doves (Columba species) to be heat

resistant in that incubation at 56°C for 90 min had no effect on the haemagglutination titre.

Sulochana *et al.* (1981) found the stability of haemagglutinin of a Newcastle disease virus isolated from ducks (NDV-D) to be 20 min. at 56°C. The haemagglutinating activity and infectivity of a mesogenic strain of NDV isolated from a mynah was reported to be labile at 56°C (Sulochana *et al.*, 1982a).

Gough and Alexander (1983) isolated a haemagglutinating virus from the caecal tonsils of dead collared doves (*Streptopelia decaocto*) which was identified as an avian paramyxovirus whose haemagglutinin was extremely thermostable compared to other prototype strains of avian paramyxoviruses.

The infectivity of two paramyxovirus type I strains isolated from the cloaca and throat of clinically diseased pigeons was not affected by five minutes exposure to 56°C, but was reduced ten times after 30 min. exposure, with haemagglutinating activity remaining intact (Wawrzkiewicz *et al.*, 1989).

Sulochana (1991a) observed that the haemagglutinating activity and infectivity of a NDV isolated from Japanese quails were completely lost at 56°C for 30 min whereas Kumanan and Venkatesan (1991) found the haemagglutinins of a strain of Newcastle disease virus isolated from Japanese quails to be stable for up to 60 min at 56°C.

Vijayarani *et al.* (1992b) characterized three field isolates of NDV and found that they belonged to the velogenic group. At 56°C, the

haemagglutinin of two of the isolates was stable for 120 min while that of the other for 60 min. Reddy *et al.* (1993) reported that the thermostability of different isolates of Newcastle disease virus belonging to the mesogenic group at 56°C varied from 6 to 120 min.

Nine pigeon paramyxovirus type I isolates from the United States and Canada were found to have a haemagglutinin thermostability for 60 min or more at 56°C (King, 1996).

Bindu (1997) observed that the infectivity of two viruses isolated from lesser whistling teals (*Dendrocygna javanica*) was completely destroyed by heating at 56°C for 30 min. Haemagglutination activity of both the isolates was also lost within 30 min.

Influenza A viruses are considered to be temperature sensitive and get inactivated at 56°C for 30 min (Merchant and Packer, 1967; Papparella *et al.*, 1969).

The infectivity and haemagglutination activity of an influenza virus isolated from turkey were destroyed rapidly by heating at 56°C, but at lower temperatures, the haemagglutination activity was more stable than infectivity (Lang *et al.*, 1968).

Buxton and Fraser (1977) reported that influenza viruses were inactivated at a temperature of 55°C for one hour or in 10 min at 60°C. However, Homme and Easterday (1970) found that influenza A/ turkey/ Wisconsin/ 66 took six hours at 56°C for inactivation. Mini (1988) found that the infectivity of four strains of influenza virus type A isolated from

ducks in Kerala was completely destroyed by heating at 56°C for 30 min.

Hess and Dardiri (1968) reported complete inactivation of duck plague virus in 10 min at 56°C and in 90- 120 min. at 50°C.

Ramkumar *et al.* (1991) reported that a haemagglutinating agent resembling EDS-76 virus isolated from poultry flocks was found to be stable at temperature of 56°C for 30 min. A similar observation was made by Swain *et al.* (1992).

Ahne *et al.* (1988) isolated a rhabdovirus from affected snake head (*Ophicephalus striatus*) which was inactivated by heat treatment at 56°C. The infectivity of a rhabdovirus isolated from penaeid shrimps was found to be sensitive to a temperature of 37°C (Lu and Loh, 1992).

2.1.3. Chloroform sensitivity

Enveloped viruses are usually susceptible to the action of lipid solvents such as ether or chloroform. Exposure to these solvents results in a marked decrease in virus infectivity. Non-enveloped viruses do not exhibit such a decrease in infectivity when treated similarly.

Ether and chloroform fragment the cell membrane that envelopes the virus and expose the nucleic acid and protein core. A given virus is considered sensitive if exposure to the lipid solvent results in a 2 log decrease in infectivity as compared to unexposed controls.

Nerome *et al.*(1978) observed that the infectivity of Kunitachi virus was completely lost on treatment with ether indicating that the virus possessed a lipid envelope.

Four Australian isolates of lentogenic NDV were tested for haemagglutination activity. None of the four isolates tested lost haemagglutinin activity on treatment with ether (Kim and Spradbrow, 1978).

Physico-chemical characterization of nine avian paramyxoviruses isolated from tracheal and cloacal swabbing of feral ducks and geese in the Atlantic flyway showed that all the isolates were sensitive to chloroform treatment (Cloud and Rosenberger, 1980).

Bindu (1997) reported that two virus isolates, T_{18} and T_{22} isolated from lesser whistling teals (*Dendrocygna javanica*) were completely inactivated on treatment with five percent chloroform indicating that both the isolates were enveloped.

Chloroform sensitivity of an influenza virus isolated from turkey was demonstrated by Lang *et al* (1968). Complete inactivation of the influenza virus when treated with five percent chloroform indicated that the virus was enveloped. Sensitivity of influenza type A viruses isolated from ducks to chloroform was also reported by Mini (1988).

Benton *et al.* (1967) found that infectious bursal disease virus resisted treatment with ether and chloroform.

Hess and Dardiri (1968) found duck plague virus to be sensitive to ether and chloroform. A herpes virus isolated from waterfowl dying of duck enteritis lost its pathogenicity to duck embryo fibroblasts completely after treatment with ether for one hour at room temperature (Lin *et al.*, 1984).

The haemagglutinating activity of three viral agents resembling EDS-76 virus isolated from poultry flocks was retained after treatment with 10 percent chloroform (Ramkumar, *et al.*, 1991). Swain *et al.* (1992) reported that a field isolate of EDS-76 virus was resistant to treatment with both ether and chloroform.

Ahne *et al* (1988) reported that a rhabdovirus isolated from snake head (*Ophicephalus striatus*) with ulcerative syndrome in Thailand was inactivated by treatment with 50 percent chloroform.

A rhabdovirus isolated from penaeid shrimps was observed to be sensitive to 20 percent ethyl ether (Lu and Loh, 1992).

2.1.4. Nucleic acid type

The drug 5-iodo-2-deoxyuridine (IuDR) is used to find out whether the nucleic acid is DNA or RNA. DNA viruses are inactivated by IuDR and so there will be no multiplication or cytopathic effect on the monolayer. IuDR has no action on RNA so that RNA viruses can grow and produce cytopathic effect on the monolayer.

The effect of IuDR on the replication of Kunitachi virus, an avian paramyxovirus was determined. There was no significant difference in

virus replication in chick embryo cells with and without IuDR indicating that Kunitachi virus contain RNA rather than DNA (Nerome, *et al.*, 1978).

The infectivity of avian paramyxoviruses was observed to be not affected by IuDR indicating that they had an RNA genome (Shortridge *et al.*,1980; Alexander *et al.*, 1981).

Alexander *et al.*(1983) opined that two paramyxoviruses isolated from waterfowl and one isolated from duck had RNA genome, because their multiplication in chick kidney cell cultures was unaffected by the presence of IuDR in the culture fluid.

The drug IuDR did not inhibit the growth of a paramyxovirus isolated from collared doves (*Streptopelia decaocto*) in chick embryo cells whereas the growth of an avian adenovirus was significantly reduced in the presence of IuDR (Gough and Alexander, 1983).

Bindu (1997) found that pretreatment of chicken embryo fibroblast cell cultures with IuDR inhibited the replication of a virus isolated from lesser whistling teals whereas another virus isolated from the same species of bird produced characteristic cytopathic effect both in drug treated as well as control tubes. These results indicated that the former was a DNA virus and the latter a RNA virus.

Kunst (1968) observed 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 apple green fluorescence indicative of DNA (Hess and Dardiri, 1968). Lin *et al.* (1984) reported that the growth of three strains of duck enteritis virus in duck embryo fibroblast culture was inhibited when 5-bromo deoxyuridine was present at a concentration of 100 μg/ ml indicating that the nucleic acid was DNA.

From studies using labeling with ^{3}H Thymidine and inhibition with iodo deoxy uridine, EDS- 76 virus was found to contain DNA (Todd and Mc Nulty, 1978; Yamaguchi *et al.*, 1981). The replication of a viral agent resembling EDS-76 virus isolated from poultry flocks was inhibited by IuDR demonstrating the nucleic acid was DNA type (Ramkumar *et al.*, 1991; Swain *et al.*, 1992).

Ahne *et al.* (1988) reported that replication of a rhabdo virus isolated from snake head (*Opicephalus striatus*) with ulcerative syndrome in Thailand was not affected by IuDR indicating a RNA genome. The replication of a rhabdovirus isolated from penaeid shrimps was not inhibited by the DNA antagonist, 5-bromo-2'-deoxyuridine at a level of 20 μg/ ml (Lu and Loh, 1992).

2.1.5. Cytopathic effects

Alexander *et al.* (1973) investigated the cytopathogencity and production of Newcastle disease virus (NDV) strain Herts, cultivated in chick embryo (CE) cells, baby hamster kidney (BHK-21), HEp-2, MDBK and L929. They found that infection at high multiplicities induced cell fusion in cultures of all cell types within 3h after infection whereas

infection at low multiplicities produced extensive cell fusion in CE cells, BHK-21, HEp-2 and L929 cultures within 24h, but MDBK cells failed to fuse.

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

Nine avian paramyxoviruses isolated from tracheal and cloacal swabs of feral ducks and geese in the Atlantic flyway were capable of replication in Vero cell cultures, resulting in varying degrees of cell monolayer degeneration, where as no cytopathology was noted in CEF cell cultures (Cloud and Rosenberger, 1980).

Infection of chicken kidney (CK) cell monolayers with three isolates of avian paramyxoviruses from poultry in Hong Kong produced cytopathic effects consisting of small discrete syncytia containing two to six nuclei which were visible from the fourth hour post infection (Shortridge *et al.*, 1980).

Alexander *et al.* (1981) reported that infection of primary chick kidney cell monolayers with a paramyxovirus isolated from doves resulted in the release of virus into the culture medium by 96 h after infection, although no evidence of cytopathic effect was seen.

Confluent monolayers of chick embryo (CE) and chick kidney (CK) cells infected with a paramyxovirus isolated from collared doves failed to show any cytopathic effect during the 10 day observation period (Gough and Alexander, 1983).

Three paramyxoviruses isolated from waterfowls in the USA and Japan failed to show any marked cytopathic effect in chick kidney cell cultures over a 72h incubation period, in particular no evident syncytial formation was observed (Alexander *et al.*, 1983).

Schemera *et al.* (1987) isolated two paramyxoviruses of serotype 3 from finches which induced cytopathic effects consisting of syncytia formation and foci of round cells in chicken embryo fibroblasts and chicken embryo kidney cell cultures four days post infection.

Singh (1993) reported that an avian paramyxovirus type I isolated from pigeons formed clear plaques in chicken embryo fibroblast cultures.

Bindu (1997) observed that two viruses isolated from lesser whistling teals (*Dendrocygna javanica*) produced characteristic cytopathic effect in chicken embryo fibroblast monolayer consisting of rounding and clumping of cells and formation of syncytia.

Niven *et al.* (1962) reported that the cytopathic effects of influenza virus in cell culture consisted of rounding and eventual detachment of cells. Cytopathic changes produced by avian influenza virus A/ turkey/ Ontario/ 7732/65 in chicken fibroblast have been described by Narayan *et al.* (1969).

Kocan *et al.* (1978) propagated influenza type A viruses in mallard duck tracheal organ cultures and observed cytoplasmic

vacuolation, nuclear swelling and sloughing of the epithelial cells following infection.

The cytopathic effects in chicken embryo liver cells of an Indian isolate of egg drop syndrome-76 virus was marked by the presence of round and refractile cells and detachment of cells from the glass surface. Intranuclear eosinophilic inclusion bodies were observed between 24 to 48h after infection (Swain *et al.*, 1993).

A morbilli virus isolated from Mediterranean striped dolphins *(Stenella coeruleoatta)* was inoculated onto African green monkey kidney (Vero) cell monolayers and found to produce cytopathic changes within four weeks (Van Bressem *et al.*, 1991).

Visser *et al.* (1993) observed that a morbilli virus isolated from harbour porpoises produced cytopathic changes including the development of small syncytia and foci of rounded cells both in Vero cells cultures and primary kidney cells of harbour porpoise.

2.1.6. Antigenic characterisation of the virus isolates

Alexander and Chettle (1978) reported that haemagglutination inhibition (HI) tests showed a high level of cross reaction between parakeet/ Netherlands/ 449/75(449) virus and paramyxovirus/ turkey/ Wisconsin/ 68 virus.

Representative isolates of the paramyxoviruses duck/ Hong Kong/75 and duck/ Mississippi/ 75 were shown to be closely related serologically by haemagglutination inhibition and neuraminidase inhibition tests (Alexander *et al.*, 1979).

On the basis of haemagglutination inhibition and virus neutralization tests, nine avian paramyxoviruses isolated from tracheal and cloacal swabbings of feral ducks and geese in the Atlantic flyway were separated into six antigenic groups (Cloud and Rosenberger, 1980).

Representatives of eight viruses isolated in Hong Kong were shown to be paramyxoviruses but were serologically distinct from other avian and mammalian paramyxoviruses by haemagglutination inhibition and neuraminidase inhibition tests (Shortridge *et al.*, 1980).

Alexander *et al.* (1983) showed that paramyxoviruses isolated from waterfowl in the USA were serologically closely related but distinct from other avian paramyxoviruses by haemagglutination inhibition and immunodiffusion tests.

Schemera *et al* (1987) found two viruses isolated from exotic finches to be serologically related to the reference strain of the paramyxovirus serotype 3 by haemagglutination inhibition tests.

Alexander *et al.* (1989) tested nine paramyxoviruses isolated from penguins for antigenic relationships amongst themselves and to other avian paramyxoviruses. One of the isolates was shown to be a lentogenic Newcastle disease virus (NDV) and the other eight isolates could be placed into three groups by haemagglutination inhibition and immunodiffusion tests.

Haemagglutination inhibition tests and immunodiffusion tests showed the prototype virus for PMV-7 serotype of avian paramyxovirus

and five other isolates obtained from birds of the Columbidae family to be distinct from the other avian paramyxoviruses but to have some minor relationships between each other (Alexander, *et al.,* 1991).

Bindu (1997) found that two virus isolates from lesser whistling teals (T_{18} and T_{22}) were antigenically distinct from NDV and EDS-76 virus by HI test.

2.1.7. Pathogenecity trials

Nerome *et al.* (1978) infected groups of 23 immature budgerigars intranasally and intratracheally with approximately 10^5 p.f.u of freshly propagated Kunitachi virus. After infection, 18 birds showed signs of depression, dyspnoea, diarrhoea and torticollis before death. All the budgerigars that developed signs of disease eventually died within two weeks.

Infection of six weeks old chicken with three avian paramyxovirus isolates by intranasal or intravenous routes produced no signs of disease throughout a 21 day observation period (Shortridge *et al.,* 1980).

Intranasal inoculation of nine avian paramyxoviruses isolated from tracheal and cloacal swabbings of feral ducks and geese in the Atlantic flyway into 12 day old broiler chickens and turkeys induced a mild respiratory disease during a 14 day observation period. Two of the isolates were recovered from the tracheae of inoculated birds at four and ten days post inoculation (Cloud and Rosenberger, 1980).

Sulochana *et al* (1981) infected eight-week-old white leghorn chicks with Newcastle disease virus isolated from an outbreak of acute respiratory condition in ducklings by subcutaneous and oral routes. All the birds infected by subcutaneous route died between the third and fourth day post inoculation and the virus could be isolated from the

cloacal swabs and also from the liver and spleen. The birds infected by oral routes showed respiratory signs and died with an average post infection period of 114 hours.

A mesogenic strain of Newcastle disease virus isolated from a mynah was found to be pathogenic to chicks below 3 weeks of age but six week old chicks were found to resist the infection (Sulochana *et al.*, 1982b).

Out of five finches from various species inoculated with a paramyxovirus of serotype 3 isolated from African and Australian finches, three remained clinically healthy through 6 weeks, but two died, one 5 days post inoculation after showing nervous distress, and the other suddenly 42 days post inoculation (Schemera *et al.*, 1987).

Pathogenecity trials with an avian paramyxovirus-2 from domestic and wild birds in Costa Rica in turkey poults and newly hatched chicks did not result in growth impairment or significant clinical signs of disease during a 25 day observation period. Attempts to isolate NDV were negative (Goodman and Hanson, 1988).

Bindu (1997) reported that two viruses isolated from lesser whistling teals (*Dendrocygna javanica*) did not produce any clinical signs or mortality when inoculated into day old and six-week-old chicken and ducklings over a four week observation period. But virus isolation was possible till 14^{th} day of infection from the cloacal swabs for both the isolates.

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

Alexander *et al.* (1978) carried out a detailed study on the pathogenecity of four avian influenza virus strains in two-week-old fowls, turkeys and ducks by intranasal administration. They found that three of the strains were pathogenic for fowls, turkeys and ducks.

Austin and Hinshaw (1984) infected ducks with two of their influenza virus isolates from mallards and reported virus excretion in their faeces for a period of 12 days following infection.

2.1.8. Purification of the virus isolates

Different methods of purification of Newcastle disease virus have been described.

Evans and Kingsbury (1969) treated, filtered and pelleted allantoic fluid with trifluoro ethane and then centrifuged the pellets on 30 percent and 16 percent potassium tartrate cushions.

Mountcastle *et al.* (1971) precipitated three paramyxovirus from tissue culture fluid with an equal volume of saturated ammonium sulphate and banded them twice on linear 15 to 40 percent (w/w) potassium tartrate gradients.

Moore and Burke (1974) purified Newcastle disease virus by centrifugation of pelletted virus to equilibrium on glycerol- sucrose-tartrate (GST) gradient.

Alexander (1974) concentrated four avian paramyxoviruses from allantoic fluid by high speed centrifugation and purified the pelleted virus samples on sucrose density gradients. Purification of a paramyxovirus isolated from a duck by centrifugation through linear sucrose gradients was also reported by Kessler and Aymard (1979).

Alexander and Collins (1981) purified concentrated avian paramyxovirus by centrifugation on discontinuous followed by continuos sucrose gradient.

Kumanan *et al.* (1994) purified Newcastle disease virus by precipitating the virus from clarified allantoamniotic fluid and cell culture fluid using saturated solution of ammonium sulphate and layering the concentrated virus onto gradients of 15- 45 percent sucrose.

Purification of clarified Newcastle disease virus by sucrose density gradient centrifugation was also reported by Swain *et al.* (1997).

2.1.9. SDS-PAGE analysis of structural proteins

The polypeptides of isolated and purified parainfluenza virus SV5 were dissociated with sodium dodecyl sulphate and separated by polyacrylamide gel electrophoresis. A total of six polypeptides ranging in molecular weights from 43 to 76 kDa were found in the SV5 strain (Caliguiri *et al.*, 1969).

Electrophoresis of Newcastle disease virus (NDV) proteins in polyacrylamide gels containing sodium dodecyl sulphate resolved three major components and five minor components. The three major protein components had molecular weights of 90, 62 and 42 kDa respectively (Evans and Kingsbury, 1969).

The polypeptides of three paramyxoviruses (Simian virus5, Newcastle disease virus and Sendai virus) were separated by polyacrylamide gel electrophoresis. The protein patterns revealed similarities among the three viruses with each virus containing at least five or six proteins. Four of the proteins had features common with corresponding proteins in the other two viruses, including similar molecular weights. These four proteins were the nucleocapsid protein (molecular weight 56 to 61.2 kDa), a larger glycoprotein (molecular weight 65 to 74 kDa), a smaller glycoprotein (molecular weight 53 to 56 kDa) and a major protein which is the smallest protein in each virion (molecular weight 38 to 41 kDa) (Mountcastle *et al.*, 1971).

Waters and Bussell (1973) studied the polypeptide composition of measles and canine distemper viruses by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Six polypeptides ranging in molecular weights from 79 to 37 kDa were detected in each virus which had similarities among themselves and also with the polypeptides of Sendai virus, Simian virus 5 and Newcastle disease virus.

Alexander (1974) compared the structural polypeptides of four paramyxovirus separated by polyacrylamide gel electrophoresis under reduced and non-reduced conditions. The protein patterns showed

basic similarities among the four viruses- under reduced conditions, seven polypeptides were revealed for each virus of which two were glycopolypeptides. The polypeptides of two of the viruses appeared identical but distinct differences between the viruses in the molecular weights of polypeptides and other migration properties were evident.

Moore and Burke (1974) characterized the structural proteins of different strains of NDV by polyacrylamide gel electrophoresis under reduced and non reduced conditions. Nonreducing conditions were found to be better for differentiating the strains.

Kessler and Aymard (1979) compared the polypeptide composition of a new strain of paramyxovirus isolated from wild ducks with that of Yucapia and Newcastle disease virus. Similarity was observed with NDV with five polypeptides being present,two of which were glycoproteins and having molecular weights ranging from 76 to 43 kDa.

The structural polypeptides of avian paramyxoviruses isolated from ducks in Hong Kong and USA were found to be similar. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate under reduced conditions revealed a similar polypeptide pattern, consisting of seven polypeptides with apparent molecular weights ranging from 46 to 190 kDa (Alexander *et al.*, 1979).

Shortridge *et al.* (1980) examined the structural polypeptides of an avian paramyxovirus isolated from poultry in Hong Kong by SDS-PAGE. Seven polypeptides were detected, with molecular weights ranging from 180 to 40 kDa.

McCarthey and Johnson (1980) compared the polypeptide patterns of five strains of mumps virus under similar electrophoretic conditions. A similar pattern of six polypeptides ranging in molecular weights from 200 to 39 kDa was obtained.

The polypeptide patterns of two paramyxoviruses isolated from doves was found to be identical, consisting of seven polypeptides, when examined by polyacrylamide gel electrophoresis in the presence of SDS (Alexander et *al.*, 1981).

The structural polypeptides of twenty-three avian paramyxoviruses from five serotypes were examined by SDS-PAGE under reducing and non-reducing conditions. All virus polypeptide proteins consisted of seven to ten polypeptides of which two were glycosylated (Alexander and Collins, 1981).

Alexander *et al.* (1983) reported the polypeptide profiles produced by polyacrylamide gel electrophoresis in the presence of SDS of three viruses isolated from water fowls to be typical of paramyxoviruses, consisting of five to seven polypeptides of which two were glycosylated.

Rima (1983) observed that morbilli viruses such as measles virus and canine distemper virus contained six proteins analogous to those of paramyxovirus.

Polypeptide analysis of paramyxoviruses isolated from penguins in Antarctica and sub-Antarctica by SDS-PAGE under reducing conditions showed typical paramyxoviruses polypeptide profiles with

the number and sizes of the major polypeptides similar to those of PMV-1 (Alexander *et al.* , 1989).

Vijayarani *et al.* (1992a) fractionated the proteins of three velogenic field isolates of NDV by PAGE and found differences in the number and nature of the protein fractions.

Five field isolates of Ranikhet disease virus were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, both before and after adaptation to chicken embryo fibroblast culture and BHK-21 cells for studying their protein fractions. The pattern of protein fractions was identical and contained eight fractions ranging from 108 to 44 kDa (Kumanan *et al.*, 1994).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of a velogenic NDV revealed six structural polypeptides with molecular weights ranging from 198 to 37 kDa (Swain *et al.*, 1997).

A field isolate of egg drop syndrome 76 (EDS 76) virus isolated from a layer flock was found to contain 12 viral proteins ranging from 10 –126 KDa by SDS –PAGE (Swain *et al.*, 1992).

The structural proteins of hirame rhabdovirus (HRV) was analysed by SDS-PAGE. Five proteins were identified and based upon their relative mobilities, molecular weights were estimated to range from 156 kDa to 19.9 KDa (Nishizawa *et al.*, 1991).

Protein profile of three rhabdoviruses from snake head fish (*Ophicephalus striatus*) was determined and compared to five known

fish rhabdo viruses and one mammalian rhabdo virus. The snake head rhabdovirus exhibited a Lyssa virus type protein profile whereas the ulcerative disease rhabdovirus isolates exhibited a vesiculovirus type protein profile (Kasornchandra *et al.*, 1992).

SDS-PAGE analysis of the structural proteins of a rhabdovirus isolated from European lake trout (*Salmotrutta lacustris*) indicated that the virus belonged to the vericulovirus genus of the family Rhabdoviridae (Bjorklund *et al.*, 1994).

Polyacrylamide gel electrophoresis of seven rhabdoviruses isolated from fish suffering from epizootic ulcerative syndrome (EUS) demonstrated that six of them had virtually identical protein profiles, whereas one differed in the number of protein bands and in their migration pattern (Liley and Frerichs, 1994).

By SDS-PAGE, the number of major bands identified and the profile of the viral polypeptides of rhabdovirus of penaeid shrimp (RPS) resembled that of the mammalian vesicular stomatitis virus (VSV) and rhabdovirus carpio (RC), but were different from that of infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) (Lu and Loh, 1994).

*

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

All the chemicals used in this study were of analytical grade obtained from Hi-Media, Mumbai and Sisco Research Laboratory (SRL), Mumbai.

3.1.1. Tryptose phosphate broth with antibiotics (TPB-A)

Ready made medium was purchased from Hi-Media, Bombay, reconstituted as per manufacturer's instructions and sterilised by autoclaving at 121°c at 15 lbs pressure for 15 min. Penicillin (250 IU/ml) and streptomycin (250 μg/ml) were added to the medium before it was used for dilution of virus samples for titration.

3.1.2. Antibiotic solutions

3.1.2.1. Streptopenicillin

Penicillin	-	10,00,000 IU
Streptomycin	-	1g

Sterile triple distilled water - 40 ml

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

3.1.2.2. Nystatin

Nystatin	-	1 g
Dimethyl sulfoxide (DMSO)	-	40ml

0.2 ml of this solution was added to 100 ml of medium to give a final concentration of 50 μg of nystatin per ml.

3.1.3. Normal saline

Normal saline solution was prepared by dissolving 0.9g of sodium chloride in double glass-distilled water and sterilised by autoclaving at 121°C at 15 lbs pressure for 15 min.

3.1.4. Phosphate buffered saline (PBS)

Solution A.

Sodium chloride	-	8.0g
Potassium chloride	-	0.2g
Disodium hydrogen phosphate	-	1.608 g
Potassium dihydrogen phosphate	-	0.2 g
Distilled water	-	800 ml

Solution B

Calcium chloride	-	0.1 g
Distilled water	-	100 ml

Solution C

Magnesium chloride	-	0.1 g
Distilled water	-	100 ml

When all salts were dissolved, solutions A, B and C were separately autoclaved at 121°C at 15lbs pressure for 15 min, mixed and pH adjusted to 7.2.

3.1.5. Calcium magnesium free phosphate buffered saline (CMF-PBS)

Ready made media purchased from Hi-Media, Bombay were reconstituted as per manufacturer's instructions and sterilised by autoclaving at 15 lbs pressure at 121°C for 15 min.

3.1.6. Citrate phosphate buffer pH- 3.2

Two stock solutions , solution A (0.1 M solution of citric acid) and solution B (0.2 M solution of dibasic sodium phosphate) were prepared in double glass distilled water. Working solution was prepared by mixing 37.7 ml of solution-A with 12.3 ml of solution B and diluted to 100 ml with distilled water. It was sterilised by autoclaving at 121°C at 15 lbs pressure for 15 min.

3.1.7. Phosphate buffers of pH 7.2 and 9.0

A 0.2 M solution of monobasic sodium phosphate (solution A) and a 0.2M solution of dibasic sodium phosphate (solution B) were prepared as stock solutions. Working solution of pH 7.2 was prepared by mixing 87.7 ml of solution A and 12.3 ml of the solution B and diluted to make a total volume of 200 ml.

Phosphate buffer of pH 9.0 was prepared by mixing 85 ml of phosphate buffered saline with 15 ml of 0.1N sodium hydroxide.

Both the buffers were sterilised by autoclaving at 121°C at 15 lbs pressure for 15 min.

3.1.8. Hank's balanced salt solution (HBSS)

Ready made media purchased from Hi-Media, Bombay were reconstituted as per manufacturer's instructions and sterilised by filtration.

3.1.9. Lactalbumin Hydrolysate

Lactalbumin hydrolysate was added to HBSS at a level of 0.5 per cent before sterilisation.

3.1.10. Yeast extract.

Yeast extract was added to HBSS at a rate of 0.2 per cent before filtration.

3.1.11. Calf serum

Blood was collected aseptically from healthy calves below two weeks of age and allowed to clot. Serum was separated, sterilised by filtration, dispersed in 10ml quantities in sterile vials, inactivated at 56°C for 30 minutes and stored at –20°C until used.

3.1.12. Sodium bicarbonate solution (7.5 per cent)

Sodium bicarbonate solution was prepared by dissolving 7.5g of sodium bicarbonate in 100 ml of triple glass distilled water and sterilised by filtration.

3.1.13. Trypsin

A 0.25 per cent trypsin solution (1:250) was prepared in CMF-PBS and sterilised by filtration.

3.1.14. Minimum Essential Medium (MEM)

Ready-made medium purchased from Hi-Media, Bombay was reconstituted as per manufacturer's instructions and sterilised by autoclaving at 115°C at 10 lbs pressure for 45 min.

3.1.15. Trypsin- Versene- Glucose Solution (TVG)

Sodium chloride	-	0.8 g
Potassium chloride	-	0.02 g
Na_2HPO_4	-	0.115 g
KH_2PO_4	-	0.02 g
Trypsin 1:250	-	0.25 g
EDTA	-	0.002 g
Phenol Red	-	0.001 g
Glucose	-	0.1 g
Triple glass distilled water		100 ml

Sterilised by passing through membrane filter of 0.2 μm pore size.

3.1.16. Cell culture growth medium

HBSS containing 0.5 per cent lactalbumin hydrolysate, 0.2 per cent yeast extract and 10 per cent calf serum constituted the growth medium. Antibiotics were added at the rate of 250 IU of penicillin and 250 μg of streptomycin per ml of the fluid. Nystatin was added as antifungal agent at the rate of 50μg per ml of the medium.

3.1.17. Maintenance medium

Same as growth medium except that the calf serum concentration was reduced to five per cent.

3.1.18. Growth medium for BHK-21 cell line

MEM containing 10 per cent calf serum constituted the growth medium for BHK-21 cell line. Antibiotics were added at the rate of 250 IU of penicillin, 250 μg of streptomycin and 50μg of nystatin per ml of the medium.

3.1.19. Maintenance medium for BHK-21 cell line.

Same as growth medium except that the serum concentration was reduced to five per cent.

3.1.20. May-Grunwald Giemsa Stain.

May-Grunwald stain was prepared by dissolving 2.5g of stain powder in 100 ml of absolute methanol and allowing to age for one month. Giemsa stain was prepared by dissolving one gram of stain powder in 66ml of glycerol at 55°C overnight and then adding 66ml of absolute methanol.

3.1.21. Agarose.

Agarose obtained from SRL was prepared as a 0.8 per cent solution in normal saline.

Agarose for pre-coating of slides was prepared as a 0.5 per cent solution in distilled water.

3.1.22. Staining solution

Amido black 10B	-	1.0 g
Sodium chloride	-	8.5 g
Distilled water	-	1000 ml

3.1.23. Decolouriser I

Methanol	-	40 parts
Acetic acid	-	10 parts
Distilled water	-	10 parts

3.1.24. Decolouriser II

Absolute alcohol	-	35 parts
Acetic acid	-	5 parts
Distilled water	-	10 parts

3.1.25. Saturated ammonium sulphate solution (SAS)

SAS solution was prepared by adding 760g of ammonium sulphate (SRL) to one litre of triple glass distilled water and heating to 56°C for 30 min in a water bath, with continuous stirring to dissolve. It

was filtered while still hot to remove insoluble impurities and then cooled to room temperature. The pH was adjusted to 7.0 with ammonium hydroxide solution just prior to use.

3.1.26. Sucrose solution

Sucrose solutions of concentrations 15, 25, 35, 45 and 65 per cent were prepared in sterile triple glass distilled water using sucrose obtained from SRL.

3.1.27. Reagents needed for SDS-PAGE.

3.1.27.1. Solution A

Acrylamide	-	30.0g
N,N methylene bis acrylamide	-	0.8 g
Distilled water	-	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C in amber coloured bottle.

3.1.27.2. Solution B (pH- 8.8)

Tris base	-	12.1 g

pH was adjusted to 8.8 with 1N hydrochloric acid and made up to 100 ml with double glass distilled water, filtered and stored at 4°C.

3.1.27.3. Solution C (pH- 6.8)

Tris base - 6.06 g

pH was adjusted to 6.8 with 1N HCl. Final volume was made up to 100 ml, filtered and stored at 4°C.

3.1.27.4. Sodium dodecyl sulphate (10 per cent)

SDS - 10 g

Distilled water - 100 ml

Filtered and stored at room temperature.

3.1.27.5. Ammonium persulphate (10 per cent)

Ammonium persulphate - 100 mg

Distilled water - 1 ml

Prepared freshly before use.

3.1.27.6. Electrode Buffer

Tris base - 3.03 g

Glycine - 14.4 g

SDS - 1.0 g

Distilled water - 800 ml

pH was adjusted to 8.3 with 1N HCl and final volume was made up to one litre with double distilled water, filtered and stored at room temperature.

3.1.27.7. Resolving gel (10 per cent)

Solution A	-	13.4 ml
Solution B	-	11.0 ml
SDS	-	4.0 ml
Distilled water	-	10.4 ml
TEMED	-	20 µl
Ammonium persulphate	-	200 µl
Total volume	-	40.00 ml

3.1.27.8. Stacking gel (5 per cent)

Solution A	-	3.3 ml
Solution C	-	5.0 ml
SDS	-	2.0 ml
Distilled water	-	3.5 ml
TEMED	-	10 µl
Ammonium persulphate	-	100 µl
Total volume	-	20.00 ml

3.1.27.9. Sample buffer

Solution C	-	8.5 ml
SDS	-	0.2 ml
Glycerol	-	1.0 ml
2- mercaptoethanol	-	0.5 ml

Pinch of bromophenol blue

3.1.27.10. Staining Solution

Coomassie Brilliant Blue R250-		200 mg
Methanol	-	50 ml
Acetic acid	-	10 ml
Distilled water	-	40 ml

Filtered through Whatman No.1 filter paper and stored at room temperature.

3.1.27.11. Destaining Solution I.

Methanol	-	500 ml
Acetic acid	-	100 ml
Distilled water	-	400 ml
Total volume	-	1000 ml

3.1.27.12. Destaining solution II.

Methanol	-	50 ml
Acetic acid	-	100 ml
Distilled water	-	850 ml
Total volume	-	1000 ml

3.1.28. Freund's Complete Adjuvant

Obtained from Difco Laboratories, Michigan, USA was used for the study.

3.1.29. Chicken eggs.

Hatching eggs and nine day old embryonated eggs from White

Leghorn / Austrawhite breeds obtained from the University Poultry Farm, Mannuthy and Commercial hatchery, Mannuthy were used for this work.

3.1.30. Chicks

Day old unvaccinated male chicks and six week old chicks obtained from Revolving Fund Hatchery, Mannuthy were used for pathogenecity studies.

3.1.31. Ducklings

Day old ducklings and six week old ducklings obtained from local market were used for pathogenecity studies.

3.1.32. Rabbits

Four rabbits, aged three months and weighing 1.5 to 2.0 kg purchased from the Small Animal Breeding Station, Mannuthy were used for preparation of antiserum.

3.1.32. Chicken red blood cells.

Blood was collected from the wing vein of healthy cockerels using sterile needle and syringe, into Alsever's solution. It was then centrifuged at 800 x g for 10 min. The pelleted RBC were washed thrice in normal saline and resuspended in fresh normal saline each time, to get a 0.5 per cent / one per cent concentration for haemagglutination test.

3.2. METHODS

Two virus isolates each, from lesser whistling teals (T_{18} and T_{22}) and Channa species of fishes (F_6 and F_{12}) which were isolated and preserved in the Department of Microbiology were used for this study. Of these T_{18} and T_{22} were partially characterised by Bindu (1997).

3.2.1. Revival of stock virus isolates.

The stock viruses were revived by inoculation into allantoic cavity of nine-day-old embryonated chicken eggs.

3.2.1.1. Allantoic cavity inoculation

The procedure described by Bishai *et al.* (1974) was followed. Nine-day-old embryonated chicken eggs were candled and the position of the air cell and embryo was marked. The air cell region was swabbed with tincture iodine and a hole was drilled 0.5 cm towards the center from the rim of air cell. Using a sterile tuberculin syringe and a 22 gauge needle, 0.2 ml of inoculum was introduced into the allantoic cavity. The hole was then sealed with melted paraffin and the eggs were incubated at 37°C in an upright position. All the eggs were candled daily. Those embryos which died within 24 h of inoculation were discarded. The embryos which died after 24 h and those which were alive after 6 days were transferred to the refrigerator for chilling.

3.2.1.2. Harvesting of the inoculated eggs.

The air cell region of the eggs was disinfected with 70 per cent alcohol and was cut and removed with sterile scissors and forceps. The inner shell membrane and chorioallantoic membrane were peeled off and the allanto-amniotic fluid collected aseptically using sterile pipette. The fluid from each embryo was tested for haemagglutination by rapid plate agglutination using one per cent chicken RBC and the embryos were inspected for the presence of any lesions. The allanto-amniotic fluid was clarified at 800 x g for 10 min at 4°C and the supernatant distributed in small aliquots and stored at –20°C until further studies.

3.2.2. Haemagglutination

Two fold dilutions of the virus isolates were made in normal saline in Perspex haemagglutination plates. Equal quantity of 0.5 per cent washed chicken RBC was added to each well. RBC control was prepared simultaneously. The plates were then incubated at room temperature for 45 min and the readings recorded after the red cells in the control well had settled.

3.2.3. Embryo infective dose 50 (EID 50)

Serial ten fold dilutions of the isolates were made in TPB-A and each dilution was inoculated through the allantoic route into

nine-day-old embryonated chicken eggs at the rate of 0.2 ml/ embryo 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 eggs was tested for haemagglutinating activity by rapid plate agglutination test and the embryos inspected for the presence of any lesions. The embryo infective dose of the isolates was calculated as per the method of Reed and Muench (1938).

3.2.4. pH Sensitivity.

Serial ten fold dilutions of the isolates were made in citrate phosphate buffer (pH 3.2) and phosphate buffers (pH 7.2 and pH 9.0) and kept at room temperature for one hour. Virus titrations were made in nine day old embryonated chicken eggs (0.2 ml per embryo) using four eggs per dilution. Untreated samples were also titrated simultaneously. The embryos were incubated at 37°C, candled daily and harvested as described earlier.

3.2.5. Thermostability

The isolates were distributed in one ml volume into screw capped vials and kept in a water bath at 56°C for 30 min. The treated and untreated samples were titrated as described above using four eggs per dilution. The isolates exposed to 56°C for 30 min were

tested for haemagglutinating activity and infectivity and compared with that of the untreated sample.

3.2.6. 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 800x g 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 infectivity and haemagglutination activity as described above.

3.2.7. Brine Sensitivity

Sea water was collected from the beach at Vadanappally and centrifuged to remove the coarse materials. The centrifuged water was then filtered through a membrane filter of pore size 0.2μm.

Serial ten fold dilutions of the isolates were made in the sterile sea water and kept at room temperature for one hour. Virus titrations were made in nine day old embryonated chicken eggs (0.2 ml per embryo) using four eggs per dilution. Untreated samples were also

titrated simultaneously. The embryos were incubated at 37°C, candled daily and harvested as described earlier.

The isolates treated with citrate phosphate buffer (pH 3.2) and phosphate buffer (pH 7.2 and 9.0) and brine were tested for haemagglutination and compared to that of the untreated sample. The embryos were also inspected for the presence of any lesions.

3.2.8. Propagation in chicken embryo fibroblasts.

3.2.8.1. Chicken embryo fibroblast cultures.

The method of Cunningham (1966) was followed. Ten to eleven day old healthy embryonated chicken eggs were selected and the air cell region was marked after candling. The air cell region was swabbed with tincture of iodine and it was then cut with a sterile scissors and removed. The inner shell membrane and the chorio allantoic membrane were cut and the embryo was lifted with sterile forceps and transferred to a sterile Petri dish containing CMF-PBS with antibiotics. The head and limbs were cut off and the embryos were eviscerated. The embryos were then transferred into sterile centrifuge tubes and cut into small pieces. The pieces were washed twice with CMF-PBS containing antibiotics and transferred to a beaker containing cold 0.2 per cent trypsin. After allowing to remain for a few min, the supernatant

was poured off and the sediments transferred into a trypsinisation flask containing magnetic pellet. Warm trypsin at 37°C was added and the trypsinisation flask placed on a magnetic stirrer and allowed to run for 5 min. The supernatant was removed and kept at 4°C after adding calf serum to a final concentration of five per cent. The procedure was repeated, the supernatant collected and pooled and serum added. The resulting cell suspension was filtered through a double layered muslin cloth. The filtrate was then centrifuged at 800xg for 10 min, the supernatant was discarded and the cell pellet resuspended in growth medium and recentrifuged. This process was repeated three times and the cells were finally suspended in growth medium containing five to ten per cent serum to get a final concentration of $1x10^6$ cells/ml.

The above cell suspension was seeded into test-tube containing coverslips at the rate of two ml per tube and incubated at 37°C in a slanting position. When a satisfactory monolayer was obtained, it was used for studying the cytopathic effect (CPE).

3.2.8.2. Inoculation of the cell culture.

Tubes with satisfactory monolayers were selected, the growth medium was poured off, the monolayer washed with maintenance medium and 0.2 ml each of the isolates was added to the tubes and

incubated at 37°C for one hour to facilitate adsorption. The inoculum was then poured off, the monolayer again washed and maintenance medium was added at the rate of 2 ml per tube. Control tubes were prepared simultaneously in which instead of the inoculum, 0.2 ml of the maintenance medium was added. The tubes were the examined at 24h intervals under an inverted microscope for evidence of any cytopathic effect. When any CPE was evident, coverslip cultures were collected at intervals of 24h, 48h, 72h and 96h fixed in methanol for 24h and stained with May-Grunwald-Giemsa stain for 30 min to demonstrate CPE and /or inclusion bodies.

3.2.8.3. Staining of coverslip cultures

The coverslip cultures were fixed overnight in methanol. They were then stained for ten min in May-Grunwald stain and for 20 min in dilute Giemsa stain (1:10) in distilled water. The coverslips were then rinsed rapidly in two changes of acetone and again in two parts acetone and one part xylol for five seconds. They were then placed in one part acetone and two parts xylol for one minute and cleared in two changes of xylol for two min each, dried and mounted in DPX on a clean glass slide and examined for CPE / inclusion bodies under oil immersion objective.

3.2.8.4. Nucleic acid type. Sensitivity to 5-iodo-2-deoxy uridine (IuDR)

Test tubes with confluent monolayers were selected and the growth medium was poured off. After washing twice with maintenance medium , the monolayer was replaced with maintenance medium containing 100 μg per ml IuDR. Control tubes were also kept with the same medium but without the drug. After incubating all the tubes at 37°C for four hours, they were inoculated with 0.2 ml of the virus isolates. The tubes were again incubated for one hour at 37°C. The inoculum was poured off and maintenance medium was added and again kept for incubation.

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.

3.2.9. BHK-21 propagation

BHK-21 cell line obtained from the Department of Animal Biotechnology, Madras Veterinary College, Chennai was used for this work.

The maintenance medium was poured off from a bottle containing a confluent monolayer. The monolayer was washed twice with minimum essential medium not containing serum.

The bottle was then incubated at 37°C for one minute after adding 2 ml of trypsin versene glucose solution. The TVG solution was discarded when cells started dislodging from the monolayer. The monolayer was again washed with MEM without serum and the cells were then mechanically disrupted using a pipette attached with a bulb. A split ratio of 1:3 to 1:4 was employed but instead of seeding into other bottles, the cells were seeded into test tubes containing coverslips after adding serum at a ten per cent level.

The tubes were then incubated at 37°C and observed daily for the formation of monolayer on the coverslips. When a suitable growth of the cells was obtained, the tubes were used for studying the cytopathic effects of the virus isolates as described earlier.

3.2.10. Pathogenecity studies.

Pathogenecity testing of the viral isolates were carried out in day old chicks/ day old ducklings and six weeks old chicks/ ducklings.

3.2.10.1. Experiment I

Twenty five, day old chicks were divided into five groups (A-E) of five each. Groups A and B received 10^6 EID_{50} of T_{18} and F_6 respectively and groups C and D received 10^5 EID_{50} of T_{22} and F_{12} respectively both by oral (one ml) and subcutaneous (0.5 ml) routes. Chicks in group E served as uninfected controls. After inoculation, the 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 four weeks. The birds, which died, were subjected to postmortem examination and the lesions noted. Liver and spleen were collected from the dead birds and used for reisolation of the viruses.

3.2.10.2. Experiment II.

Fifteen, one-week-old ducklings were used for this study, which were divided into groups of five each. One of the groups served as control. The birds in the first and second groups were given 10^6 EID_{50} of T_{18} and F_6 respectively via oral (one ml) and subcutaneous routes (0.5 ml), while the chicks in the third and fourth groups were given 10^5 EID_{50} of T_{22} and F_{12} respectively via the same routes. The birds were kept separately taking care to prevent cross infection and were observed daily for a period of four weeks for the development of any clinical symptoms or death, as above.

3.2.10.3. Experiment III

Twenty five, six week old chicks were divided into five groups of five each. The first four groups were inoculated with one ml of undiluted samples $T_{18,}$, T_{22}, F_6 and F_{12} respectively via the subcutaneous route. Birds in the fifth groups were given sterile saline injection and were kept as controls. All the birds were kept in separate cages and extreme care was taken too prevent cross contamination. The birds were observed twice daily for the development of any clinical symptoms. Cloacal and throat swabs were collected at 3, 5, 7, 14, 21 and 28 days post infection (PI) for virus isolation.

3.2.10.4. Experiment IV

A total of 15, six-week-old ducklings were purchased and divided into five groups of three each.

All the birds were infected with the same doses of different virus isolates, *ie.*, one ml by the oral route and 0.5 ml by subcutaneous route. The birds in the first group received 10^6 EID_{50} of T_{18}, the second group 10^5 EID_{50} of T_{22}, third group 10^6 EID_{50} of F_6 and those in the fourth group 10^5 EID_{50} of F_{12}. The fifth group served as uninfected controls and was inoculated with sterile saline. The different groups

were kept separately taking care to avoid cross contamination for four weeks as described earlier.

3.2.11. Preparation of antiserum

Four, three month old healthy rabbits weighing about 1.5 to 2.0 kg were used for raising antiserum against the virus isolates. One ml each of the virus isolate was homogenised with an equal quantity of Freund's Complete Adjuvant (FCA) and one ml injected deep intramuscularly into the thigh muscles of the rabbits. Two booster doses of the antigen without the adjuvant were given at seven day intervals. Meanwhile the rabbits were monitored for the presence of specific antibodies at periodic intervals. When sufficient level of antibody was found in the serum, which was about one week after the second booster injection, 20 ml of blood was collected from each rabbit by cardiac puncture, serum was separated and stored at –20°C until used.

3.2.12. Haemagglutination Inhibition (HI) Test

Antiserum obtained from rabbits was used for HI test using the corresponding isolate as antigen. Beta procedure of HI test was employed through out the study. The haemagglutination titre of the virus isolates was ascertained by the HA test and four HA units of the

virus isolate was prepared in normal saline. Serial double fold dilutions of the serum was prepared in normal saline in Perspex haemagglutination plates. 0.2 ml of four HA units of the virus was added to each well and incubated at room temperature for 30 min, 0.4 ml of 0.5 per cent suspension of washed chicken RBC was added to each well and mixed. Virus and RBC controls were also set simultaneously. At the end of the incubation period of one hour, the HI antibody titre was noted as the highest dilution of the serum in which there was complete inhibition of HA.

3.2.13. Immunodiffusion test

Clean microscopic slides were pre-coated with 0.5 per cent agarose in distilled water and dried in air by keeping the slides horizontally over glass rods. About three to four ml of melted 0.8 per cent agarose in normal saline at about 50 °C was poured onto each agarose coated slide kept on a leveled surface. The agarose was allowed to solidify initially at room temperature and subsequently at 4°C. Five wells were cut in the agarose, one at the centre and four wells surrounding it. After removing agarose from the wells, the central well was filled with antiserum and the surrounding wells with the different virus isolates. The slides were incubated overnight in a humid

chamber and examined against a light source for the development of precipitation lines. Uninfected allantoic fluid was also tested against the various antisera and this was taken as control.

The slides were washed after soaking in two changes of normal saline for 24 hours each and then in distilled water for a further 24 hours to remove the unreacted proteins. They were then dried slowly, stained with amido black stain for 15 min and decolourised with decolouriser I and decolouriser II for 20 min each. The slides were again dried at 37°C for one hour and mounted in DPX.

3.2.14. Purification of the virus isolates

The method of Kumanan *et al.* (1994) was followed.

The allanto amniotic fluid was clarified at 500 g for 20 min at 4°C. The virus was precipitated out by adding saturated ammonium sulphate solution (two parts of allanto amniotic fluid and one part of saturated ammonium sulphate solution) drop by drop, while continuously stirring the fluid. It was then centrifuged at 5000 g for 20 min at 4°C. The precipitate was then re-suspended in phosphate buffered saline and centrifuged at 75000 g for 40 min over 0.3 ml cushion of 65 per cent sucrose in an ultracentrifuge. The viral pellet was then re-suspended in the sucrose cushion together with little of supernatant fluid and clarified at 5000 g for 10 min and re-pelleted on

to sucrose cushion as described above. The concentrated virus was then layered onto gradients of 15 to 45 per cent sucrose and centrifuged at 125000 g in an ultracentrifuge. The visible band was then collected and dialyzed overnight against PBS at 4°C and stored at –20°C till used.

3.2.15. SDS-PAGE of proteins

3.2.15.1. Preparation of sample

Equal volumes of the purified viral samples and the sample buffer were mixed and heated in a water bath at 100°C for one min, cooled and stored at 4°C until used. Standard protein markers and purified, uninfected allantoic fluid to be used as control were also prepared in the same way.

3.2.15.2. Electrophoresis

The discontinuous system of polyacrylamide gel electrophoresis was employed according to the procedure of Laemmli (1970).

The gels were prepared in between 16 x 10 cm glass plates supplied with the vertical gel electrophoresis apparatus. One mm thick spacers were used in between the glass plates.

Ten per cent resolving gel was prepared (according to the composition given earlier) and poured in between the glass plates. Over this, three ml of distilled water was added to get a level surface and allowed to polymerize for 30 min. After polymerization was completed the water was poured off and five per cent stacking gel was added and allowed to polymerize for 30 min after inserting the comb. The comb was removed when the polymerization was completed and the wells were loaded with 20µl quantities each of the different samples. The standard protein markers and the uninfected allantoic fluid were also loaded in the same way.

Electrophoresis was initially done at 100V till the dye reached the surface of the resolving gel and then the voltage was changed to 150V and was continued till the dye reached the bottom of the gel.

The gel was removed and stained overnight with Coomassie Brilliant blue staining solution and then destained with decolouriser I for 3h. followed by decolouriser II with frequent changes to complete destaining.

After destaining, the gel was placed in the Alpha imager and photographed. The molecular weights of the different viral proteins

were directly obtained by comparing the distance migrated by the viral proteins with that of the standard marker proteins, whose molecular weights were already known.

Bands having the same molecular weight as that found in the control were not taken into account and molecular weights of the remaining bands only were taken.

RESULTS

4. RESULTS

4.1. Revival of the virus isolates

Two partially characterised virus isolates (T_{18} and T_{22}) from lesser whistling teals (*Dendrocygna javanica*) and another two isolates (F_6 and F_{12}) from Channa species of fish which were preserved in the Department of Microbiology were revived by allantoic cavity inoculation of nine day old embryonated chicken eggs.

The isolates $T_{18,}$, F_6 and F_{12} killed the embryos within four days of inoculation. The embryos were highly congested (Fig. 1 and 3). There was also congestion of chorioallantoic membrane (CAM). Characteristic lesions were also produced in the embryos like suboccipital and inter digital haemorrhages. The allantoic fluid collected showed haemagglutination with one percent chicken RBC.

The isolate T_{22} did not produce any change in chicken embryos at first and had to be passaged twice in duck embryos before it could be adapted to chicken embryos. Thereafter it started producing congestion of the embryos and CAM. The embryos appeared stunted when compared with the controls (Fig. 2). Yellowish brown patches could be observed in the liver. Allantoic fluid collected agglutinated one percent chicken RBC.

4.2. Haemagglutination

All the four isolates showed haemagglutination with chicken RBC with rapid plate agglutination test. The haemagglutination titre of the isolates after the third passage in chicken embryos is given in the

Fig. 1. Nine day old chicken embryos infected with isolate T_{18}

Fig. 2. Nine day old chicken embryo infected with isolate T_{22}
(infected and control)

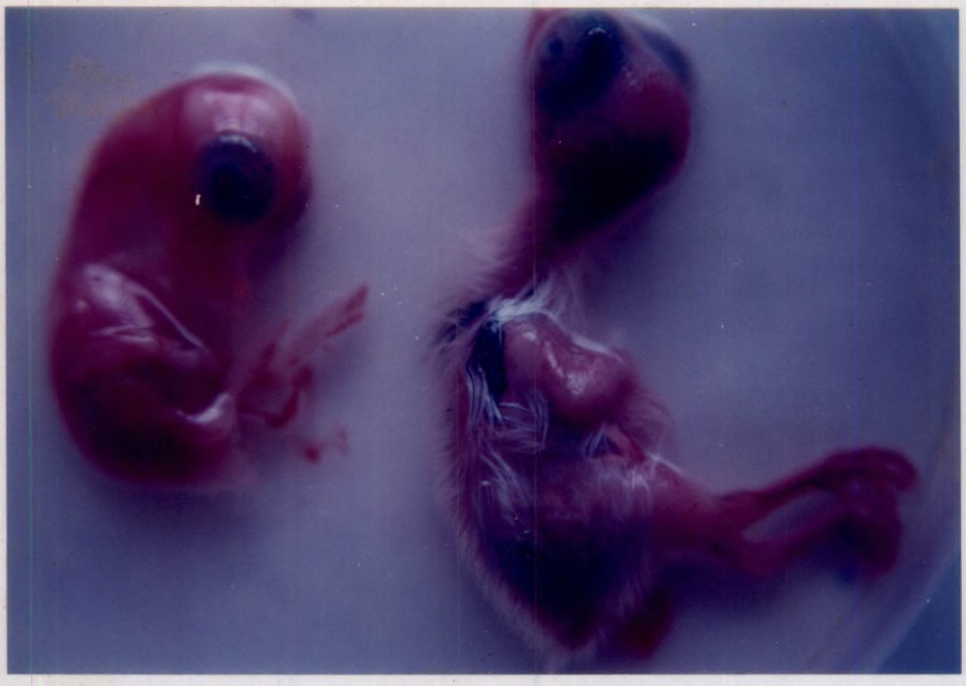
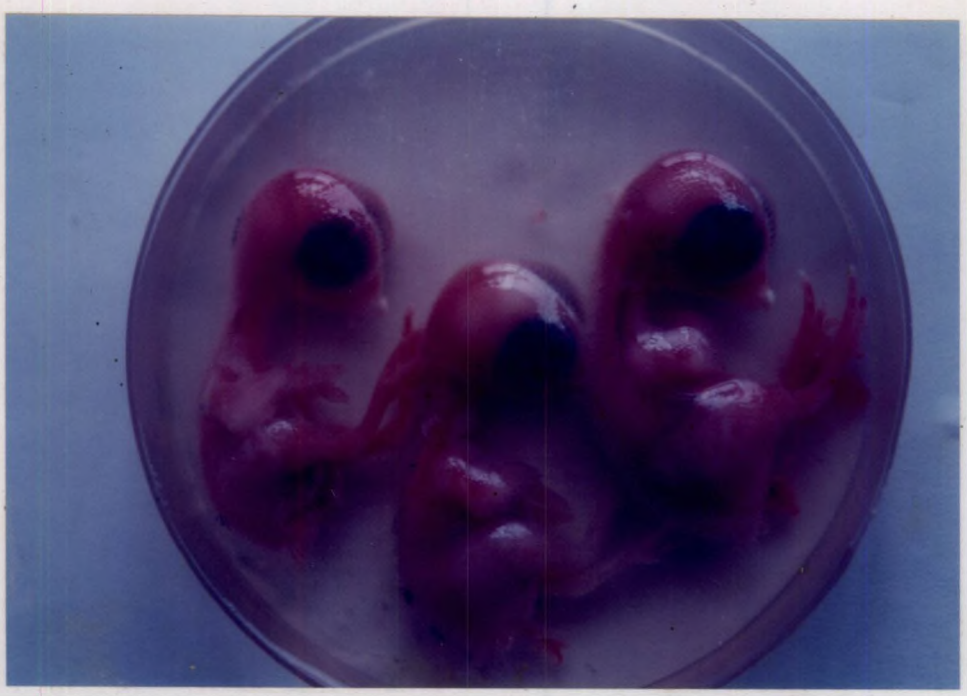


Fig. 3. Nine day old chicken embryos infected with isolate F_6

Fig. 4. Cytopathic effects in chicken embryo fibroblast at 96 h - syncytia formation
May Grunwald-Giemsa stain (100x)



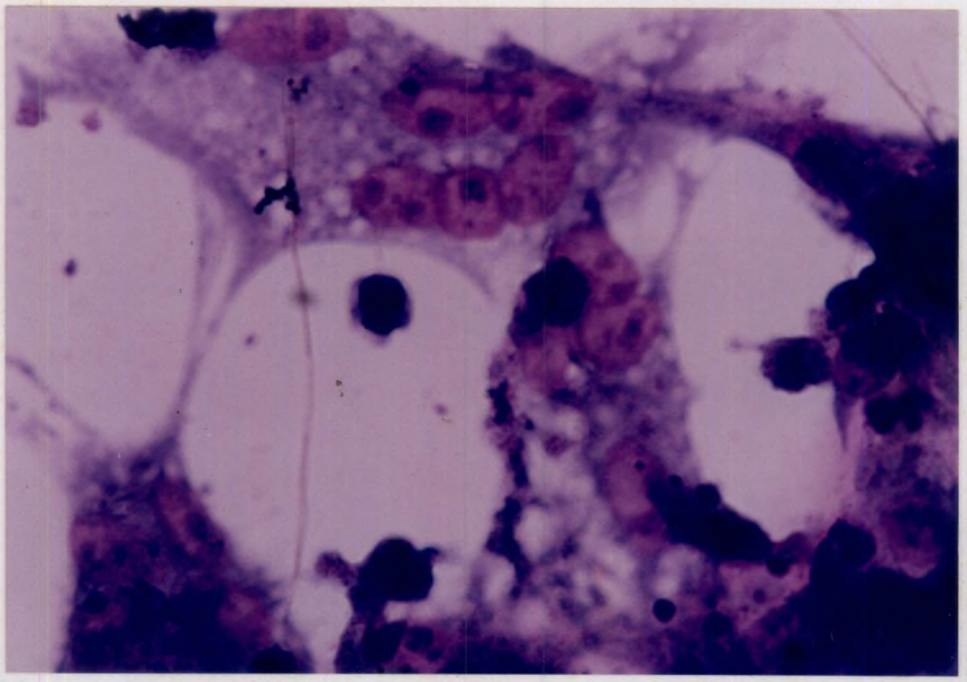


Table 1. It was seen that the isolates T_{18} and F_6 showed the highest haemagglutination titre of 512 while F_{12} had the lowest titre of 128.

Table 1. Haemagglutination titre of the isolates.

Isolate	Haemagglutination titre
T_{18}	512
T_{22}	256
F_6	512
F_{12}	128

4.3. Embryo Infective Dose 50 (EID_{50})

The virus isolates were quantified in nine day old embryonated chicken eggs. The EID_{50} of the four isolates were calculated according to the method described by Reed and Muench (1938). The titre of the different samples after the third passage in chicken embryos is given in the Table 2. It was seen that the highest titre was given by the isolate F_6 and the lowest by the isolate F_{12}.

Table 2. Infectivity titre of the different isolates.

Virus isolate	Embryo infective dose 50 (EID_{50}) / ml of allantoic fluid
T_{18}	3.2×10^6
T_{22}	5.62×10^5
F_6	1.65×10^7
F_{12}	3.16×10^5

4.4. pH Sensitivity

The sensitivity / stability of the four isolates was tested at three different pH values, namely pH 3.2, 7.2 and 9.0. The results of sensitivity of the isolates to the various pH levels are given in the Table 3.

The results obtained showed that the infectivity and haemagglutinating activity of all the isolates were unaffected at pH 7.2 for one hour. At pH 3.2 and 9.0, there was complete loss of both the infectivity and haemagglutinating properties which was evidenced by failure of the samples to produce any lesions in the chicken embryos as well as failure of the allantoic fluid to show haemagglutination with chicken embryos.

Table 3. Effect of pH on viability of the isolates, T_{18}, T_{22}, F_6 and F_{12}

Virus isolate	pH 3.2		pH 7.2		pH 9.0	
	HA	Infectivity	HA	Infectivity	HA	Infectivity
T_{18}	Nil	-*	512	$3.2 \times 10^{6+}$	Nil	-
T_{22}	Nil	-	256	5.6×10^{5}	Nil	-
F_6	Nil	-	512	1.65×10^{7}	Nil	-
F_{12}	Nil	-	128	3.16×10^{5}	Nil	-

*- Complete loss of infectivity
+ - Embryo infective dose 50

4.5. Thermostability

Thermostability of the isolates was assessed by keeping the isolates at 56°C in a water bath for 30 min. It was observed that both the infectivity and haemagglutinating activity of all the isolates were completely lost at 56°C for 30 min. Thermoinactivation of the isolates is presented in the Table 4.

Table 4. Effect of temperature at 56°C for 30 min. on the virus isolates

Virus isolate	Haemagglutination titre		Infectivity titre in chicken embryo	
	Control	Treated	Control	Treated
T_{18}	512	No HA	$3.2 \times 10^{6*}$	-
T_{22}	256	No HA	5.6×10^{5}	-
F_6	512	No HA	1.65×10^{7}	-
F_{12}	128	No HA	3.16×10^{5}	-

*- EID_{50}
- Complete loss of infectivity

4.6. Chloroform sensitivity

Sensitivity to lipid solvents and thereby the presence of an envelope, was studied by exposing the isolates to five percent chloroform for a period of ten minutes at room temperature. It was seen that three of the isolates were sensitive to treatment with chloroform, which was evidenced by almost complete loss of haemagglutinating activity and infectivity. Infectivity of the isolate F_6 was completely lost, but the haemagglutinating activity showed a decrease in titre from 512 to 128. The results of sensitivity of the isolates to chloroform is shown in the Table 5.

Table 5. Chloroform sensitivity of the isolates.

Virus isolate	Infectivity titre in chicken embryo		HA titre	
	Control	Treated	Control	Treated
T_{18}	$3.2 \times 10^{6*}$	-	512	16
T_{22}	5.6×10^{5}	-	256	8
F_6	1.65×10^{7}	-	512	128
F_{12}	3.16×10^{5}	-	128	0

*- EID_{50}
- Complete loss of infectivity

4.7. Brine sensitivity

The sensitivity of the four isolates to brine was tested after treating them with sterile sea water and keeping at room temperature for one hour. All the four isolates retained both their infectivity and haemagglutinating activity. Table 6 shows the effect of brine on the isolates.

Table 6. Brine sensitivity of the isolates

Virus isolate	Infectivity titre in chicken embryo		HA titre	
	Control	Treated	Control	Treated
T_{18}	10^{6+}	10^{6+}	512	512
T_{22}	10^{5}	10^{5}	256	256
F_{6}	10^{7}	10^{7}	512	512
F_{12}	10^{5}	10^{5}	128	128

+ - EID_{50}

4.8. Nucleic acid type

No inhibitory effect was produced on the replication of any of the isolates by pre-treatment of chicken embryo fibroblast cultures with 100μg per ml of 5-iodo- 2- deoxyuridine (IuDR). Characteristic cytopathic effects such as rounding and clumping of cells and syncytia formation were observed both in control as well as drug treated tubes. A known RNA virus, Newcastle Disease Virus was also not inhibited by treatment of the monolayers with IuDR as CPE was evident in both drug treated and untreated tubes. This showed that all the four isolates had RNA as their nucleic acid. Sensitivity of the isolates towards IuDR is given in Table 7.

Table 7. Effect of IuDR on the isolates in chicken embryo fibroblasts.

Virus isolate	IuDR treatment	
	Treated	Untreated
T_{18}	CPE produced	CPE produced
T_{22}	"	"
F_6	"	"
F_{12}	"	"
NDV	"	"

The results of characterisation of the four isolates are given in Table 8.

Table 8. Characterization of the virus isolates

Characteristic	T_{18}	T_{22}	F_6	F_{12}
Thermostability (56°C for 30 min.)	Complete inactivation	Complete inactivation	Complete inactivation	Complete inactivation
Sensitivity to pH				
3.0	Complete inactivation	Complete inactivation	Complete inactivation	Complete inactivation
7.2	Unaffected	Unaffected	Unaffected	Unaffected
9.2	Complete inactivation	Complete inactivation	Complete inactivation	Complete inactivation
Chloroform sensitivity	Sensitive	Sensitive	Sensitive	Sensitive
Brine sensitivity	Unaffected	Unaffected	Unaffected	Unaffected
Type of Nucleic acid	RNA	RNA	RNA	RNA

4.9. Propagation in CEF culture

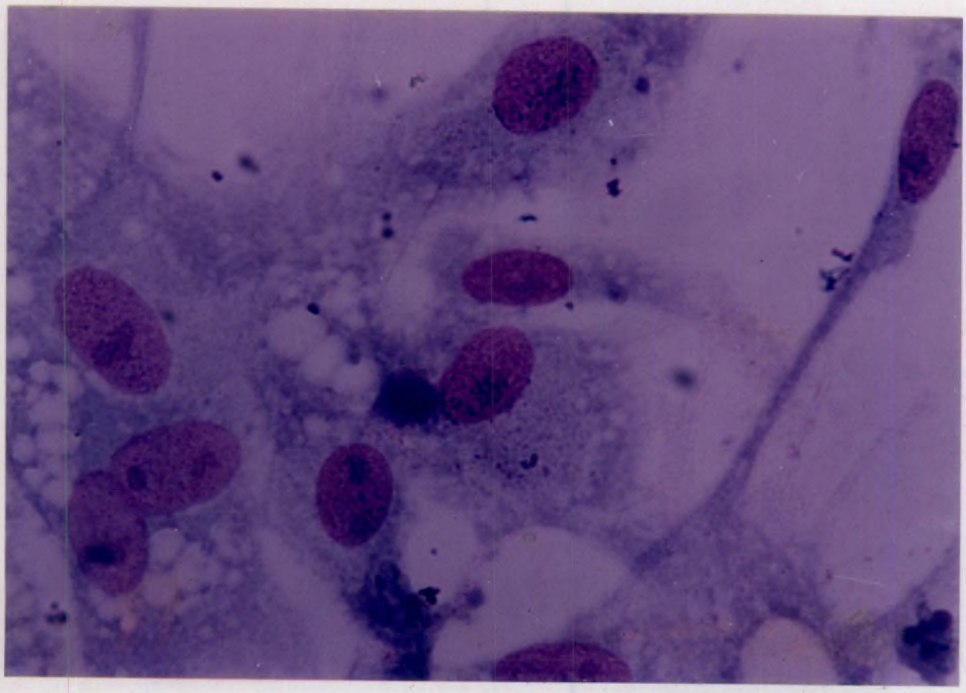
Primary chicken embryo fibroblast culture was grown in Hank's balanced salt solution supplemented with 0.5 percent lactalbumin hydrolysate, 0.2 percent yeast extract and ten percent calf serum. Satisfactory monolayers were formed in 24 h. Morphologically the cells were more or less spindle shaped and in areas of high density were oriented with their long axis parallel to one another. The monolayers were inoculated with the four isolates T_{18}, T_{22}, F_6 and F_{12}. CPE was observed in the monolayer infected with the isolates T_{18} and F_6 within 24h and was complete within 96h. CPE was less prominent for the isolates T_{22} and F_{12} and was seen only after 72h. In unstained cultures, the initial changes were noticed from the periphery and subsequently the changes were uniformly distributed and finally the whole monolayer was affected and cells got dislodged from the glass surface in the case of isolates T_{18} and F_6. For the isolates T_{22} and F_{12}, there was no dislodging of cells from the glass surface. The control tubes in which instead of the isolates, sterile HBSS was inoculated showed no changes during the observation period and remained as confluent monolayers.

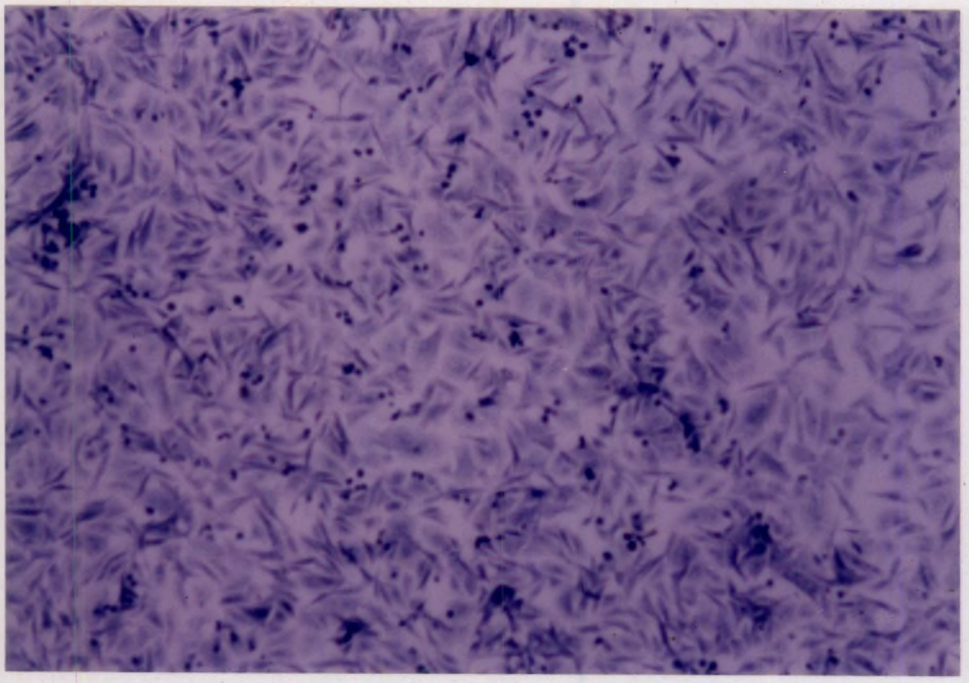
4.9.1. Examination of coverslip cultures

The coverslips showing CPE and the controls were fixed in methanol overnight and stained with May Grunwald Giemsa stain. In the case of isolates T_{18} and F_6, 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. There were syncytia (Fig. 4) with four or five nuclei without any cytoplasmic demarcation. Marked cytoplasmic vacuolation (Fig. 5) was another constant feature

Fig. 5. Cytopathic effects in chicken embryo fibroblast at 96h – vacuolation
May Grunwald-Giemsa stain (100x)

Fig. 6. BHK –21 cell line monolayer (control) at 24h
May Grunwald-Giemsa stain (10x)





of most of the infected cells. Inclusion bodies were not detected either in the nucleus or cytoplasm.

When the isolates T_{22} and F_{12} were infected, the cytopathic effects were not so marked and developed later only. There was rounding of the cells, but without much separation. Syncytium formation was noticed by about 72h after inoculation but involved only two or three cells. Cytoplasmic vacuolation though present was less marked. No inclusion bodies, either intranuclear or intra cytoplasmic could be detected.

No changes were noticed in the stained control tubes with the cells in the monolayer retaining their original morphology. There was no rounding or separation of the cells through out the observation period.

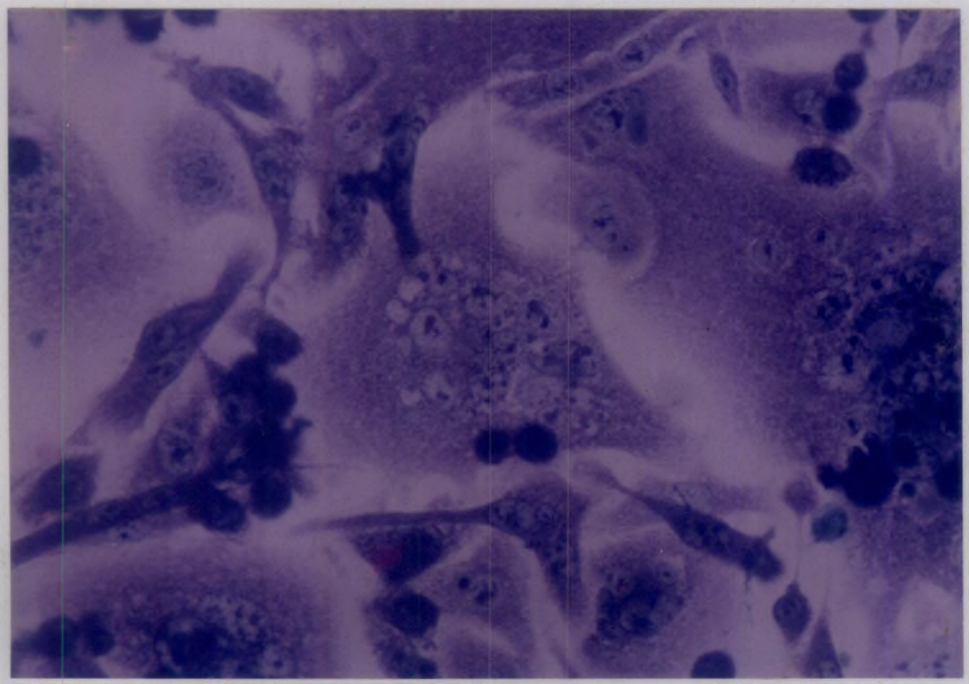
4.10. Propagation in BHK-21 cell line

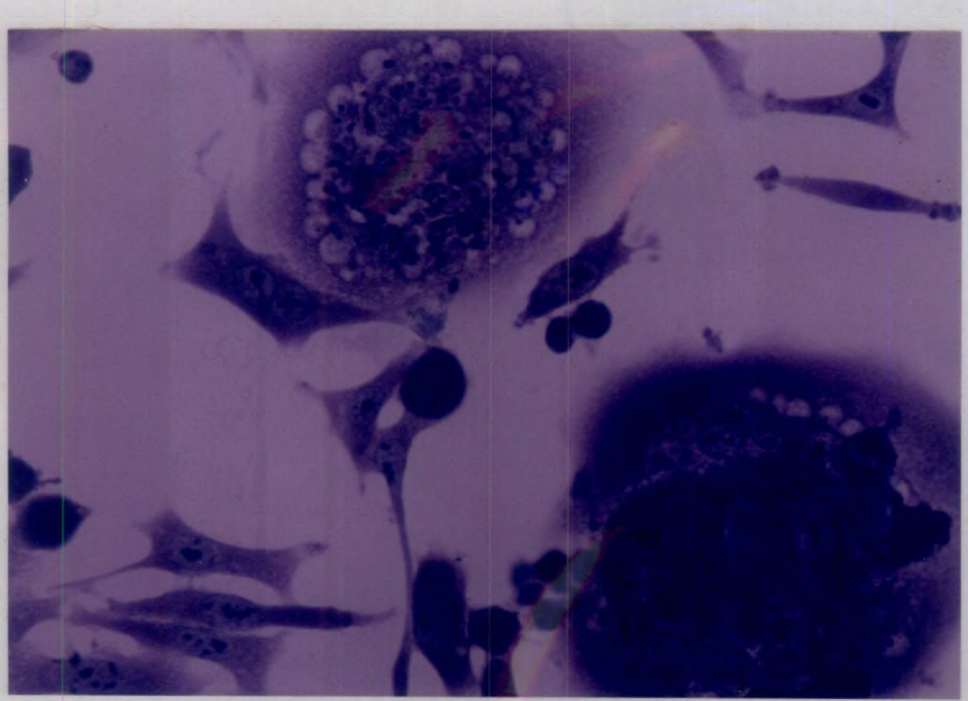
BHK-21 cells were grown in Minimum essential medium (MEM) containing 10 percent calf serum in test tubes containing coverslips. A good monolayer was formed within 24 hours and consisted of spindle shaped cells arranged parallel to each other. On staining with May Grunwald –Giemsa stain, the nucleus stained dark blue while the cytoplasm was seen faint blue. The nuclei were more or less oval in shape and centrally placed and had one or two, sometimes more nucleoli (Fig. 6).

In infected cells, CPE was produced by the isolates T_{18}, T_{22} and F_{12}, while F_6 failed to produce any visible CPE. CPE produced by the isolates T_{18}, T_{22} and F_{12} were more or less similar. Within 24h after

Fig. 7. Cytopathic effects in BHK –21 cell line at 72h – syncytia formation
May Grunwald-Giemsa stain (40x)

Fig. 8. Cytopathic effects in BHK –21 cell line at 96h – syncytia formation
May Grunwald-Giemsa stain (40x)





infection, the cells started separating from each other with many of them getting rounded. Fusion of cells to form polykaryocytes was also visible by 24h. Between 48- 72 h, these polykaryocytes fused with other polykaryocytes to form large syncytia. By 96h, the monolayer consisted mainly of large syncytia and few individual cells scattered around (Fig. 7 and 8). These changes were seen along the whole course of the monolayer. Intracytoplasmic inclusion bodies could also be observed by 24h after infection, which was quite prominent by 96h (Fig. 9). There was no peeling off of the monolayer even after 96h of infection.

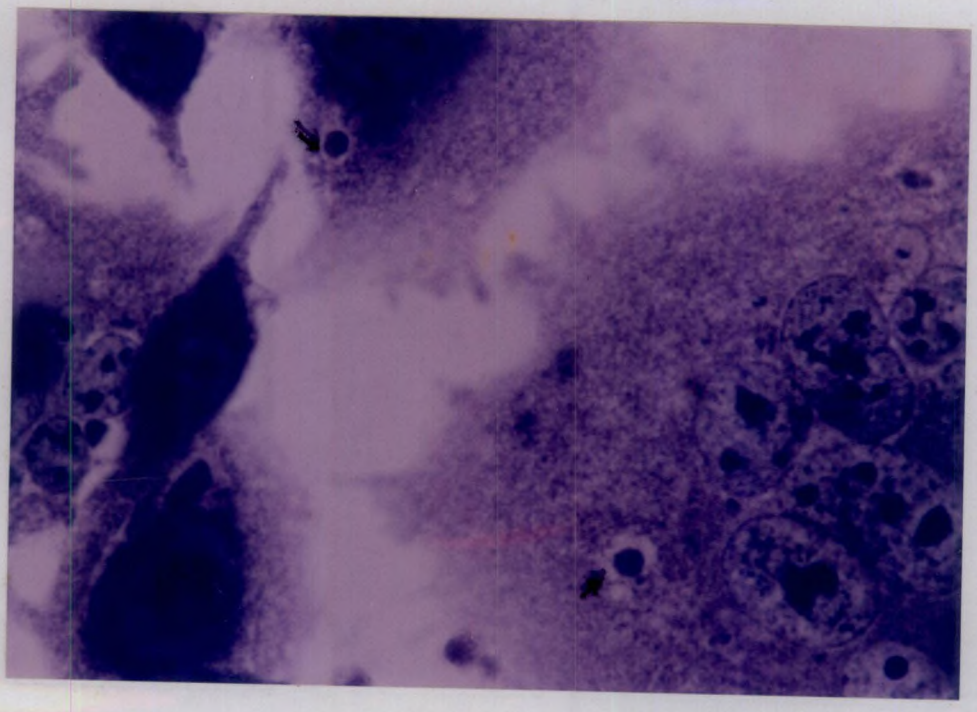
Control tubes, which were stained and observed during the same time intervals as the inoculated tubes retained their original appearance with out any changes.

4. 11. Pathogenecity

4.11.1. Experiment I

Each of the four isolates was given to groups of day old chicks (five numbers in each group) at the rate of 0.5 ml subcutaneously and one ml orally. Control birds were given 0.5 ml of sterile normal saline subcutaneously and one ml orally and kept separately. The birds were monitored daily for any signs of disease or mortality for a period of four weeks. One of the birds given the isolate T_{22} and two birds given the isolate F_{12} died on the 2^{nd} day after inoculation. But postmortem examination of the dead birds did not reveal any lesions and no virus could be isolated even after three passages in embryonated eggs. All the other birds exhibited no clinical signs or mortality during the period of observation. Postmortem examination of the birds sacrificed at the end of the observation period did not reveal any lesions. Liver and

Fig. 9. Cytopathic effects in BHK –21 cell line at 96h– inclusion bodies
May Grunwald-Giemsa stain (100x)



spleen were collected from the birds and passaged in embryonated eggs, but no virus could be isolated even after three passages. The results of experimental infection of day old chicks is given in the Table 9.

Table 9. Experimental infection of day old chicks with the four isolates

Virus isolate	No. of birds inoculated	No. with clinical symptoms	No. dead
T_{18}	5	-	-
T_{22}	5	-	1
F_6	5	-	-
F_{12}	5	-	2
Control	5	-	-

4.11.2. Experiment II.

Fifteen numbers of one week old ducklings were separated into groups of three each and the first four groups were given 0.5 ml each of the isolates subcutaneously and one ml each orally. The fifth group which served as control was given 0.5 ml saline subcutaneously and one ml orally. None of the birds showed any clinical symptoms during a four week observation period nor was there any mortality. Postmortem examination of the birds sacrificed at the end of four weeks did not reveal any characteristic changes. Virus isolation was not possible even after three passages of processed liver and spleen in embryonated chicken eggs. The details of the experimental infection are given in the Table 10.

Table 10. Experimental infection of one week old ducklings with the four isolates.

Virus isolate	No. of birds inoculated	No. with clinical symptoms	No. dead
T_{18}	3	-	-
T_{22}	3	-	-
F_6	3	-	-
F_{12}	3	-	-
Control	3	-	-

4.11.3. Experiment III

Five numbers each of six week old chicks were infected with the four isolates at the rate of one ml subcutaneously. None of the birds showed any clinical symptoms or mortality till the 28th day after inoculation. Tracheal and cloacal swabs were collected from all the birds at 3rd, 5th, 7th, 14th , 21st and 28th day post infection and processed for viral isolation. The results are given in Tables 11, 12, 13 and 14.

Virus excretion was found to be present on 3rd, 5th, 7th and 14th day after infection with the isolate T_{18} as the virus could be isolated from the cloacal swabs of all of the birds. After the 14th day, the number of isolations decreased and all the birds were negative by the 28th day. Tracheal swabs collected at the same time as the cloacal swabs were found to be negative.

For the isolate T_{22}, cloacal swabs collected at 3rd and 5th day post infection were positive for virus isolation from all the five infected birds, while on the 7th day cloacal swabs collected from four of the birds were positive. Between the 7th and 14th day, the number of isolation

Table 11. Experimental infection of six week old chicks with isolate T_{18}

Time of collection / observation in days	No. of birds inoculated	Virus isolation		No. showing clinical symptoms	No. of birds dead	Percentage of isolation	
		Cloacal swab No. +ve/ No. screened	Tracheal swab No. +ve/ No. screened			Cloacal Swab	Tracheal Swab
0	5	-	-	-	-	-	-
3	-	5/5	0/5	-	-	100	0
5	-	5/5	0/5	-	-	100	0
7	-	5/5	0/5	-	-	100	0
14	-	5/5	0/5	-	-	100	0
21	-	2/5	0/5	-	-	40	0
28	-	0/5	0/5	-	-	0	0

Table 12. Experimental infection of six-week-old chicks with isolate T_{22}

Time of collection / observation in days	No. of birds inoculated	Virus isolation		No. showing clinical symptoms	No. of birds dead	Percentage of isolation	
		Cloacal swab No. +ve/ No. screened	Tracheal swab No. +ve/ No. screened			Cloacal Swab	Tracheal Swab
0	5	-	-	-	-	-	-
3	-	5/5	0/5	-	-	100	0
5	-	5/5	0/5	-	-	100	0
7	-	4/5	0/5	-	-	80	0
14	-	0/5	0/5	-	-	0	0
21	-	0/5	0/5	-	-	0	0
28	-	0/5	0/5	-	-	0	0

Table 13. Experimental infection of six-week-old chicks with isolate F_6

Time of collection / observation in days	No. of birds inoculated	Virus isolation		No. showing clinical symptoms	No. of birds dead	Percentage of isolation	
		Cloacal swab No. +ve/ No. screened	Tracheal swab No. +ve/ No. screened			Cloacal Swab	Tracheal Swab
0	5	-	-	-	-	-	-
3	-	0/5	0/5	-	-	0	0
5	-	0/5	0/5	-	-	0	0
7	-	0/5	0/5	-	-	0	0
14	-	0/5	0/5	-	-	0	0
21	-	0/5	0/5	-	-	0	0
28	-	0/5	0/5	-	-	0	0

Table 14. Experimental infection of six-week-old chicks with isolate F_{12}

Time of collection / observation in days	No. of birds inoculated	Virus isolation		No. showing clinical symptoms	No. of birds dead	Percentage of isolation	
		Cloacal swab No. +ve/ No. screened	Tracheal swab No. +ve/ No. screened			Cloacal Swab	Tracheal Swab
0	5	-	-	-	-	-	-
3	-	0/5	0/5	-	-	0	0
5	-	0/5	0/5	-	-	0	0
7	-	0/5	0/5	-	-	0	0
14	-	0/5	0/5	-	-	0	0
21	-	0/5	0/5	-	-	0	0
28	-	0/5	0/5	-	-	0	0

decreased and was nil by the 14th day. Tracheal swabs collected during the same period were found to be negative.

Both tracheal and cloacal swabs collected at the same time as above from the birds inoculated with the isolates F_6 and F_{12} and processed were found to be negative for the presence of any viruses.

4.11.4. Experiment IV

Three numbers each of six week old ducklings given the isolates T_{18}, T_{22}, F_6 and F_{12} respectively, both by oral and subcutaneous routes, failed to show any clinical symptoms or mortality over an examination period of four weeks. Uninfected controls also showed no clinical signs or mortality. Postmortem examination of the birds sacrificed at the end of the observation period did not show the presence of any macroscopic lesions. Attempts to isolate virus from the liver and spleen were negative. Results of experimental infection of six-week-old ducklings are given in Table 15.

Table 15. Experimental infection of six-week-old ducklings with the different isolates.

Virus isolate	No. of birds inoculated	No. with clinical symptoms	No. dead
T_{18}	3	-	-
T_{22}	3	-	-
F_6	3	-	-
F_{12}	3	-	-
Control	3	-	-

4.12. Haemagglutination Inhibition (HI) test

All the four isolates were tested against their corresponding antisera and also against the antisera to the other three isolates. Beta procedure was employed. Results of HI test are given in the Table 16. The isolates gave a good antibody response to their corresponding antiserum. The HI titre of the isolate T_{18} against its corresponding antiserum was 256. The isolate T_{22} did not give any titre against T_{18} antiserum and the isolates F_6 and F_{12} gave titres of 64 and 16 respectively against T_{18} antiserum. Against T_{22} antiserum the HI titres of the isolates T_{18}, T_{22}, F_6 and F_{12} were 16, 128, 32 and 32 respectively. The isolate F_6 gave a titre of 128 against its homologous antiserum, while the isolates T_{18}, T_{22} and F_{12} gave titres of 64, 18 and 32 respectively. The HI titres of the four isolates T_{18}, T_{22}, F_6 and F_{12} against F_{12} antiserum were 32, 64, 32 and 128 respectively.

Table 16. HI titres of the isolates against different antisera.

Virus isolate	Antiserum			
	T_{18}	T_{22}	F_6	F_{12}
T_{18}	256	16	64	32
T_{22}	-	128	16	64
F_6	64	32	128	32
F_{12}	16	32	32	128

4.13. Immunodiffusion Test

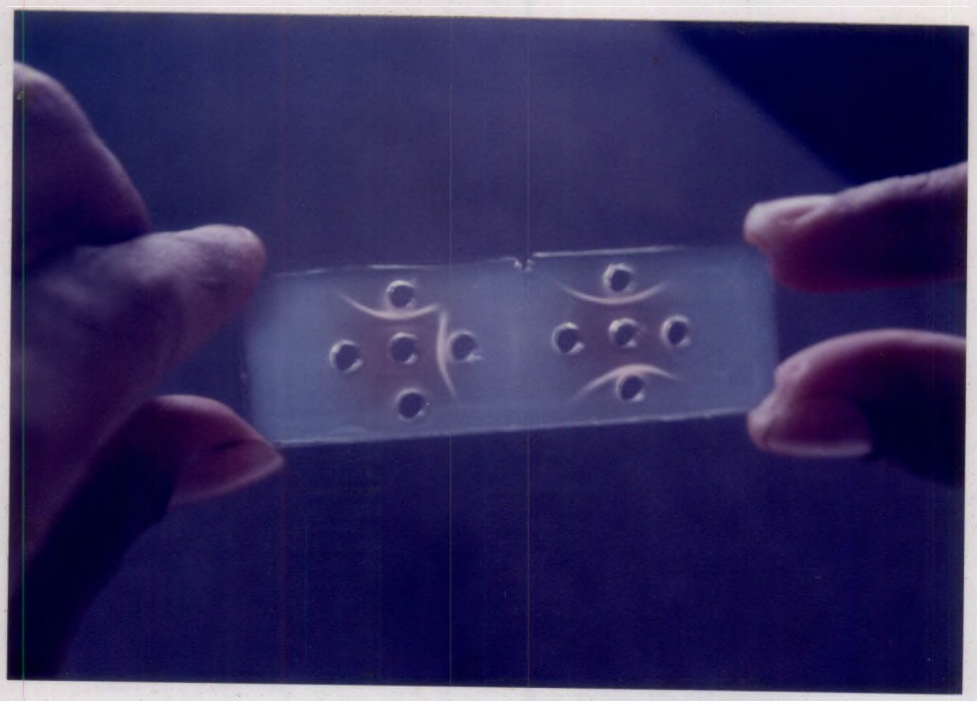
Immunodiffusion test in 0.8 percent agarose gel on clean microscopic slides was performed to determine the antigenic similarities /dissimilarities between the isolates. Antisera against the four isolates were raised in rabbits. The central well contained the antiserum and the four surrounding wells contained the different isolates.

When antiserum against the isolate T_{18} was placed in the central well, three precipitin lines could be observed against the corresponding antigen T_{18} (Fig. 10). Of these the line curved towards the antigen well was bold, but as a similar line fusing with this line was present in the control, it was not taken into consideration. The other two lines were very thin and curved more towards the central well. They appeared separate and did not fuse with the line of any other isolate. Against the isolate F_6 also there were three lines of which the outer one was bold and curved towards the antigen well. This bold line also was not considered, because a similar line was present in the control. The middle one was thin, quite straight and towards the central well was another thin line which was seen fused with the innermost line against isolate T_{22} as well as with the single line against isolate F_{12}. There were three lines against isolate T_{22} also, the outer and middle ones being straight and not fusing with any other lines. The innermost line was seen fused with the single line against isolate F_{12} and the innermost line against isolate F_6. Only a single line was present against isolate F_{12}, which was fused with the inner lines of both the isolates T_{22} and F_6.

Three precipitin lines could be observed against the corresponding antigen T_{22}, when antiserum against isolate T_{22} was placed in the central well (Fig. 11). Of these, the outer most and innermost lines were straight and did not fuse with any other precipitin lines. The middle line was seen fused with the single line against isolate F_{12} as well as with the inner of the two lines against isolate F_6. There were two thin precipitin lines against isolate T_{18} which were curved towards the central well and remained unfused. Two lines were observed against isolate F_6, the inner line of which was fused both with the middle line against isolate T_{22} and the single line against isolate F_{12} while the outer

Fig. 10. Immunodiffusion of the isolates against T_{18} antiserum

1. T_{18}
2. T_{22}
3. F_6
4. F_{12}
5. T_{18} Antiserum



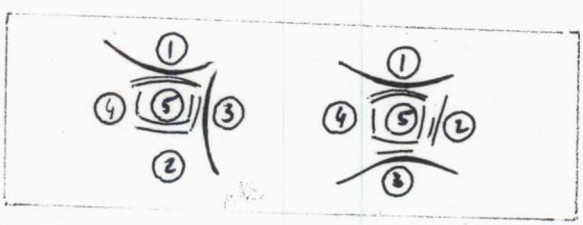
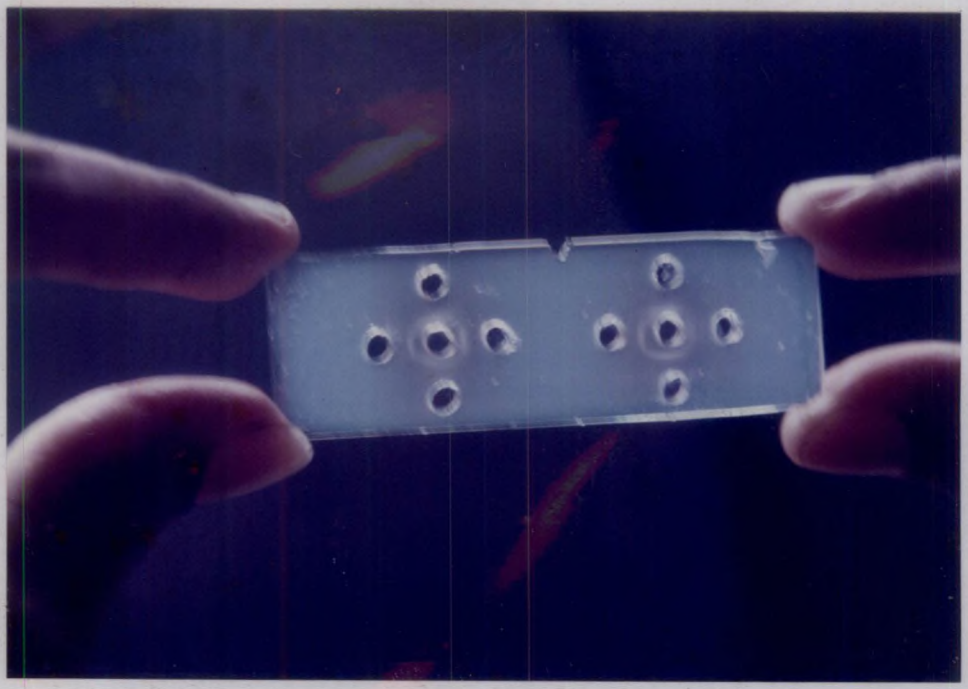
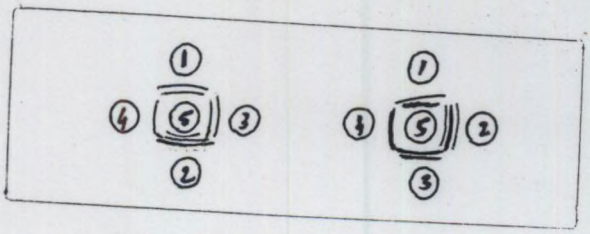

1
4
5
3
2
1
4
5
2
3

Fig. 11. Immunodiffusion of the isolates against T_{22} antiserum

1. T_{18}
2. T_{22}
3. F_6
4. F_{12}
5. T_{22} Antiserum




1
4
5
3
2
1
4
5
2
3

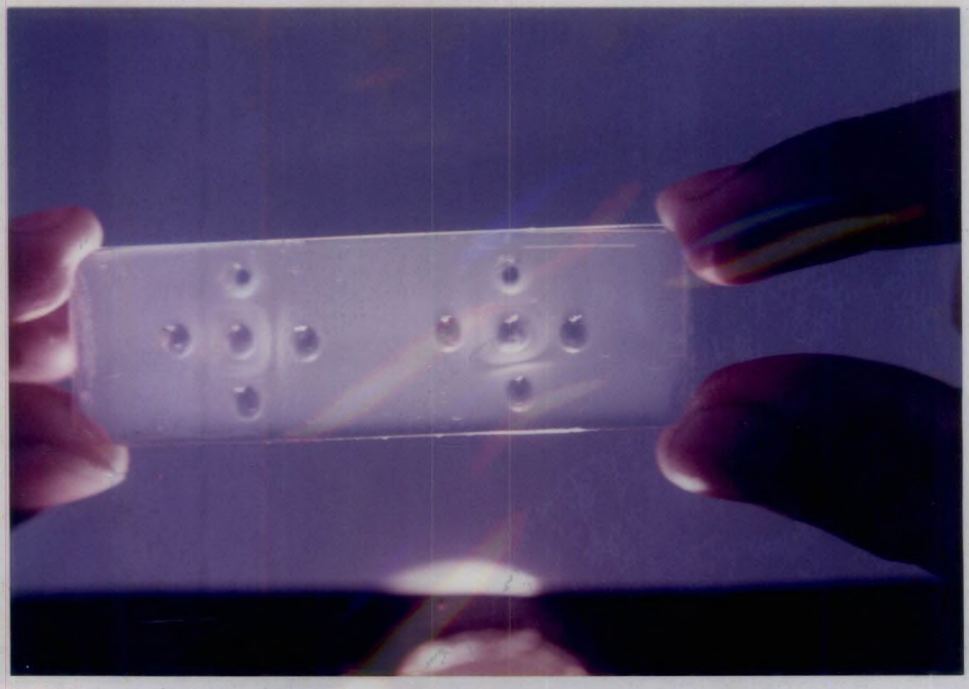
one was seen to be straight and separate. Against isolate F_{12}, a single line alone was observed seen fused with the inner line of isolate F_6 and the middle line of isolate T_{22}.

Only two lines could be observed against the homologous antiserum of isolate F_6 (Fig. 12). Of these, the inner one was bolder than the outer one and fused with he innermost line against isolate T_{22} and the outer line against isolate F_{12}. The outer one was straight and not fusing with any other lines. Against isolate T_{18} also there were two lines, the outer one curved towards the antigen well and the inner one curved towards antiserum well. Both the lines were seen separate and not joining with any other lines. There were three lines against isolate T_{22}, the innermost of which fused with the inner line against isolate F_6 and the outer one against isolate F_{12}. The other two lines were towards the antigen well, being straight and separate. Two lines could be observed against isolate F_{12}, the outer of which found with the innermost line against isolate T_{22} and the inner line against isolate F_6. The inner line was quite thin and straight and seen towards the antiserum well.

With F_{12} antiserum in central well, the isolate F_{12} produced two precipitin lines, of which the outer one fused with the innermost line of isolate T_{22} and the inner line of isolate F_6 (Fig. 13). The inner line was straight and very thin and separate. Against isolate T_{18}, two lines could be observed, the outer one of which was fused with the outer line of F_6 and the middle line of T_{22}. Two lines were observed against isolate F_6, the outer one of which fused with the outer line of T_{18} and middle line of T_{22}. The inner line fused with the inner line against isolate T_{22}, and with the outer line against isolate F_{12}. There were three lines against isolate T_{22}, the outermost of which was seen very close to the antigen well and

Fig. 12. Immunodiffusion of the isolates against F_6 antiserum

1. T_{18}
2. T_{22}
3. F_6
4. F_{12}
5. F_6 Antiserum



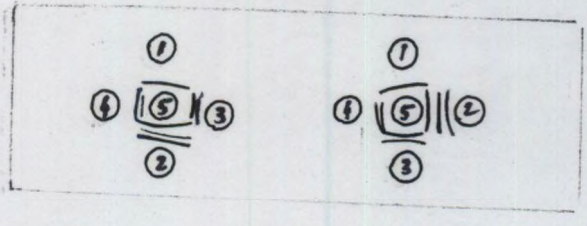
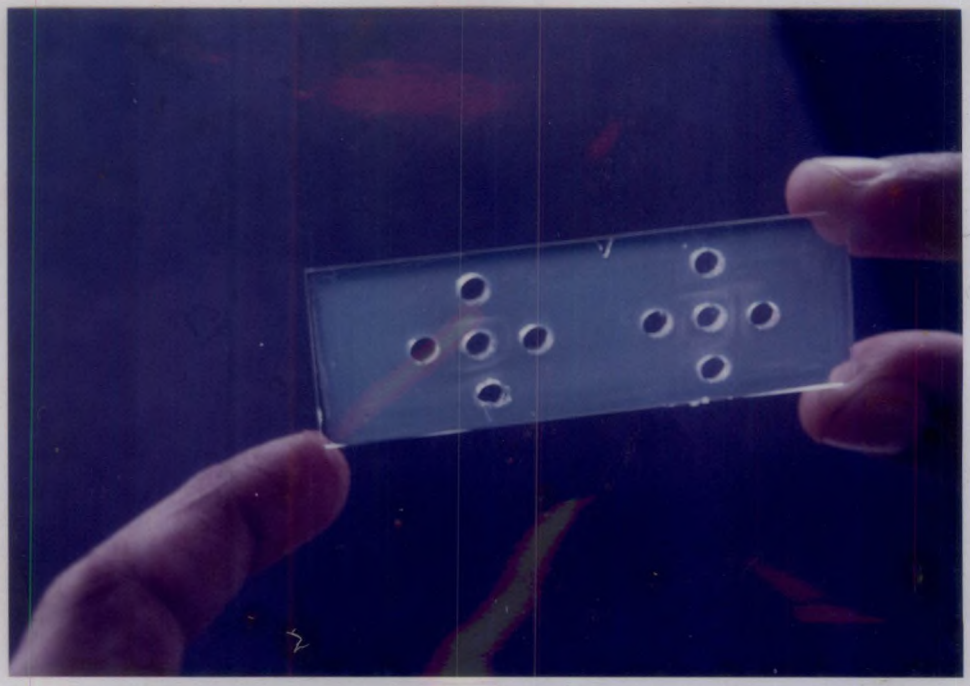
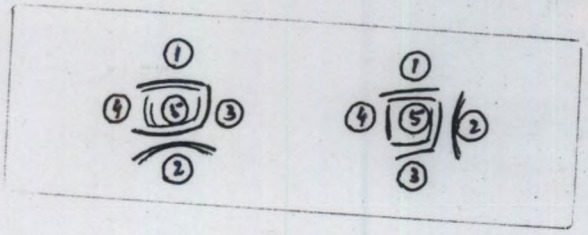

1
4
5
3
2
1
4
5
2
3

Fig. 13. Immunodiffusion of the isolates against F_{12} antiserum

1. T_{18}
2. T_{22}
3. F_6
4. F_{12}
5. F_{12} Antiserum




1
4
5
3
2
1
4
5
2
3

curved towards it. At the tip, the line showed splits giving a suggestion that it may actually be two lines. The middle line was curved towards the central well and fused with the outer lines of T_{18} and F_6. This line also showed a very fine split at one end. The innermost line was curved towards the central well and fused with the outer line of F_{12} and inner line of F_6.

4.14. SDS-PAGE of the virus isolates

Purified virus samples, standard protein marker and pelleted, uninfected allantoic fluid were subjected to the discontinuous system of SDS-PAGE.

The standard protein marker (Sigma) yielded 13 bands ranging in molecular weights from 205 kDa to 6.5 kDa. Based on the position of the bands of the standards, the molecular weights of the control and the four isolates were calculated directly by placing the stained gel in the Alpha Imager (Alpha Innotech-2000).

The control, which was pelleted allantoic fluid yielded two bands of molecular weights 98 kDa and 83 kDa. When bands having similar molecular weights to those seen in the control were observed in any of the isolates, they were not taken into account as being present in the isolates. The number of bands that could be resolved in the four isolates and their molecular weights are shown in Table 17.

The isolates T_{18}, T_{22}, F_6 and F_{12} produced 10, 11, 8 and 7 bands respectively. The molecular weights of the ten bands in the isolate T_{18} ranged from 182 to 18 kDa. Isolate T_{22} had 11 bands and isolate F_6 had eight bands with molecular weights of both ranging from 181 kDa to 18 kDa. Only seven bands were observed in isolate F_{12} which

Fig. 14. SDS-PAGE analysis of proteins of isolates

Lane 1. Marker
Lane 2. Control
Lane 3. T_{18}
Lane 4. T_{22}
Lane 5. F_6
Lane 6. F_{12}

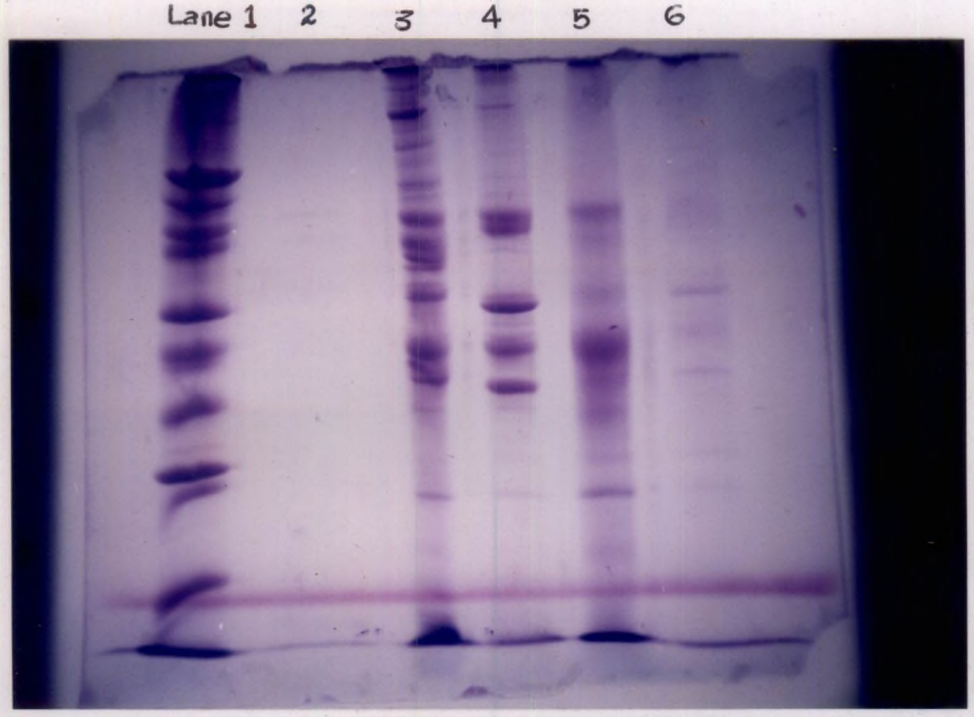
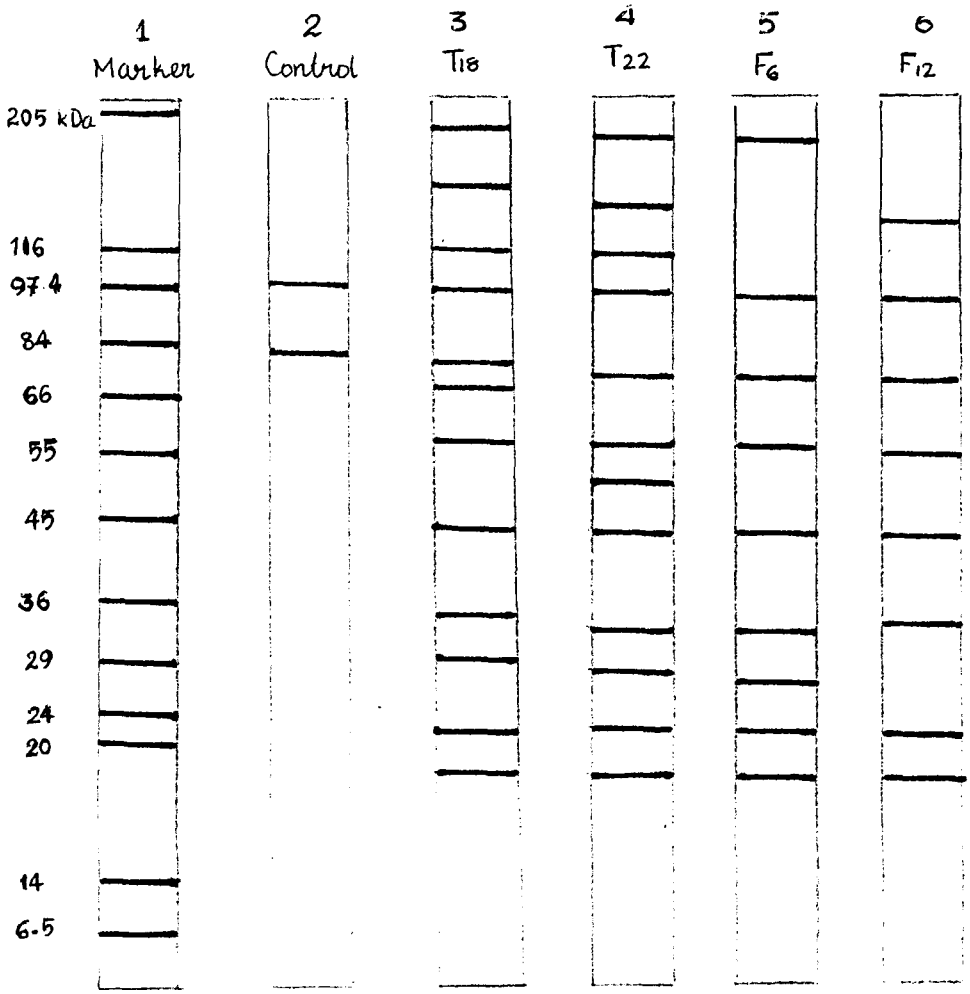

Lane 1 2 3 4 5 6

Fig. 15. Graphical representation of SDS-PAGE

Lane 1. Marker
Lane 2. Control
Lane 3. T_{18}
Lane 4. T_{22}
Lane 5. F_6
Lane 6. F_{12}


1
Marker
2
Control
3
T18
4
T22
5
F6
6
F12
205 kDa
116
97.4
84
66
55
45
36
29
24
20
14
6.5

ranged in molecular weight from 135 kDa to 18 kDa (Fig. 14 and 15). The band of molecular weight 149 kDa was unique for T_{18} and that of molecular weight 48 kDa for T_{22}.

Table 17. SDS-PAGE analysis of proteins of the virus isolates

Protein fraction	Molecular weights of the proteins (kDa) of the isolates			
	T_{18}	T_{22}	F_6	F_{12}
I	182	-	-	-
II	-	181	181	-
III	149	-	-	-
IV	-	137	-	-
V	-	-	-	135
VI	118	118	-	-
VII	-	77	77	77
VIII	70	-	-	-
IX	60	60	60	-
X	-	-	-	58
XI	-	48	-	-
XII	44	44	44	44
XIII	-	-	-	35
XIV	34	34	34	-
XV	30	30	-	-
XVI	-	-	28	-
XVII	-	22	22	22
XVIII	21	-	-	-
XIX	18	18	18	18
Total number of bands in each isolate	10	11	8	7

DISCUSSION

5. DISCUSSION

5.1. Revival of the virus isolates

The isolates T_{18}, F_6 and F_{12} could be propagated in nine day old embryonated chicken eggs by the allantoic route of inoculation and produced characteristic lesions or mortality in the embryos. The haemagglutination titres of the allantoic fluid were 512, 512 and 128 respectively. The isolate T_{22} also after initial propagation in embryonated duck embryos, produced lesions in chicken embryos with the allantoic fluid giving a haemagglutination titre of 256.

The lesions seen in the embryos were almost similar for all the isolates, all the embryos being congested. Congestion of chorio allantoic membrane (CAM) was also noted with the isolates T_{18}, T_{22} and F_6. For the isolate F_{12}, congestion of the embryo was not so marked but sub-occipital haemorrhages could be observed. Embryos infected with isolate T_{22} appeared stunted and had yellowish brown patches in the liver.

From the above observations it could be seen that the four isolates under study were capable of producing mortality of chicken embryos with congestion and allantoic fluid showed haemagglutination with chicken RBC. Congestion of chicken embryos and sub-occipital and inter digital haemorrhages are features characteristic of viruses belonging to the orthomyxo/ paramyxo group.

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

dark red skin, petechial haemorrhages on the head, congestion of the muscle and punctiform haemorrhages on the sternum in embryos infected with influenza virus.

Gough and Alexander (1983) could observe no mortality in ten day old embryonated chicken eggs, but at the end of the observation period of seven days, embryos infected by allantoic route with a paramyxovirus isolated from collared doves appeared inert and stunted with necrotic areas in the liver and kidneys.

5.2. pH sensitivity

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 all the four isolates was lost when exposed to pH 3.2 for a period of one hour at room temperature. There was also complete loss of haemagglutinating activity at pH 3.2. Same was the case when the isolates were exposed to a pH of 9.0. At pH 7.2 both infectivity and agglutinating activity of all the four isolates were retained.

All the four isolates were labile at pH 3.2 and pH 9.0 and could survive only within a narrow range of pH. Usually enteric viruses are resistant to an acidic pH, because they have to survive in the highly acidic pH of the stomach. In the present study all the viruses were found to be sensitive to an acidic pH indicating that they do not belong to the above group.

Sensitivity of avian paramyxoviruses to pH 3.0 has been reported by Sulochana *et al.* (1981) and Bindu (1997). But

Nerome *et al.* (1978) found that the infectivity of a virus designated Kunitachi virus and identified to be a member of the paramyxo group was found not to be affected by acid treatment (pH 2.9). Stability of avian paramyxovirus to a pH of 3.0 have also been reported by Kumanan and Venkatesan (1991), Vijayarani *et al.* (1992b), Kumanan *et al.* (1992) and Kamaraj *et al.* (1998).

At an alkaline pH of 10, a NDV isolated from ducks (NDV-D) was found to be labile (Sulochana *et al.* 1981). Sensitivity of two viruses isolated from lesser whistling teals to a pH of 9.0 was also reported by Bindu (1997).

Most of the orthomyxo and paramyxo viruses are considered to be stable at an alkaline pH between 8 to 10.9 (Merchant and Packer, 1967; Mini, 1988).

Inactivation of a rhabdovirus isolated from snakehead (*Ophicephalus striatus*) with ulcerative syndrome by treatment with acid (pH 3.0) was reported by Ahne *et al.* (1988). A similar finding of loss of infectivity of a rhabdovirus isolated from penaeid shrimps at a low pH was observed by Lu and Loh (1992).

5.3. Thermostability

All the four isolates lost their infectivity and haemagglutinating activity by heating at 56°C for 30 min., suggesting that these two properties of the isolates are thermolabile. Similar findings of susceptibility of avian paramyxovirus to heating to 56°C for 30 min. have also been reported by other workers (Nerome *et al.*, 1978: Shortridge *et al.*, 1980; Sulochana *et al.*, 1982a; Sulochana, 1991a and

Bindu, 1997). But conflicting results of thermostability of avian paramyxoviruses at 56°C have been reported by Lomniczi (1975), Alexander *et al.* (1981), Gough and Alexander (1983), Kumanan and Venkatesan (1991), Vijayarani *et al.* (1992b) and King (1996).

Hanson *et al.* (1949) conducted detailed thermostability studies on the haemagglutinins of Newcastle disease virus (NDV) strains of different virulence and concluded that at 56°C haemagglutinins of lentogenic strains were heat labile whereas those of mesogenic and velogenic strains maintained their activity at least for 30 min. But Hanson and Spalatin (1978) reported the isolation of viscerotropic velogenic strains of NDV having a thermosensitive haemagglutinin and of a lentogenic strain with an unusually thermostable haemagglutinin, which confirmed that virulence for chicken was not related to thermostability of haemagglutinin.

Eight out of nine paramyxoviruses 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 virus was stable between 15 and 30 min. (Cloud and Rosenberger, 1980).

Moses *et al.* (1948) reported that the infectivity of fowl plague virus was destroyed by exposure to 60°C for a period of five min or less. Similar inactivation of influenza A virus was also reported by Merchant and Packer (1967) and Papparella *et al.* (1969). Inactivation of influenza viruses at a temperature of 55°C within one hour or in 10 min. at 60°C has been reported by Buxton and Fraser (1977).

Hess and Dardiri (1969) reported complete inactivation of duck plague virus in 10 min at 56°C and in 90- 120 min at 50°C.

Ahne *et al.* (1988) observed that a rhabdovirus isolated from affected snakehead (*Ophicephalus striatus*) in Thailand was readily inactivated by heat treatment at 56°C. A rhabdovirus isolated from penaeid shrimps was found to be sensitive to a temperature of 37°C (Lu and Loh, 1992).

5.4 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 and haemagglutinating activity are completely lost or greatly reduced. In the present study, all the four isolates were completely inactivated on treatment with five percent chloroform indicating that they were all enveloped.

Sensitivity of avian paramyxoviruses to ether has been reported by Nerome *et al.* (1978) and to chloroform by Cloud and Rosenberger (1980) and Bindu (1997). But Kim and Spradbrow (1978) reported that none of four lentogenic Australian NDV isolates tested lost haemagglutinin activity on treatment with ether.

Complete inactivation of an influenza virus isolated from turkey when treated with five percent chloroform was demonstrated by Lang *et al.* (1968). Hess and Dardiri (1968) found duck plague virus to be sensitive to ether and chloroform.

Ahne *et al.* (1988) reported inactivation of a rhabdovirus isolated from Snakehead (*Ophicephalus striatus*) with ulcerative syndrome in Thailand when treated with 50 percent chloroform. Complete loss of activity of a rhabdovirus isolated from penaeid shrimp on treatment with 20 percent ethyl ether was also reported by Lu and Loh (1992).

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).

5.5 Brine sensitivity

Sensitivity of the isolates towards brine was employed to find out the reaction of the isolates towards high salt concentrations. There are no published reports on the brine sensitivity of paramyxoviruses. The isolates were treated with sterile seawater for one hour and the infectivity and haemagglutination titre recorded. It was observed that there was no reduction either in infectivity or haemagglutination activity of any of the isolates indicating that they were capable of surviving at high salt concentrations.

5.6. Nucleic acid type.

Certain halogenated deoxyuridines such as the drug 5-iodo-2 deoxyuridine (IuDR) are used to find out whether the nucleic acid is DNA or RNA. DNA viruses are inactivated by IuDR and so there will be no multiplication or cytopathic effect on the monolayer. IuDR has no action on RNA so that RNA viruses can grow and produce cytopathic effect on the monolayer.

All the four isolates were inoculated into coverslip cultures of chicken embryo fibroblast which were pretreated with IuDR at the level of 100 μg /ml. All treated and untreated cell cultures exhibited CPE. This indicated that they were all RNA viruses. When a known RNA virus, Newcastle disease virus was grown in drug treated cells also no inhibitory effect on its multiplication was observed.

Kunst (1968) observed that the replication of duck plague virus and the development of CPE could be inhibited by IuDR suggesting that its nucleic acid was DNA.

The replication of a viral agent resembling EDS-76 virus isolated from poultry flocks was inhibited by IuDR demonstrating that the nucleic acid was DNA type (Ramkumar, *et al.* 1991; Swain, *et al.* 1992).

That the nucleic acid of avian paramyxoviruses is RNA has been reported by a number of workers (Nerome *et al.* 1978; Shortridge *et al.* 1980; Alexander *et al.* 1983 and Gough and Alexander, 1983). Bindu (1997) found that pretreatment of chicken embryo fibroblast cell cultures with IuDR inhibited the replication of a virus isolated from lesser whistling teals, whereas another virus isolated from the same species of bird produced characteristic cytopathic effect both in drug treated as well as control tubes indicating that the former was a DNA virus and the latter was a RNA virus.

Ahne *et al.* (1988) reported that replication of a rhabdovirus isolated from snake head (*Ophicephalus striatus*) with ulcerative

syndrome was not affected by IuDR indicating an RNA genome. A similar observation with a rhabdovirus isolated from penaeid shrimps towards the DNA antagonist 5-bromo-2'-deoxyuridine at a level of 20 µg/ ml was made by Lu and Loh (1992).

From the initial physico- chemical characterisation of the isolates, it was observed that all the isolates were enveloped, having RNA as their nucleic acid and were capable of producing haemagglutination with chicken RBC. They could survive at high salt concentration and were thermolabile. They were sensitive to both acidic and alkaline pH and could survive only at a pH of 7.2. From the above observations, it could be tentatively inferred that they belong to either the orthomyxo or paramyxo group of viruses.

5.7. Propagation in cell cultures

When the four isolates were propagated in chicken embryo fibroblast cultures, marked CPE was produced by the isolates T_{18} and F_6 only. The CPE was characterised by rounding and clumping of cells and formation of syncytia which was seen along the whole course of the monolayer. The CPE started by 24h and by 96, the monolayer had started peeling off. Isolates T_{22} and F_{12} produced less pronounced CPE which started only after 72h and consisted of rounding of cells and syncytia formation.

In BHK-21 cell line, the isolates T_{18}, T_{22} and F_{12} produced marked CPE but CPE was absent for the isolate F_6. The CPE consisted of rounding of cells and fusion of cells to form large polykaryocytes. Intracytoplasmic inclusion bodies could be observed by 24h after inoculation which was quite prominent by 96h. Formation of

large polykaryocytes and syncytia were observed all along the monolayer but there was no sloughing off of the monolayer.

Production of CPE in chick embryo (CE) cells and baby hamster kidney (BHK) cells by Newcastle disease virus (NDV) strains have been suggested to be caused by an accumulation of virus products (Reeve and Alexander, 1970; Reeve and Poste, 1971; Reeve *et al.* 1972). Alexander *et al.* (1973) observed that infection of Herts strain of NDV both at high and low multiplicities induced cell fusion in chick embryo and BHK-21 cell cultures.

Rosenberger *et al.* (1975) observed that isolates of NDV from Canada geese produced plaques in chicken embryo fibroblast cultures. Cytopathic effects consisting of rounding and clumping of cells and formation of syncytia by avain paramyxoviruses in chicken embryo fibroblast have been observed by Schemera *et al.* (1987) and Bindu (1997). But conflicting results that no cytopathology could be observed in CEF cell cultures infected with paramyxoviruses isolated from feral ducks and geese and collard doves have been reported by other workers (Cloud and Rosenberger, 1980 ; Gough and Alexander, 1983).

Production of cytopathic effect consisting of small discrete syncytia containing two to six nuclei and foci of round cell in chick kidney cells infected with avian paramyxoviruses was reported by Shortridge *et al.* (1980) and Schemera *et al.* (1987). But no evidence of cytopathic effect could be observed in chick kidney cells infected with paramyxoviruses isolated from doves by Alexander *et al.* (1981) and Gough and Alexander (1983).

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

Van Bressem *et al.* (1991) observed that a morbilli virus isolated from Mediterranean striped dolphins (*Stenella coeruleoatta*) and inoculated onto Vero cell monolayers produced cytopathic changes within four weeks. A similar observation was made by Visser *et al.* (1993) with a morbilli virus isolated from harbour porpoises and the cytopathic changes included the development of small syncytia and foci of rounded cells.

Failure of the isolate F_6 to produce any cytopathic effect in BHK-21 monolayer may be because it requires adaptation to grow in mammalian cells. It was passaged only once in the BHK-21 cell line and if it had been passaged repeatedly, it may have become adapted and started producing CPE.

5.8. Pathogenecity

To study the pathogencity of the four isolates, different age groups of chicks and ducklings were used and two different routes of inoculation, subcutaneous and oral were tried.

All the four isolates were found to be non-pathogenic to day old chicken and one week old ducklings. The birds did not show any clinical symptoms during the observation period of four weeks. One, day old chicken inoculated with the isolate T_{22} and two inoculated with the isolate F_{12} died suddenly on the second day of inoculation without showing any clinical symptoms. On postmortem examination, no

characteristic lesions could be observed and materials collected from the birds were negative for the presence of virus even after three passages in embryonated chick eggs. Bacterial isolation was tried with materials collected from the dead birds, but no specific pathogen could be identified. So it may be inferred that death of these three birds must be due to some stress factors arising from handling of the birds and management problems.

Six-week-old ducklings were resistant to all the four isolates which was evidenced by failure of the isolates to produce any clinical signs or mortality in the birds during the observation period of four weeks.

Six-week-old chicken infected with the four isolates did not exhibit any clinical symptoms or mortality even after 28 days of inoculation. However, the virus could be isolated from the cloacal swabs of birds inoculated with the isolate T_{18} and T_{22} from the third day of infection onwards. Isolation was possible from all birds until 14^{th} day for isolate T_{18} and the number excreting the virus diminished to zero by the 28^{th} day. For isolate T_{22}, isolation was possible from all the birds only on 3^{rd} and 5^{th} day and by 7^{th} day isolation was possible only from 80 percent of birds and was nil by the 14^{th} day. No virus isolations were obtained from the tracheal swabs indicating that the viruses multiplied well only in the cells lining the intestinal tract.

For the isolates F_6 and F_{12}, no virus isolation was possible from either cloacal or tracheal swabs collected during the same period showing that they were not capable of multiplying in organs of birds. These two isolates were from epizootic ulcerative syndrome in fishes.

So, even if birds are involved in transmission of these viruses as opined by Sulochana (1992), this could only be by mechanical means. It was reported that primary isolation of these two viruses was possible only by yolk sac inoculation (Sulochana, 1992), but subsequently got adapted to allantoic route. It is a known fact that embryos below ten days of age are highly susceptible to a wide range of viruses (Betts, 1967) including known avian viruses.

Shortridge *et al.* (1980) reported that infection of six-week-old chicken with three avian paramyxovirus isolates by intranasal or intravenous routes produced no signs of disease throughout a 21 day observation period. A mesogenic strain of NDV isolated from a mynah was found to be pathogenic to chicks below three weeks of age, but six-week-old chicks resisted infection (Sulochana, *et al.* 1982b). Gough and Alexander (1984) isolated PMV-4 from a ringed teal, which was non-pathogenic for ducklings and chicken. Bindu (1997) reported that two viruses isolated from lesser whistling teals did not produce any clinical signs or mortality in day old and six-week-old chicken and ducklings over a four week observation period. But virus isolation was possible till 14^{th} day of infection from the cloacal swabs for both the isolates.

The persistence of the virus isolates T_{18} and T_{22} in the system indicate the possibility of its transmission through contaminated water. Though the virus isolates F_6 and F_{12} failed to produce any clinical signs or mortality in the experimental birds, chances of their transmission by wild birds can't be ruled out. In orthomyxo and paramyxo virus, host adaptation is considered to be one of the factors that determine the virulence / pathogenecity (Russel and Edington, 1985). It is possible

that when these avirulent isolates get a chance to infect a different species, they may acquire virulence. Though transmissibility could not be established experimentally, in natural habitat this may not be the case when the environment, especially the water is heavily contaminated and the birds are under stress due to various factors.

5.9. Antigenic relationships between the isolates

Immuno diffusion tests have been used to assess the antigenic relatedness of avian paramyxoviruses and it has been suggested that such tests are far less likely to detect non-specific reactions than HI tests (Kida and Yanagawa, 1991). Alexander *et al.* (1981) reported that typical avian paramyxoviruses against homologous antisera produced three major precipitin lines, viz. n, h and m, which corresponded to the three major virus protein – nucleoprotein (NP), haemagglutinin – neuraminidase (HN) and the matrix protein (M) respectively. The HN protein was represented by the most prominent precipitin line and appeared between the NP and M protein, the latter lines being closed to the antigen and antiserum respectively. Often a fourth line close to h and near to n was also seen.

The isolate T_{18} produced two precipitin lines against its homologous antiserum. The outer one could be supposed to be the precipitin line due to NP and the inner one the line due to the HN protein. Line due to M protein was not noticed. The two lines appeared separately showing that the isolate T_{18} did not have any antigenic similarity with the other isolates. By HI test also it could be seen that the isolate T_{18} did not have any antigenic similarity with the other isolate, because its HI titres against heterologous antisera showed a four-fold difference.

Three precipitin lines were produced by isolate T_{22} against its homologous antiserum of which the middle line possibly HN showed complete identity with precipitin lines of F_6 and F_{12}. But by HI test , the isolate T_{22} could be seen as having antigenic similarity to the isolate F_{12} only and being quite distinct from T_{18} with whose antiserum, it produced no inhibition at all and also from F_6 were there was greater than four fold decrease in the HI titre.

Only two precipitin lines could be seen against corresponding antiserum for the isolate F_6, of which the outer line suggestive of NP was seen separate. The inner line (possibly HN) was identical with the precipitin lines of both T_{22} and F_{12}. Line suggestive of M protein was not observed. All the other precipitin lines were seen separate, without showing any identity.

Even though a similarity between the isolates F_6, F_{12} and T_{22} could be observed on the basis of immunodiffusion tests, such a similarity could not be observed on doing the HI test. The isolate F_6 was distinct from F_{12} and T_{22} and showed similarity with isolate T_{18} only. Kida and Yanagawa (1991) had put forward a suggestion that identity of M proteins in immunodiffusion tests should be used as the criteria for serogrouping avian paramyxoviruses. Here the reasons for contradicting results between the two tests may be because there was no line suggestive of M protein, so that the antigenic relationship shown in immunodiffusion tests may not be enough to establish a similarity.

Isolate F_{12} also showed only two precipitin lines, the lines suggestive of HN protein and M protein based on their position. The

inner precipitin line (possibly M protein) was separate while the outer line (HN) was identical with the HN lines of T_{22} and F_6. The outer line of T_{18} (NP) was seen to be identical with those of F_6 and T_{22}. So antigenic similarity was noticed between all the isolates when the antiserum to F_{12} was placed in the central well. A bold line, which could quite possibly be two lines, was seen close to the antigen well in case of T_{22}. Alexander *et al* (1981) had observed that lines in varied position could be obtained in the case of avian paramyxoviruses.

In the case of isolate F_{12} also, against homologous antiserum the M protein was seen separate from the other isolates and the antigenic similarity was observed only due to HN protein and NP. The results of HI tests shoed that F_{12} was quite distinct from all the three isolates.

It could be seen that the isolate T_{18} was quite distinct from the other three isolates both by immunodiffusion and HI tests. The isolate T_{22} showed antigenic similarity to F_{12} by both tests. F_6 showed similarity to T_{18} by HI test, but not by immunodiffusion test. Isolate F_{12} was found to be distinct from the other three isolates by HI tests, but showed some similarity with them by immunodiffusion test.

Alexander *et al.* (1991) tested the prototype virus for the PMV seven serotypes and five other isolates obtained from birds of Columbidae family for antigenic relationships between themselves and to other avian paramyxoviruses. One virus showed high levels of homology in HI test and at least one line of identity in immunodiffusion test with all the other five isolates.

The antigenic relationship of the fish viruses with the teal viruses suggest that they may be antigenic variants of the teal viruses, supporting the view of Sulochana *et al.* (1992), that the viruses causing EUS in fresh water fishes in Kerala may be disseminated by waterfowls.

5.10. SDS-PAGE of proteins

The virus isolates T_{18}, T_{22}, F_6 and F_{12} consisted of 10, 11, 8 and 7 protein fractions respectively. Of these six bands were common in all the four isolates. The presence of 7- 10 polypeptides in NDV and other paramyxoviruses have been reported by a number of workers (Moore and Burke, 1974; Shortridge *et al.* 1980; Alexander and Collins, 1981; Kumanan *et al.*, 1994). But other workers have shown NDV and other avian paramyxoviruses to contain 5-7 polypeptides (Kessler and Aymard, 1979; Alexander *et al.,* 1983; Vijayarani, *et al.,* 1992a; Swain *et al.,* 1997). When the structural proteins of hirame rhabdovirus (HRV) was analysed by SDS-PAGE, five proteins were identified (Lu and Loh, 1994).

Kumanan *et al.* (1994) and Swain *et al.* (1997) resolved a glycoprotein of molecular weight 76 kDa in Newcastle disease virus which they opined to be the HN protein of paramyxoviruses. The band of molecular weight 70- 77 kDa observed in all the four isolates in the present study could therefore correspond to the HN protein of NDV and other avian paramyxoviruses reported by various other workers (Moore and Burke, 1974; Kessler and Aymard, 1979; Shortridge *et al.* 1980; Alexander and Collins, 1981; Vijayarani, *et al.* 1992a).

Presence of a minor polypeptide of molecular weight 180 kDa in NDV and other avian paramyxoviruses has been reported by Moore and Burke (1974), Shortridge *et al.* (1980) and Alexander and Collins (1981). Swain *et al.* (1997) could identify a band of molecular weight 198 kDa in a velogenic strain of NDV which they opined could be the L protein based on the molecular weight. So the 181- 182 kDa bands resolved in the isolates T_{18}, T_{22} and F_6 could be similar to the L protein of avian paramyxoviruses. This band was not seen in the isolate F_{12}, which could be due to some strain variation.

The isolate T_{18} had a protein fraction of molecular weight 149 kDa and the isolates T_{22} and F_{12} both had fractions with molecular weight 137 kDa and 135 kDa respectively which have not been previously described to be present in avian paramyxoviruses by any workers.

The isolates T_{18} and T_{22} had two bands of similar molecular weight of 118 kDa which were not observed in the other two isolates. The presence of a band of 110 kDa in a strain of PMV-1 had been reported by Alexander and Collins (1981) but they had opined that this result should be treated with some caution as a polypeptide of similar molecular weight was frequently seen in inadequately purified preparations of viruses.

Presence of a polypeptide having a molecular weight of 60 kDa in a paramyxovirus isolated from poultry in Hong Kong has been reported by Shortridge *et al.* (1980). The presence of a glycoprotein of 55 kDa in Newcastle disease virus and other avian paramyxoviruses has also been reported by Moore and Burke (1974) and Alexander and

Collins (1981). Swain *et al.* (1997) could resolve a protein fraction of molecular weight 54 kDa in a velogenic Newcastle disease virus which they suggested to be the nucleocapsid (N) protein. So the band of 60 kDa observed in the three isolates T_{18}, T_{22} and F_6 and of 58 kDa in F_{12} in the present study could be the protein of avian paramyxoviruses. Vijayarani *et al* (1992a) and Kumanan *et al.* (1994) detected bands of molecular weight 59 kDa and 56 kDa respectively in Newcastle disease virus strains which they opined to be the N protein.

The band of molecular weight 48 kDa observed in the isolate T_{22} alone would correspond to the P protein of molecular weight 50 kDa in velogenic NDV reported by Swain *et al.* (1997). Presence of bands of molecular weight ranging from 48- 49 kDa in avian paramyxoviruses have also been reported by other workers (Moore and Burke, 1974; Shortridge *et al.* 1980; Alexander and Collins, 1981; Vijayarani, *et al.* 1992a).

Kumanan *et al.* (1994) could resolve a 44 kDa low molecular weight fraction in five field isolates of Ranikhet disease virus which resembled the matrix (M) protein in its electrophoretic mobility. A band of molecular weight 44 kDa could be resolved in all the four isolates in the present study which could be supposed to represent the M protein of avian paramyxoviruses. But Swain *et al.* (1997) opined that a band of molecular weight 37 kDa observed in a velogenic NDV would correspond to the M protein.

The low molecular weight fractions ranging from 35 kDa to 18 kDa observed in all the isolates have not been reported by any other

workers but could correspond to some minor polypeptides of paramyxoviruses.

The presence of the protein bands of molecular weights 181-182 kDa, 70- 77 kDa, 58- 60 kDa and 44 kDa in all the isolates (except for absence of the band of 181- 182 kDa in isolate F_{12}) would suggest that the protein profiles of the isolates are almost similar to those of avian paramyxoviruses. Even the viruses isolated form fishes showed a similar protein profile to that of avian paramyxoviruses. The number of polypeptides identified ranged from 7-11 which is also in accordance with the number of bands identified in avian paramyxoviruses by other workers. At the same time, presence of unique bands of 149 kDa and 48 kDa in T_{18} and T_{22} respectively suggest some dissimilarities between the teal viruses and the fish viruses.

The isolates T_{18} and T_{22} were send for monoclonal antibody typing to the Central Veterinary Laboratory, Surrey, England. Both were identified as belonging to the paramyxovirus – I group. T_{18} gave a binding pattern with monoclonal antibody placing it in group C (velogenic) viruses. T_{22} had binding pattern placing it in group E (B_1 and LaSota) viruses (Sulochana, 1999).

Isolates F_6 and F_{12} have to be send for typing to confirm their identity. The similarities in physico- chemical characteristics and protein profile to the above isolates suggest that they also belong to the paramyxo virus group. The possibility of these two isolates being fish adapted strains of paramyxoviruses cannot be ruled out as in the case of pestes des petits virus of ruminants in sheep and goat, which is very closely related to rinderpest virus and lumpy skin disease virus in cattle which again is considered as a cattle adapted strain of sheep pox virus.

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SUMMARY

6. SUMMARY

There have been frequent outbreaks of poultry diseases leading to significant losses in the recent years in our state. A decade ago an outbreak of epizootic ulcerative syndrome (EUS) occurred in fresh water fishes leading to heavy losses to the fish farmers also. Free flying waterfowls were suspected to be an important source for the introduction of disease agents to both the domestic poultry and fishes. On screening of the waterfowls, two viral agents (T_{18} and T_{22}) could be isolated and from fishes with EUS also two viral agents could be isolated (F_6 and F_{12}). Of these T_{18} and T_{22} were partially characterised in an earlier study. The present study was undertaken to completely characterise all the four isolates and to find out whether the migratory waterfowls have any role in the transmission of viruses to domestic poultry and fish. The isolates preserved in the Department of Microbiology were revived by passaging through embryonated chicken / duck eggs. Primary passage in embryonated duck eggs was needed for the isolate T_{22} before it could be adapted to grow in chicken embryos.

The allantoic fluid collected from the chicken embryos inoculated with the isolates showed marked haemagglutination with chicken RBC. The HA titre of the allantoic fluid after the third passage in chicken embryos was 512, 256, 512 and 128 respectively for the isolates T_{18}, T_{22}, F_6 and F_{12}. All the isolates killed the embryos within 4 days time when inoculated via the allantoic route. With T_{18} and F_6, the embryos were highly congested and the CAM was also congested. Congestion of embryo was less marked for the isolate F_{12}. No specific lesions could be seen in the internal organs of embryos given the isolates T_{18}, F_6 and F_{12}. Chicken embryos inoculated with isolate T_{22} were stunted and

congested, with the liver of the embryos showing yellowish brown patches.

The infectivity titres in chicken embryos were $3.2x10^6$, 5.6×10^5, $1.65x\ 10^7$ and $3.16x\ 10^5$ respectively for T_{18}, T_{22}, F_6 and F_{12}. All the isolates were thermolabile, as evidenced by inactivation at 56°C for 30 min. At pH 7.2, the infectivity and haemagglutinating activity of all the isolates were unaffected, but at pH 3.2 and 9.0 complete loss of infectivity and reduction in haemagglutinating activity were noted. The enveloped nature of the isolates was evidenced by their sensitivity to chloroform. Both infectivity and haemagglutinating activity of the isolates were unaffected by brine treatment, indicating their capability to survive at high salt concentration.

Marked CPE was exhibited by the isolates T_{18} and F_6 in chicken embryo fibroblast, culture with the CPE being complete within 96h. In stained preparations, rounding and clumping of cells, syncytia formation and severe cytoplasmic vacuolation were observed. Inclusion bodies could not be detected either in the nucleus or cytoplasm. For the isolates T_{22} and F_{12} CPE developed later only and was not as prominent as for T_{18} and F_6. There was rounding of cells and syncytia formation by about 72h involving only two or three cells. Cytoplasmic vacuolation though present was less marked. No inclusion bodies, either intracytoplasmic or intranuclear could be detected.

In BHK-21 cell line, CPE was produced by the isolates T_{18}, T_{22} and F_{12}, while F_6 failed to produce any visible CPE. Within 24h after inoculation, cells started fusing to form polykaryocytes. Between

48- 72h large syncytia were found. Intracytoplasmic inclusion bodies could be observed by 24h after infection, which was quite prominent by 96h.

Nucleic acid type of the isolates was detected by pretreating chicken embryo fibroblast cultures with 5-iodo-2-deoxyuridine which led to the conclusion that all the isolates were RNA viruses.

Pathogenecity tests were carried out in day old and six-week-old chicken and ducklings. All the four isolates were found to be non-pathogenic to chicken and ducklings when given by the oral/ subcutaneous route or by both. In all cases, neither clinical symptoms nor mortality was observed. But virus isolation was possible from the cloacal swabs of six-week-old chicken for the isolates T_{18} and T_{22} up to the 14^{th} day and 7^{th} day post infection respectively.

Antigenic relationship between the isolates was tested by immunodiffusion and haemagglutination inhibition tests. It was found that the isolate T_{18}, did not show any antigenic similarity with any of the other three isolates. Antigenic similarity was noticed between the other three isolates by either the immunodiffusion or HI tests.

SDS-PAGE of the isolates yielded 10, 11, 8 and 7 bands respectively for the isolates T_{18}, T_{22}, F_6 and F_{12} with the bands corresponding to the HN protein, nucleoprotein and matrix protein being common for all the isolates. The polypeptide profile of the isolates resembled that of avian paramyxoviruses, indicating their similarity to the paramyxovirus group.

The isolates T_{18} and T_{22} were sent to the Central Veterinary Laboratory, Surrey, England for further typing. It was found that both the viruses belonged to the PMV group with T_{18} belonging to the group C (velogenic) and T_{22} to group E (B_1 and LaSota) viruses.

It was concluded from the study that all the isolates were enveloped RNA viruses, with T_{18} and T_{22} being paramyxoviruses belonging to Group I. The properties of the isolates F_6 and F_{12} resembled these paramyxoviruses and hence can also be inferred to be paramyxoviruses.

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REFERENCES

7. REFERENCES

*Ahne, W., Jorgensen, P.E.V., Olesen, N.J. and Wat-Tanavijarn, W. (1988). Serological examination of a rhabdovirus isolated from snake head (*Ophicephalus striatus*) in Thailand with ulcerative syndrome. *J. Applied Ichthyology.* **4**: 194- 196.

Alexander, D.J. (1974). Comparison of the structural polypeptides of four avian paramyxoviruses. *Arch. ges. Virusforsch.* **46**: 292-301.

Alexander, D.J. (1982). Avian paramyxoviruses- other than Newcastle disease virus. *World Poult. Sci. J.* **38**: 97- 104.

Alexander, D.J. and Chettle, N.J. (1978). Relationship of parakeet/ Netherlands/449/75 virus to other avian paramyxoviruses. *Res. Vet. Sci.*, **25**: 105-106.

Alexander, D.J. and Collins, M.S. (1981). The structural polypeptides of avian paramyxoviruses. *Arch. Virol.* **67**: 309- 323.

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

Alexander, D.J., Aymard, M., Kessler, N. and Collins, M.S. (1979). Antigenic and structural relationships between avian paramyxoviruses isolated from ducks in Hongkong and Missisippi. *J. gen. Virol.* **44**: 839- 842.

Alexander, D.J., Hewlett, G., Reeve, P. and Poste, G. (1973). Studies on the cytopathic effects of Newcastle disease virus: the cytopathogenecity of strain Herts 33 in five cell types. *J. gen. Virol.* **21**: 323- 327.

Alexander, D.J., Hinshaw, V.S. and Collins, M.S. (1981). Characterization of viruses from doves representing a new serotype of avian paramyxoviruses. *Arch. Virol.* **68**: 265- 269.

Alexander, D.J., Hinshew, V.S., Collins, M.S. and Yamane, N. (1983). Characterization of viruses which represent further distinct serotypes (PMV-8 and PMV-9) of avian paramyxoviruses. *Arch. Virol.* **78**: 29- 36.

Alexander, D.J., Manvell, R.J., Collins, M.S. and Brockman, S.J. (1991). Evaluation of relationships between avian paramyxoviruses isolated from birds of the family Columbidae. *Arch. Virol.* **116**: 267- 276.

Alexander, D.J., Manvell, R.J., Collins, M.S., Brockman, S.J., Westbury, H.A., Morgas, I. and Austin, F. J. (1989). Characterization of paramyxoviruses isolated from penguins in Arctica and sub-Antarctica during 1976- 1979. *Arch. Virol.* **109**: 135- 143.

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

Benton, W.J., Cover, M.S., Rosenberger, J.K. and Lake, R.S. (1967). Physicochemical properties of infectious bursal agent. *Avian Dis.* **11**: 438- 445.

Betts, A.O. (1967). The cultivation of viruses- In "Viral and Rickettsial infections of animals". (A.O. Betts and C.J. York, eds.) Vol. I. pp- 69- 117, Academic Press, New York.

Bindu, M.S. (1997). Isolation and identification of viruses from waterfowls seen in Kerala. M.V.Sc. thesis submitted to Kerala Agricultural University.

Bishai, F., Blaskovic, P., Hamvas, J.J., Iwakata, S., Labzoffsky, N.A., Subrahmanyan, T.P. and Zalan, E. (1974). Virology Manual. pp- 23, 136. Ministry of Health, Ontario.

*Bjorklund, H.V., Olesen, N.J. and Jorgensen, P.E.V. (1994). Biophysical and serological characterisation of rhabdovirs 903/ 87 isolated from European lake trout *Salmo trutta lacustris*. *Dis. Aquatic. Organisms*. **19**: 21- 26.

Breese, S.S. Jr. and Dardiri, A.H. (1968). Electron microscopic characterization of duck plague virus. *Virology*. **34**: 940- 949.

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

Caliguiri, L.A., Klenk, H. and Choppin, P.W. (1969). The proteins of the parainfluenza virus SV5 1. Separation of virion polypeptides by polyacrylamide gel electrophoresis. *Virology.* **39**: 460- 466.

Cloud, S.S. and Rosenberger, J.K. (1980). Characterization of nine avian paramyxoviruses. *Avian Dis.* **24**: 139- 152.

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

Evans, M.J. and Kingsbury, D.W. (1969). Separation of Newcastle disease virus proteins by polyacrylamide gel electrophoresis. *Virology.* **37**: 597- 604.

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

Goodman, B.B. and Hanson, R.P. (1988). Isolation of avian paramyxovirus –2 from domestic and wild birds in Costa Rica. *Avian Dis.* **32**: 713-717.

Gough, R.E. and Alexander, D.J. (1983). Isolation and preliminary characterization of a paramyxovirus from collared doves (*Streptopelia decaocto*). *Avian Path.* **12**: 125- 134.

Gough, R.E. and Alexander, D.L. (1984). Avian paramyxovirus type 4 isolated from a ringed teal (*Calonetta leucophrys*). *Vet. Rec.* **115**: 653.

Gulka, C.M., Piela, T.H., Yates, V.J. and Bagshaw, C. (1984). Evidence of exposure of waterfowl and other aquatic birds to the haemagglutinating duck adenovirus identical to EDS-76 virus. *J. Wildl. Dis.* **20**: 1-5.

Hanson, R.P. and Spalatin, J. (1978). Thermostability of the haemagglutinin of Newcastle disease virus as a strain marker in epizootiologic studies. *Avian Dis.* **22**: 659- 665.

Hanson, R.P., Upton, E., Brandley, C.A. and Winslow, N. S. (1949). Heat stability of haemagglutinin of various strains of Newcastle disease virus. *Proc. Soc. Exp. Biol.* (N.Y.) **70**: 283- 287.

*Hess, W.R. and Dardiri, A.M. (1968). Some properties of the virus of duck plague. *Arch. ges. Virusforsch.* **24**: 148- 153.

Hinshaw, V.S., Wood, J.M., Webster, R.G., Deibel, R. and Turner, B. (1985). Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. *Bull. World Health Org.* **63**: 711- 719.

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

Kamaraj, G., Nachimuthu, K. and Kumanan, K. (1998). Isolation and characterization of Newcastle disease virus strains from *desi* birds. *Indian J. Anim. Sci.* **68**: 43-45.

*Kasornchandra, J., Engelking, H.M., Lannan, C.N., Rohovec, J.S. and Fryer, J.L. (1992). Characteristics of three rhabdoviruses from snake head fish (*Ophicephalus* (Channa) *striatus*). *Dis. Aquatic. Organisms.* **13**: 89- 94.

Kessler, N. and Aymard, M. (1979). Study of a new strain of paramyxoviruses isolated from wild ducks: structural polypeptides. *J. gen Virol.* **43**: 411- 415.

Kida, H. and Yanagawa, R. (1991). Classification of avian paramyxoviruses by immunodiffusion on the basis of antigenic specificity of their M protein antigens. *J. gen. Virol.* **52**: 103-111.

Kim, S.J. and Spradbrow, P.B. (1978). Some properties of lentogenic Newcastle disease virus . *Vet. Microbiol.* **3**: 129-141.

King, D.J. (1996). Avian paramyxovirus type I from pigeons: Isolate characterisation and pathogenecity after chicken or embryo passage of selected isolates. *Avian Dis.* **40**: 707- 714.

Kocan, A.A., Hinshaw, V.S. and Daubney, G.A. (1978). Influenza A viruses isolated from migratory ducks in Oklahoma. *J. Wildl. Dis.* **16**: 281- 289.

Kumanan, K. and Venkatesan, R.A. (1991). Characterization of a strain of Ranikhet disease virus isolated from Japanese quails. *Indian J. Anim. Sci.* **61**: 495- 500.

Kumanan, K., Elankumaran, S., Vijayarani, K., Palaniswami, K.S., Padmanaban, V.D., Manvell, R.J. and Alexander, D.J. (1992). Characterisation of Newcastle disease viruses isolated in India. *J. Vet. Med.* Series B. **319**: 333- 337.

Kumanan, K., Mustaq, A.N. and Venkatesan, R.A. (1994). Protein profile of some strains of Ranikhet disease virus. *Indian J. Anim. Sci.* **64**: 319- 321.

*Kunst, H. (1968). Duck plague virus investigation *in vitro*. Neth. *J. Vet. Sci.* **1**: 197- 200.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*. **227**: 680- 685.

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

Leibovitz, L. (1991). Duck virus enteritis (duck plague). In "Disease of Poultry". Ninth Edition. Eds. M.S. Hofstad, B.W. Calnek, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder Jr. Iowa State University Press. Ames. pp 609- 618.

*Liley, J.H. and Frerichs, G.N. (1994). Comparison or rhabdoviruses associated with epizootic ulcerative syndrome (EUS) with respect to their structural proteins, cytopathology and serology. *J. Fish. Dis.* **17**: 513- 522.

Lin, W., Lam, K.M. and Clark, W.E. (1984). Isolation of a pathogenic immunogenic strain of duck enteritis virus from waterfowl in California. *Avian Dis.* **28**: 641- 650.

Lomniczi, B. (1975). Thermostability of Newcastle disease virus strains of different virulence. *Arch. Virol.* **47**: 249- 255.

*Lu, Y. and Loh, P.C. (1992). Some biological properties of a rhabdovirus isolated from penaeid shrimps. *Arch. Virol.* **127:** 339- 343.

*Lu, Y. and Loh, P.C. (1994). Viral structural proteins and genome analysis of the rhabdovirus of penaeid shrimp (RPS). *Dis. Aquatic Organisms.* **19**: 187- 192.

McCarthy, M. and Johnson, R.T. (1980). A comparison of the structural polypeptide of five strains of mumps virus. *J. gen. Virol.* **46**: 15- 27.

McFerran, J.B. and Nelson, R. (1971). Some properties of an avian Newcastle disease virus. *Arch. ges. Virusforsch.* **34**: 64- 74.

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

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

Mini. M. (1988). Characterisation and immunogenecity of influenza virus type A isolated from ducks in Kerala. M.V.Sc. thesis submitted to Kerala Agricultural University.

Moore, N.F. and Burke, D.C. (1974). Characterization of the structural proteins of different strains of Newcastle disease virus. *J. gen. Virol.* **25**: 275-289.

Moses, H.E., Brandly, C.A., Jones, C.E. and Jungherr, E.L. (1948). The isolation and characterisation of fowl plague virus. *Am. J. Vet. Res.* **14**: 314- 330.

Mountcastle, W.E., Compans, R.W. and Choppin, P.W. (1971). Proteins and glycoproteins of paramyxoviruses: a comparison of simian virus 5, Newcastle disease virus and Sendai virus. *J. Virol.* **7**: 47-52.

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

Nerome, K., Nakayama, M., Ishida, M. and Fukumi, H. (1978). Isolation of a new avian paramyxovirus from budgerigar (*Melopsittacus undulatus*) *J. gen. Virol.*. **38**: 293- 301.

*Nishizawa, T., Yoshimizu, M., Winton, J., Ahne, W. and Kimura, T. (1991). Characterisation of structural proteins of hirame rhabdovirus HRV. *Dis. Aquatic Organisms.* **10**: 167- 172.

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

*Papparella, A., Zampino, V., Vaccaro, A. and De Rogatis, A. (1969). Isolation of type A influenza virus on a turkey farm in Camponia. *Acta. Med. Vet. Nepoli.* **15**: 105- 113.

Proctor, S.J. Pearson, G. L. and Leibovitz, L. (1975). Colour atlas of wildlife pathology. 2. Duck plague in freeflying waterfowl observed during the Lake Andes epizootic. *J. Wildl. Dis.* **67**: 59.

Ramkumar, J. M., Kataria, J.M. and Mohanty, G.C. (1991). Isolation and characterisation of haemagglutinating viral agents resumbling EDS-76 virus from poultry flocks. *Indian J. Virol.* **7**: 53- 58.

Reddy, Y.K., Koteeswaran, A. and Dorairajan, N. (1993). Characterization of Ranikhet disease virus isolates from Namakkal. *Indian Vet. J.* **70**: 1097- 1100.

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

*Reeve, P. and Alexander, D.J. (1970). Plague formation, cell fusion and haemadsorption by Newcastle disease virus. *Cytobiosis.* **2**: 55-57.

Reeve, P. and Poste, G. (1971). Studies on the cytopathogenecity of Newcastle disease virus: relation between virulance , polykaryocytes and plaque size. *J. gen. Virol.* **11**: 17- 24.

Reeve, P., Poste, G., Alexander, D.J. and Pope, G. (1972). Studies on the cytopathic effects of Newcastle disease virus: metabolic requirements. *J. gen. Virol.* **15**: 219- 225.

Rima, B.K. (1983). The proteins of morbilli viruses. *J. gen. Virol.* **64**: 1205- 1219.

Rithombar, V., Reddy, R. and Rao, P.V. (1986). A survey study of duck farming and hatcheries in Andhra Pradesh. *Indian J. Poult. Sci.* **21**: 180- 185.

Rosenberger, J.K., Klopp, S. and Krauss, W.C. (1975). Characterisation of Newcastle disease virus isolated from migratory waterfowl in Atlantic flyway. *Avian. Dis.* **19**: 142- 149.

Russel, P.H. and Edington, N. (1985). Veterinary viruses. First Ed. Burlington Press. pp 138- 155.

Schemera, B., Toro, H., Kaleta, E.F. and Herbst, W. (1987). A paramyxovirus of serotype 3 isolated from African and Australian finches. *Avian Dis.* **31**: 921- 925.

Shortridge, K.F., Alexander, D.J. and Collins, M.S. (1980). Isolation and properties of viruses from poultry in Hong Kong which represent a new (sixth) distinct group of avian paramyxoviruses. *J. gen. Virol.* **49**: 255- 262.

Singh (1993). Biological properties of avian paramyxovirus type I isolated from pigeons. *Infect. Dis.* **14**: 34-35.

Smit, J., Roozelaar, D. and Hoekstra, J. (1980). Diagnosis, epidemiology and control of infectious hepatitis in water fowl. *Stichting voor Diergeneskunding Onderzoek Jaarverslag.* **1977**: 43-45.

Sulochana, S. (1991a). Isolation and characterization of Newcastle disease virus from Japanese quails (*Coturnix coturnix japonica*). *J. Vet. Anim. Sci.* **22**: 103- 105.

Sulochana, S. (1991b). Isolation of Newcastle disease virus from lesser whistling teals (*Dendrocygna javanica*). *Zoos Print.* **6**: 23- 24.

Sulochana, S. (1999). Personal communication.

Sulochana, S. and Nair, G.K. (1979). A note on the isolation of Newcastle disease virus from a duckling. *Kerala J. Vet. Sci.* **10**: 337- 338.

Sulochana, S., James, P.C., George, M.C. and Nair, K.M. (1992). Epizootic ulcerative syndrome in fishes in Kerala. Isolation of a paramyxovirus. Paper presented at the annual conference and national symposium on Biotechnology in the diagnosis of viral diseases, Hyderabad. pp

Sulochana, S., Pillai, R.M., Nair, G.K. and Abdulla, P.K. (1981). Characterisation and pathogenecity of an isolate of Newcastle disease virus (NDV-D) from ducks. *Kerala J. Vet. Sci.* **12**: 23-30.

Sulochana, S., Pillai, R.M., Nair, G.K. and Abdulla, P.K. (1982a). Isolation and characterization of a mesogenic strain of Newcastle disease virus from a mynah (*Acridotheres tristis tristis*). *Kerala J. Vet. Sci.* **13**: 199- 203.

Sulochana, S., Pillai, R.M., Nair, G.K. and Abdulla, P.K. (1982b). Pathogenecity and immunogenecity of a mesogneic strain of Newcastle disease virus isolated from mynah. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **3**: 109- 115.

Sulochana, S., Sudharma, D., Jayaprakasan, V., Mathew, K.S. and Mini, M. (1987). Isolation of influenza virus type A from ducks in Kerala. *Indian J. Poult. Sci.* **22**: 255-260.

Suss, J., Schafer, J., Sinnecker, H. and Webster, R.G. (1994). Influenza virus subtypes in aquatic birds of Eastern Germany. *Arch. Virol.* **135**: 101- 114.

Swain, P., Kataria, J.M. and Verma, K.C. (1993). Biological characterisation of an Indian isolate of egg drop syndrome- 76 virus. *Res. Vet. Sci.* **55**: 396- 397.

Swain, P., Kataria, J.M., Verma, K.C. and Satishkumar. (1992). Characterisation of a field isolate of egg drop syndrome 76 (EDS-76) virus. *Indian J. Virol.* **8**: 8- 14.

Swain, P., Verma, K.C. and Kataria, J.M. (1997). Characterization of structural polypeptides of velogenic Newcastle disease virus. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **18**: 125- 129.

Todd, D. and McNutty, M.S. (1978). Egg drop syndrome 1976. In "Diseases of poultry". Ninth edition. Eds. M.S. Hofstad, B.W. Calnek, H.J. Barnes, C.W. Beard, W.H. Reid and H.W. Yoder Jr. Iowa State Univ. Press. Ames, Iowa. pp- 516- 523.

Tumova, B., Turek, R., Kubinova, I., Stumpa, A. and Ciampor, F. (1984). Incidence of paramyxoviruses in free living birds in 1978- 1982. *Acta. Virol.* **28**: 114- 121.

Van Bressem, M.F., Visser, I.K.G., Van De Bildt, M.W.G., Teppema, J.S., Raga, J.A. and Osterhaus, A.D.M.E. (1991). Morbilli virus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*). *Vet. Rec.* **129**: 471- 472.

Vijayarani, K., Kumanan, K. and Padmanabhan, V.D. (1992a). Studies on the proteins of three field isolates of Newcastle disease virus. *Indian Vet. J.* **69**: 975- 977.

Vijayarani, K., Kumanan, K., Elankumaran, S., Albert, A. and Padmanabhan, V.D. (1992b). Characterization of field isolates of Newcastle disease virus. *Indian Vet. J.* **69**: 584- 588.

Visser, I.K.G., Van Bressem, M.F., de Swart, R.L., Van de Bildt, M.W.G., Vos, H.W., Van Der Heijden, R.W.J., Saliki, J.T., Orvell, C., Kitching, P., Kuiken, T., Barrett, T. and Osterhaus, A.D.M.E. (1993). Characterisation of morbilli viruses isolated from dolphins and porpoises in Europe. *J. gen. Virol.* **76**: 631- 641.

Waters, D.J. and Bussell, R.H. (1973). Polypeptide composition of measles and Canine distemper viruses. *Virology.* **55**: 554- 557.

*Wawrzkiewicz, J., Majer- Dziedzic, B. and Pochodyla, A. (1989). Physicochemical and biological properties of paramyxovirus type I strains isolated from pigeons blood. *Medycyna weterynaryjna.* **45**: 464- 468.

Yamaguchi, S., Imada, T., Kawamura, S., Taniguchi, S., Saio, H. and Shimamatsu, K. (1981). Egg drop syndrome 1976. In "Diseases of poultry". Ninth edition. Eds. M.S. Hofstad, B.W. Calnek, H.J. Barnes, C.W. Beard, W.H. Reid and H.W. Yoder Jr. Iowa State Univ. Press. Ames, Iowa. pp- 516- 523.

*- Originals not seen

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CHARACTERISATION OF VIRUS ISOLATES FROM LESSER WHISTLING TEALS *(Dendrocygna javanica)* AND CHANNA SPECIES OF FISH

By

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ABSTRACT OF A THESIS

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ABSTRACT

Characterisation of virus isolates (T_{18} and T_{22}) from lesser whistling teals (*Dendrocygna javanica*) and Channa species of fish (F_6 and F_{12}) was carried out to determine the similarities if any between the isolates and to identify the role of waterfowls in dissemination of these viruses.

The virus isolates, preserved in the Department of Microbiology were revived by passaging through embryonated chicken/ duck eggs through allantoic route. After the third passage, all the isolates were found to produce death of the embryos and the allantoic fluid collected agglutinated one percent chicken RBC. The isolates T_{18}, F_6 and F_{12} produced congestion of the embryo and CAM and the embryos showed sub-occipital and interdigital haemorrhages. Isolate T_{22} also produced congestion of the embryo and CAM and the embryos were stunted. Liver of the embryos had yellowish brown patches.

The EID_{50} of isolates were $3.2x10^6$, 5.6×10^5 , $1.65x\ 10^7$ and 3.16×10^5 respectively for the isolates T_{18}, T_{22}, F_6 and F_{12}. The infectivity and haemagglutinating activity of all the isolates were retained at pH 7.2, but were completely lost at pH 3.2 and 9.0 and also by treatment at 56°C for 30 min. All the isolates were sensitive to chloroform indicating their enveloped nature. Pretreatment of chicken embryo fibroblast cultures with 100μg/ ml of IuDR did not inhibit the multiplication of any of the isolates indicating they all had RNA genomes. All the isolates were resistant to treatment with brine indicating that they were capable of surviving at high salt concentration.

The isolates T_{18} and F_6 produced marked CPE in chicken embryo fibroblast culture with rounding and clumping of cells and syncytia formation. Marked cytoplasmic vacuolation was also observed. Inclusion bodies could not be detected either in nucleus or cytoplasm. For isolates T_{22} and F_{12}, CPE developed later only and was not as prominent as for T_{18} and F_6. Rounding of cells and their fusion forming syncytia was noticed by 72 h. Cytoplasmic vacuolation though present was much less marked. Inclusion bodies were absent.

Large polykaryocytes were produced by the isolates T_{18}, T_{22} and F_{12} in BHK-21 cell line within 24h after inoculation. Between 48-72h large syncytia were formed. Intracytoplasmic inclusions could be observed by 24h after infection, which were quite prominent by 96h. The isolate F_6 failed to produce any CPE in BHK-21 cell line.

Pathogenecity tests in day old and six-week-old chicken and ducklings showed that all the four isolates were non-pathogenic when given by the oral /subcutaneous route or by both. Neither clinical signs or mortality could be observed in the birds. Virus isolation was possible from the cloacal swabs of six-week-old chicken for the isolates T_{18} and T_{22} up to the 14^{th} and 7^{th} day respectively.

Antigenic relationship between the isolates was tested by gel diffusion and haemagglutination inhibition tests, which showed that the isolate T_{18} did not have any similarity with any of the other three isolates. The isolate T_{22} showed antigenic similarity by both the tests. F_6 showed similarity to T_{18} by HI test but not by immunodiffusion test.

Isolate F_{12} was found to be distinct from the other three by HI test, but showed some similarity with them by immunodiffusion test.

By sodium dodecyl sulphate – polyacrylamide gel electrophoresis on 10 percent gels, 7-11 bands could be resolved for the different isolates. Three of the bands were common for all the four isolates and were having molecular weights similar to the three major proteins HN, NP and MP of avian paramyxoviruses, suggesting that the isolates belonged to the paramyxovirus group.

Monoclonal antibody typing of the isolates T_{18} and T_{22} at the Central Veterinary Laboratory, Surrey, England confirmed that both belonged to the paramyxovirus group with T_{18} belonging to group C (velogenic) and T_{22} to group E (B_1 and LaSota) viruses. The isolates F_6 and F_{12} need to be further typed.

It was concluded from the study that all the isolates were enveloped RNA viruses with T_{18} and T_{22} being paramyxoviruses belonging to Group I. The properties of the isolates F_6 and F_{12} resembled the paramyxoviruses and from the similarly in protein profile with the other two viruses can also be concluded to be paramyxoviruses.

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