CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES FROM DESI CHICKEN

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Dedicated to my

Parents,

Teachers,

Profession and

Nation

TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY, MADRAS

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Newcastle Disease Virus isolates from Desi Chicken"** submitted in part fulfilment of the requirements of the degree of **Master of Veterinary Science in Animal Biotechnology** to the Tamil Nadu Veterinary and Animal Sciences University, Madras is a record of bonafide research carried out by **Thiru. V.S. RAGHAVAN** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles of prizes and that work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES FROM DESI CHICKEN

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The present study has been undertaken to isolate and characterize Newcastle Disease Virus (NDV) strains from Desi chicken.

A total of eleven strains of NDV were isolated in embryonated chicken eggs and identified as NDV by haemagglutination inhibition test (HI) and Virus Neutralization test (VNT). The isolates were characterized by Mean Death Time (MDT), Intracrebral Pathogenicity Index (ICPI), Intravenous Pathogenicity Index (IVPI), Stability of haemagglutinins at 56°C, Agglutination of mammalian erythrocytes and adsorption of haemagglutinins by chicken brain cells. The results of these tests indicated that these isolates are not lentogenic. The isolates were also charaterized by Monoclonal antibody typing which placed them in groups C_1, E and L. The NDV isolates were passaged in MDBK cells also for differentiating them as velogenic and lentogenic. A 21 mer oligonucleotide probe (complementary to the conserved region of the fusion protein), labelled with γ^{32}P-ATP was also used to differentiate the isolates. The results of these experiments indicated that the eleven isolates made in this study are of either velogenic or mesogenic in nature but not lentogenic.

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TITLE

LIST OF ABBREVIATIONS

AAF	:	Amnio allantoic fluid
CAM	:	Chorio allantoic membrane
CPE	:	Cytopathic effect
EDTA	:	Ethylene diamine tetra acetic acid
ELD_{50}	:	Embryo lethal dose, 50 per cent end point.
DEPC	:	Diethylpyrocarbonate
g	:	Gram
HA	:	Haemagglutination
HI	:	Haemagglutination inhibition
hrs	:	Hours
ICPI	:	Intracerebral pathogenicity index
IVPI	:	Intravenous pathogenicity index
M	:	Molar
MAb	:	Monoclonal antibody
MDBK	:	Madin darby bovine kidney
MDT	:	Mean death time
mM	:	Milli Molar
min	:	Minutes
MOPS	:	Morpholino propane sulphonic acid
NDV	:	Newcastle disease virus
PAGE	:	Poly Acrylamide Gel Electrophoresis
rpm	:	revolutions per minute
RNA	:	Ribonucleicacid
SDS	:	Sodium Dodecyl Sulphate
SSC	:	Saline Sodium Citrate
TGDW	:	Triple Glass Distilled Water
VNT	:	Virus neutralization test

Introduction

CHAPTER - I

INTRODUCTION

Newcastle disease (ND) which affects poultry and other aves is an economically important disease because of its high morbidity, mortality and drop in production (Alexander, 1990). This disease was first reported in England in 1926 (Doyle, 1927). Eversince its first report in India (Edwards, 1928), the disease has gained significant importance due to the industrialization of poultry farming.

Many wild birds, free roaming birds and water fowls are said to harbour the disease producing virus and help in the spread of the disease to commercial flocks. Luthgen (1981) lists about 117 species of birds belonging to various orders of the class aves found to harbour the virus and spread the disease. Moreover, it has also been reviewed by many authors that village flocks are the richest source of Newcastle disease virus (NDV) and play a vital role in shedding the virus continuously (Alexander, 1988; Kamaraj, 1993). The panzootic Newcastle disease outbreak in 1969-73 in Europe and North America has been linked to NDV isolated from backyard chicken (Allan *et al.*, 1978). Traditional village flocks seldom receives ND vaccination and are known to carry different NDV strains (Buxton and Frazer, 1978). India has about 172.6 millions of Non-descript, otherwise known as desichicken which is nearly 68% of its total poultry population as per 1987 census (Indian poultry Industry year book, 1994). It has been observed that all these desi birds are reared in backyard as a means of household poultry farming. It has been pointed out by several workers that, these backyard poultry could transmit the disease and act as

reservoirs of NDV (Allan *et al.*, 1978; Majiyagbe and Nawathe, 1981; Bell and Mouloudi, 1988; Kamaraj, 1993). Since vaccination is the only means of controlling the disease, many vaccines are being tried and complete eradication of this disease still remains a question.

Although several factors are responsible for such a scenario, the role of desi chicken as carriers of virulent viruses could be one of the reasons for the existence of this disease as it has been rightly pointed out by Higgins and Shortridge (1988).

At this juncture, it has become necessary to assess the role played by desi chicken in the spread of Newcastle disease. The mere presence of Newcastle disease virus in the system and its excretion may not pose a serious threat as long as the virus present is a vaccine virus. However, there are many instances wherein the Newcastle disease virus isolated from apparently healthy desi chicken were of velogenic in nature (Kamaraj, 1993) necessitating a thorough study on the nature of the NDV present in desi chicken.

Velogenicity of Newcastle disease virus isolates is assessed by a combination of tests like Mean Death Time in embryonated eggs, Intracerebral pathogenicity index in day old chicken and Intravenous pathogenicity index in six weeks old susceptible chicken (Allan *et al.*, 1978). Although other tests like agglutination of mammalian erythrocytes, chicken brain cell adsorption and stability of haemagglutinins at 56°C (Alexander, 1988) are also used to assess the virulence of an isolate to some extent, at times they do not reveal the true nature of the virus.

Antigenic diversity among NDV has also been demonstrated by classical serological methods, Polypeptide analysis (Kumanan, 1989), Monoclonal antibodies (Russel and Alexander, 1983), Oligonucleotide finger printing (McMillan and Hanson, 1982; Palmeiri, 1989) and using cell culture system (King, 1993). With the advent of the molecular techniques, oligonucleotide probes have been constructed and used in the detection and differentiation of velogenic NDV from lentogenic NDV (Jarecki Black, *et al.*, 1992; Jarecki Black and King, 1993).

Under these circumstances, the present study has been undertaken to isolate and characterize NDV strains from desi chicken in Tamil Nadu with the following objectives.

i. To isolate and identify NDV strains from desi chicken.

ii. To characterize the NDV isolates by conventional methods.

iii. To differentiate the NDV isolates using oligonucleotide probe.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1 HISTORY OF NEWCASTLE DISEASE

Newcastle disease (ND) was the name given to a highly pathogenic disease noticed in chicken in England by Doyle in 1926. In the same year, the disease also emerged in the Island of Jawa, Indonesia and Korea (Doyle, 1927). In India, the disease was first reported in the year 1927 in Ranikhet, Utterpradesh (Edwards, 1928). Since then, many reports are available on the outbreak of ND in other parts of the country and the globe.

Beard and Hanson (1981) classified the various forms of ND based on the clinical signs and mortality pattern of the affected flock into five pathotypes:-

(i) Viscerotrophic velogenic form (VV) - high mortality with intestinal lesions.

(ii) Neurotrophic velogenic (NV) - high mortality following nervous signs.

(iii) Mesogenic - low mortality, respiratory and nervous signs.

(iv) Lentogenic - mild or inapparent respiratory infections.

(v) Asymptomatic - enteric - inapparent intestinal infection.

Alexander and Allan (1973) however observed that these groupings were indicators of variable nature of the disease and guide to clinical signs suspicious of ND.

2.1.1 NEWCASTLE DISEASE IN DESI BIRDS

Newcastle disease has got great economic and ecologic impact on pet and free living as well as domestic birds. In many instances, the desi chicken do not exhibit the classical form of ND and the loss due to mortality is poorly appreciated. Velogenic Newcastle disease virus (VNDV) strains have been isolated from roaming birds and village chicken flocks and desi birds were reported to be reservoir of virulent NDV. (Majiyagbe and Nawathe, 1981; Alexander, *et al.*, 1987; Bell and Mouloudi, 1988; Kamaraj, 1993).

2.1.2 NEWCASTLE DISEASE IN WILD BIRDS

There have been many instances of the isolation of Newcastle disease virus (NDV) from feral birds. About 117 species of birds covering seventeen of the twentyfour orders of the class aves have been shown to be infected with NDV (Luthgen, 1981). Wild birds were reported to carry strains of NDV but their role in epidemiology of the disease was unknown (Alexander, 1988). NDV has been isolated from Doves (Ojeh and Okoro, 1992; Arshad *et al.*, 1994), Parrots (Panigrahy *et al.*, 1991; Arshad *et al.*, 1994), wild ducks (Kawamura *et al.*, 1987), Pigeons (Buonavoglia, *et al.*, 1991; Sulochana and Mathew, 1991), Quails (Kumanan and Venkatesan, 1991; Sridhar *et al.*, 1994), Starlings and Sparrows (Arshad *et al.*, 1988), Penguins (Alexander *et al.*, 1989), Ostriches (Samberg *et al.*, 1989; Huchzermeyer and Gerdes, 1993), Pheasants (Capua *et al.*, 1994) and many of the waterfowls including ducks, geese, cormorants, pelicans and ring billed gull (Nettles, 1991; Hlinak *et al.*, 1992; Wobeser *et al.*, 1993; Banergee *et al.*, 1994). Free roaming wild feral birds and

water fowls were found to be richest source of NDV and help in spread of the disease (Alexander, 1988).

2.2 ISOLATION AND IDENTIFICATION OF NEWCASTLE DISEASE VIRUS (NDV)

2.2.1 ISOLATION OF NEWCASTLE DISEASE VIRUS

2.2.1.1 COLLECTION OF SAMPLES

Successful isolation of NDV has been most frequently obtained from samples taken from either respiratory or intestinal tracts, cloacal swabs or droppings, regardless of clinical signs (Lancaster, 1966). Other samples taken during postmortem examinations should relate to the clinical signs and organs obviously affected. NDV isolates were reported to be fairly stable in non-putrifying tissues, organ samples and faeces, provided they are not exposed to high temperature. Bone marrow has also been reported as a useful sample for the isolation of NDV (Omojola and Hanson, 1986). Brain tissue from the infected bird was considered as a richest source of virus. It has also been reported that respiratory tracts acted as a permanent reservoir of the virus (Beach, 1943; Karzon and Bang, 1951). Vaccine and field strains of NDV were isolated from eggs also (Bivins *et al.*, 1950).

2.2.1.2 ISOLATION TECHNIQUES

Embryonated chicken eggs were the most widely used system for NDV isolation. However, successful isolations were also obtained with the use of the embryonated duck eggs and tissue culture systems (Beard and Hanson, 1981).

Initially, unfiltered suspensions of the samples were used as inocula without adding antibiotics. Later filtered suspensions were used as inocula (Iyer and Dobson, 1940; Beach, 1943). Beaudette *et al.* (1949) recommended the addition of antibiotics to the inocula to eliminate contaminants. Nine to eleven days old embryonated chicken eggs from birds free of NDV antibodies were found to be ideal for the successful isolation of NDV (Alexander, 1988).

As far as the route of inoculation is concerned, although allantoic cavity route is commonly employed for virus isolation, other methods like chorio allantoic membrane inoculation, intravenous inoculation and yolk sac inoculation were also tried (Beaudette *et al.*, 1952). A combination of chorio allantoic membrane and allantoic sac inoculation yielded better results in the primary isolation of the virus (Fabricant, 1957). In few cases several blind passages were needed since some degree of adaptation to the embryonated eggs was essential when the virus content of the inoculum was low (Beaudette *et al.*, 1948).

2.2.2 IDENTIFICATION OF NEWCASTLE DISEASE VIRUS

Identification of NDV isolates could be established based on the lesions on the embryos, haemagglutinating (HA) ability with chicken erythrocytes, inhibition of haemagglutination with known NDV antiserum and virus neutralization tests in embryonated chicken eggs (Brandly *et al.*, 1946; Lancaster, 1963).

2.2.2.1 LESIONS ON THE EMBRYOS

Lesions produced on the embryonated chicken eggs varied according to the strain of the virus inoculated. Velogenic strains produced small or large haemorrhages on the legs and wings, over the cranium and on the dorsal surface of the body (Iyer and Dobson, 1940 ; Iyer, 1943; Jungherr *et al.*, 1946). However, lentogenic strains seldom produced haemorrhages, but resulted in stunted growth with embryonic mortality commencing on the fourth and succeeding days (Hitchner *et al.*, 1951).

2.2.2.2 HAEMAGGULTINATION (HA) TEST

The property of NDV to agglutinate chicken erythrocytes has been widely employed for identification purposes. However, care should be taken to rule out other viruses like paramyxoviruses -3, egg drop syndrome virus-76 and avian influenza virus which also agglutinate chicken erythrocytes (Lush, 1943, Brandly *et al.*, 1946). Though different NDV strains have shown some variations in their HA property, this method has been serving as a rapid method for NDV identification. Use of potassium periodate has been found to increase the reading time of HA test (Lancaster, 1963). Haemagglutination test followed by haemagglutination inhibition test has been used to identify various isolates of Newcastle disease virus.

2.2.2.3 HAEMAGGLUTINATION INHIBITION (HI) TEST

Haemagglutination Inhibition test with specific antisera has found to be an useful, confirmatory diagnostic tool in the identification of NDV isolates qualitatively and quantitatively. (Brandly *et al.*, 1946; Hanson, 1972). However, the

antigen-antibody incubation period was found to be more critical in performing this test (Brugh *et al.*, 1978).

2.2.2.4 VIRUS NEUTRALIZATION TEST (VNT)

Virus neutralization test has been reported to be one of the confirmatory test for identifying the viral etiology. It has been found that a mixture of NDV and NDV antiserum, when inoculated into embryonated chicken eggs, did not kill the embryos. This test was found to be useful in differential diagnosis and to identify the field strains. VNT has been successfully employed for identification and confirmation of NDV isolates from various sources (Keogh, 1937; Beach, 1942; Cunningham, 1966; Hanson, 1972; Janakiram, 1983; Lakshminarayanan, 1983; Kumanan, 1989; Vijayarani, 1990; Kamaraj, 1993).

2.3 CHARACTERIZATION OF NDV ISOLATES

Newcastle disease virus isolates have been broadly classified as velogenic, mesogenic and lentogenic, depending upon the nature of the clinical manifestation (Hanson *et al.*, 1949). It has been observed that several viruses submitted to the International Reference Laboratory, Weybridge were found to be apathogenic, despite their isolation from birds with clinical signs similar to those produced by virulent strains of NDV (Alexander *et al.*, 1987). This has necessitated the local or international requirement for an assessment of virulence. Generally, field isolates of NDV were differentiated by the virulence and strain differentiating characters (Hanson and Brandly 1955; Schloar and Hanson, 1971; Lancaster and Alexander, 1975; Allan *et al.*, 1978; Vijayarani *et al.*, 1992; Kumanan and Venkatesan, 1991; Kamaraj, 1993).

2.3.1 VIRULENCE CHARACTERS

Currently the *in vivo* tests like Mean Death Time (MDT) in embryonated chicken eggs, Intra Cerebral Pathogenicity Index (ICPI) in day old chicks or mice and Intravenous Pathogenicity Index (IVPI) in 6 weeks old susceptible chicken were employed for assessing virulence of NDV isolates (Alexander and Allan, 1973; Janakiram, 1983; Kumanan, 1989).

2.3.1.1 MEAN DEATH TIME

Mean Death Time (MDT) was originally described by Hanson and Brandly (1955) to differentiate NDV strains as Velogenic, Mesogenic and Lentogenic based on the mean time required to kill chicken embryos. NDV strains which could kill embryos within 40-60 hrs were classified as velogenic 60-90 hrs as mesogenic and more than 90 hrs as lentogenic. However, some velogenic strains killed embryos within 40 hours. Several Indian isolates were examined for virulence and pathogenicity by assessing the MDT in chick embryos (Singh and Singh, 1970; Kumanan, 1989; Kumanan *et al.*, 1992; Kamaraj, 1993). Though the test was considered as an important tool in characterising different isolates, it has been reported to be imprecise, particularly when used to characterise isolates from hosts other than chicken (Alexander, 1988).

2.3.1.2 INTRACEREBRAL PATHOGENICITY INDEX

Intracerebral Pathogenicity Index (ICPI) in day old chicks refers to the neurovirulence of the isolates and the intracerebrally inoculated chicks were observed for 8 days (Hanson and Brandly, 1955). The index was assessed by giving appropriate scores depending on the disease signs developed or death after inoculation (Allan *et al.*, 1978). The index for lentogenic strains ranges between 0 and 0.25, mesogenic strains 0.8 and 1.46 and velogenic strains 1.75 and 2.0 (Hanson, 1956; Singh and Singh, 1970; Vijayrani, 1990; Kamaraj, 1993). Several authors have modified the test procedure by increasing the volume of inoculum or the observation period (Toth, 1965; Hanson, 1975). In general, ICPI has found to have the drawback in distinguishing the low and high virulence for the single reason that even the moderate virulent strains like Komorov and Mukhteswar would give ICPI values in excess of 1.5 and more and time of onset would result in marked differences for viruses of low virulence (Alexander, 1988). Hence a combination of ICPI, IVPI and MDT could be used for assessing the correct picture about the virulent nature of the isolate (Martone *et al.*, 1974).

Intracerebral pathogenicity index in mice has been generally used for differentiating viscerotrophic strains from neurotrophic strains (Upton, 1955; Tanwani, 1974; Vijayarani, 1990).

2.3.1.3 INTRAVENOUS PATHOGENICITY INDEX

Intravenous pathogenicity index (IVPI) involves the inoculation of infective allantoic fluid intravenously into NDV antibodies free six week old chicken (Allan

et al., 1978). IVPI was the mean score per bird per observation over a period of 10 days. The IVPI for different strains ranged from 0-2.8. For lentogenic strains, the value was 0. For mesogenic strains, 0-0.8 and for velogenic strains 1.70-2.8 (Lancaster, 1962; Janakriram, 1983; Kumanan, 1989; Vijayarani, 1990; Kamaraj, 1993). Swabing of the conjunctiva and cloaca with infective allantoic fluid was also performed to replace intravenous inoculation in order to detect viscerotropic NDV (Hanson, 1980).

2.3.2 STRAIN DIFFERENTIATING CHARACTERS

Although strain differentiating characters were not clear indicators of the virulence, they were found to play a vital role in assessing the nature of the isolate atleast to a little extent (Bell, 1986).

2.3.2.1 STABILITY OF HAEMAGGLUTININS AT 56°C

This property was used as a genetic marker in differentiating the NDV strains. It has been reported that lentogenic strains are found to be stable at 56°C for 5 to 10 min (Hanson *et al.*, 1949). Mesogenic strains like R_2B had a thermostability of less than 30 min (Chandra *et al.*, 1972). Virulent strains were stable even after 270 min exposures to 56°C (Estola, 1974). However, many authors have found that thermostability was not related to the infectivity of the isolate (Kohn and Fuchs, 1969).

2.3.2.2 HAEMAGGLUTINATION OF MAMMALIAN ERYTHROCYTES

Newcastle disease virus isolates were found to agglutinate erythrocytes of Bovine, Ovine, Swine and Equine species, apart from Human 'O' erythrocytes

(Winslow *et al.*,1950; Liu, 1952). This property was also used to differentiate NDV strains to some extent. However, variations have been reported among different strains of NDV in agglutinating equine erythrocytes. It has been found that only a few laboratory adapted lentogenic strains had the ability to agglutinate equine erythrocytes (Lancaster, 1962: Hanson *et al.*, 1967; Hanson, 1975). Vrtiak *et al.* (1960) observed that haemagglutination capacities of two strains of NDV, one grown in tissue culture and other in chick embryo were similar. This technique has been used to differentiate NDV strains isolated from commercial poultry (Vijayarani, 1990), desi birds (Kamaraj, 1993) and Japanese quails (Kumanan and Venkatesan, 1991).

2.3.2.3 ADSORPTION OF HAEMAGGLUTININS BY CHICKEN EMBRYO BRAIN CELLS

Piraino and Hanson (1960) first reported this test for differentiation of NDV strains. Since then many authors had used this test to differentiate the NDV isolates. Strains like Lasota, F and GAL were adsorbed well where as B_1 was unaffected by the chicken embryo brain cells (Hanson *et al.*, 1967). Similarly, the adsorption of mesogenic 'K' strain was also found to be poor (Tanwani, 1974). However, the adsorption of velogenic strains like Texas GB and CDF 66 were found to be good (Chowdhury *et al.*, 1966; Welch *et al.*, 1970).

2.3.2.4 STABILITY OF INFECTIVITY AT pH3

It has been observed that some strains of NDV are stable at pH3 for even four hours (Hanson *et al.*, 1967). However, this phenomenon had no relevance with the

virulence of these strains studied. Similar observations were also made for strains like F,K,CDF 66 and R_2B (Tanwani, 1974; Rajan Samuel, 1977).

2.3.2.5 AGGLUTINATION OF CHICKEN LYMPHOCYTES

Different strains of NDV were found to agglutinate and lyse chicken lymphocytes and anti-NDV sera inhibited the agglutination of chicken lymphocytes by NDV. This test was compared with HA test for characterising field isolates of NDV and it was found that HA test was easier to perform and more accurate (Lam and Hao, 1987; Kumanan, 1989; Vijayarani, 1990).

2.4 USE OF CELL CULTURE SYSTEMS FOR CHARACTERIZATION

Primary chicken embryo fibroblast (CEF) cell culture system was used to study the plaque morphology to differentiate various NDV strains (Daniel and Hanson, 1968; Estupinan and Hanson, 1969). Virulent strains, usually formed larger plaques whereas mesogenic and lentogenic strains produced smaller plaques (Daniel and Hanson 1968; Park *et al.*, 1988). Velogenic strains produced eosinophilic inclusion bodies in chicken embryonic cells (Mateva, 1962), fusion of cells as early as four hours after infection and large polykaryocytes were formed by 8 hours (Alexander and Allan, 1973). Mesogenic strains like Mukhteswar and R_2B produced changes in the monolayer only from third passage onwards with acidophilic granules in the cytoplasm of cells (Chandra *et al.*, 1972; Ramana and Sethi, 1976).

Recently, MDBK cell line has been used to identify the virulent subpopulation of NDV (King 1993). It was reported that velogenic strain (Texas GB) overgrows in

MDBK cell line as compared to lentogenic strain (Lasota) and could be used as an aid in detecting the virulent subpopulations.

It was also reported that cell line from bovine embryonic Kidney (BS/BEK) supported the growth of lentogenic Lasota virus even after third passage as compared to other cell lines like BHK_{21}, HEP_2 and QT-35 (Losio *et al.*, 1995).

2.5 DIAGNOSIS AND DIFFERENTIATION OF NEWCASTLE DISEASE VIRUS STRAINS

2.5.1 CONVENTIONAL TESTS

A battery of tests including plaque type and size, haemagglutination elusion rate, haemagglutinin's thermostability, ability to agglutinate equine erythrocytes, haemagglutination with chicken erythrocytes and hemagglutination inhibition with NDV antisera were used to characterize and distinguish between various strains of NDV. Apart from these tests some authors had also studied the biochemical properties of the infected allantoic fluid for differentiating NDV strains (Alexander, 1988; Dhir *et al.*, 1988).

2.5.2 CURRENT TECHNIQUES

The current techniques that were employed for the diagnosis of the Newcastle disease and differentiation of viral strains include Enzyme linked immunosorbent assay (ELISA), Polypeptide analysis by polyacrylamide gel electrophoresis (PAGE), Monoclonal antibody typing, Oligonucleotide finger printing, Nucleic acid sequencing, Polymerase chain reaction and use of nucleic acid probes.

2.5.2.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme linked immunosorbent assay with polyclonal antisera was widely practiced for the detection of NDV antigen. ELISA has been also widely used to detect NDV antibodies in the sera. A rapid method of detecting NDV antigens by dot ELISA has been developed and used (Oberoi *et al.*, 1993; Thirumurugan, 1995). A Micro ELISA technique was developed to detect NDV antibodies in chicks. This technique was reported to be simple and highly sensitive in detecting the NDV antibodies (Parimal Roy and Padmanaban, 1991).

2.5.2.2 POLYPEPTIDE ANALYSIS WITH POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Several workers have fractionated NDV proteins by Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS - PAGE) for differentiating various strains. Polypeptide finger printing has been used for strain characterization and could be considered as a technique for tracing specific epizootic virus (Nagy and Lomniczi, 1984). Haslam *et al.*(1969)in their earlier studies showed that NDV virions contained three major proteins 80 kd, 54 kd and 38 kd and similar findings were reported by Alexander and Reeve (1972). Mount castle *et al.*, (1971) found that NDV had six proteins, two of which were glycoproteins. Then it was found that one of the NDV proteins of size 53-56 kd was formed from a larger precursor protein of 67 kd by a post translational cleavage event. The proteins of a non-pathogenic strain of NDV were separated into eight fractions by electrophoresis in 7.5% polyacrylamide gel and they comprised of three glycoproteins, three lipoproteins and one haemagglutinating protein (Nachkov *et al.*, 1972). Structural proteins of 14 strains of NDV were studied

by SDS-PAGE and the results revealed no differences between the strains (Moore and Burke, 1974). Glycoprotein F was found in all the strains and responsible for haemolysis and cell fusion (Nagai *et al.*, 1980). SDS-PAGE analysis of NDV field isolates revealed a total of eight fractions comprising of three major and five minor proteins (Kumanan, 1989; Kumanan *et al.*, 1994). Guo *et al.* (1987) subjected 2 vaccine strains of NDV to PAGE and found no structural differences. Three local isolates of NDV were characterized by SDS - PAGE and it was found that NDV 1 contained six proteins NDV 2 and NDV 3 had five proteins each. The five proteins were common to all the isolates and the sixth which was found in NDV 1 had the highest molecular weight of 89 kd (Vijayarani *et al.*, 1992).

2.5.2.3 MONOCLONAL ANTIBODIES IN NDV CHARACTERIZATION

Monoclonal antibodies (MAbs) are widely used for diagnosis and to study the epizootiology of the Newcastle disease (Alexander *et al.*, 1987). Monoclonal antibodies produced by Russel and Alexander (1983), have proven to be useful in differentiating and grouping of NDV isolates as vaccine and field strains (Srinivasappa *et al.*, 1986; Meulemans *et al.*, 1987).

Twenty MAb's were prepared against velogenic TexasGB strain of NDV and type-1 pigeon paramyxovirus (Lana *et al.*, 1988). Eight MAbs developed against Ploufragan strain of NDV, were used to characterise fifty eight virus strains including twenty nine french isolates (Jestin *et al.*, 1989). Parede *et al.* (1992) reviewed the structural variation and antigenic variation of NDV isolates. A blocking ELISA using MAb's was designed to characterize Australian isolates of NDV (Dellaporta and Spencer, 1989). A sensitive avidin biotin amplified fluorogenic ELISA using

biotinylated MAbs for the identification of NDV was developed by Wong *et al.* (1991). Eleven field isolates from India were characterised as virulent strains using MAbs in an immunoperoxidase test (Kumanan *et al.*, 1992). Similarly five isolates from desi chicken were characterized as velogenic C_1 type by MAbs (Kamaraj, 1993).

2.5.2.4 OLIGONUCLEOTIDE FINGERPRINTING

RNA oligonucleotide finger printing has been proposed as a method of identifying strains of NDV, not distinguishable by other tests. Oligonucleotides from T_1-RNAse digests of NDV was resolved by two dimensional PAGE (McMillan and Hanson, 1980). RNA oligonucleotide fingerprinting was employed as a means of differentiating five velogenic NDV strains (Mcmillan and Hanson, 1982). The authors latter in 1986, distinguished six viral clones of Hickman strain from each other. Use of two dimensional PAGE was found to be advantageous over one dimensional PAGE for oligonucleotide fingerprinting. The two dimensional PAGE had high resolution, low input radiation and biohazard considerations (Palmieri and Perdue, 1989). Oligonucleotide fingerprinting was used to findout the genetic relationship among five strains of NDV (B1, England F_1, Nebraska, Queensland V_4, and Ulster) and was found to be useful in characterizing the NDV isolates (Palmieri, 1989). Three NDV isolates were characterized by oligonucleotide fingerprinting and no detectable difference was observed among them (Vijayarani, 1990).

2.5.2.5 USE OF NUCLEIC ACID PROBES

A synthetic oligonucleotide DNA, termed as NDV probe was used to detect 14 NDV isolates in a slot blot hybridization assay. The probe was generated from a

highly conserved region of NDV genome and found to be highly specific than the probe from cloned DNA fragments (Jarecki Black *et al.*, 1992). A synthesized oligonucleotide probe termed as Newcastle disease cleavage probe (NDV-CL) was used to complement the cleavage activation site of F gene of Texas GB strain of NDV. This probe, 21 bases in length, bound with RNA isolated from velogenic NDV strains in a slot blot hybridization assay. However, this probe recognised mesogenic strains also and did not give any signal when bound to lentogenic strains (Jarecki Black and king, 1993).

2.5.2.6 MISCELLANEOUS TECHNIQUES

2.5.2.6.1 WESTERN BLOT ANALYSIS

Western blot analysis of NDV proteins to find out specific variation in membrane glycoprotein precursors with antipeptide antibodies was developed (Gormon *et al.*, 1992; Hodder *et al.*, 1993). Field isolates of NDV were characterized using antipeptide antibodies in a western blot analysis (Hodder *et al.*, 1994).

2.5.2.6.2 POLYMERASE CHAIN REACTION (PCR)

Polymerase Chain Reaction was used to identify the NDV antigen in allantoic fluids of embryonated eggs (Jestin and Jestin, 1991).

Seal *et al.* (1995) has characterized NDV isolates by RT-PCR, coupled to nucleotide sequencing. They have created sequence data base for pathotyping and molecular epidemiological analysis of NDV isolates.

2.5.2.6.3 SEQUENCING OF NUCLEIC ACIDS

Nucleotide sequencing is an useful aid in identifying the mutants which differ by base substitution at one or few places. Millar *et al.* (1988) have sequenced 'F' and 'HN' glycoprotein gene of Ulster strain of NDV to assess the molecular basis for variation in pathogenesis among strains. Pigeon paramyxo virus-1 isolate was successfully sequenced to find out the variations, if any. Deduced aminoacid sequences were found to be different among different strains of NDV and could be used in the differentiation of various NDV isolates (Collins *et al.*, 1993; Collins *et al.*, 1994).

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 BIRDS

Day old Chicks

Day Old White Leghorn (WLH) male chicks were obtained from Poultry Research Station (PRS), Nandhanam, Madras and used for conducting ICPI tests.

Six Weeks old chicks

Six Weeks Old WLH male chicks, tested for the absence of NDV antibodies were obtained from Vaccine Research Centre, Centre for Animal Health Studies, Madhavaram and used for IVPI tests.

Adult Vaccinated Chicks

Ten to twelve weeks old chicks were obtained from PRS, Nandhanam, Madras and used for raising hyperimmune serum against NDV.

3.1.2 BIOLOGICALS

3.1.2.1 HYPERIMMUNE SERUM

Hyperimmune serum against NDV was raised in chicks at the Department of Animal Biotechnology, Madras Veterinary College, Madras, as per standard procedures.

3.1.2.2 VACCINES

Ranikhet disease vaccines, both F and K were obtained from the Institute of Veterinary Preventive Medicine, Ranipet and used for raising hyperimmune serum.

3.1.3 CHEMICALS AND REAGENTS

3.1.3.1 ALSEVER'S SOLUTION

Sodium chloride (AR, BDH)	:	4.20 g
Tri sodium citrate (AR, BDH)	:	8.00 g
Glucose (AR, BDH)	:	20.50g
Citric acid (AR, BDH)	:	0.55 g
Triple glass distilled water	:	1000ml

The pH was adjusted to 7.2 and sterilized at 110°C for 10 minutes and stored at 4°C.

3.1.3.2 PHOSPHATE BUFFERED SALINE (PBS)

Sodium chloride (AR, BDH)	:	8.000g
Disodium hydrogen phosphate (GR, Sarabhai)	:	1.150g
Potassium chloride (AR, BDH)	:	0.200g
Potassium dihydrogen phosphate (AR, Glaxo)	:	0.200g
Distilled water	:	1000ml

The pH was adjusted to 7.2 and sterilized at 121°C for 15 minutes.

3.1.4 EMBRYONATED CHICKEN EGGS

Nine to eleven days old embryonated chicken eggs obtained from PRS, Madras were used for the isolation and propagation of the NDV isolates.

3.1.5 ERYTHROCYTES

3.1.5.1 CHICKEN ERYTHROCYTES

Chicken blood was collected by wing vein puncture in Alsever's solution and washed three times in PBS. 0.5 per cent suspension of chicken erythrocytes was used for HA and HI tests.

3.1.5.2 MAMMALIAN ERYTHROCYTES

One per cent suspension of cattle, horse, goat and human 'O' erythrocytes was prepared by collecting blood in Alsever's solution and washed thrice in PBS.

3.1.6 CHICKEN EMBRYO BRAIN CELLS

Ten per cent brain cell suspension was prepared from 12 days old chicken embryos in PBS under sterile conditions and used in chicken brain cell adsorption tests.

3.1.7 CELL CULTURE MEDIA AND REAGENTS

3.1.7.1 ANTIBIOTIC STOCK SOLUTION

Benzyl penicillin 10 lakhs unit x 1 vial (Alembic Chemicals Ltd., Baroda)

Streptomycin sulfate 1g x 1 vial (Sarabhai Chemicals Ltd, Baroda)

The contents of the vials were dissolved in sterile distilled water and made upto 100ml, checked for sterility and stored at 4°C until use.

3.1.7.2 GROWTH MEDIUM

Growth medium for MDBK cell line was prepared as given below

Minimum essential medium (MEM) with Earle's salts (Sigma, USA)	:	14.2g
Goat serum	:	100ml
Antibiotic stock solution	:	10ml

Sterile TGDW to make upto 1000ml

The pH of the medium was adjusted to 7.4 with 8.5 percent sodium bicarbonate solution and sterilised by filtering through membrane filters (Sartorius, Germany) with a pore size of 0.22 μm.

3.1.7.3 MAINTENANCE MEDIUM

Maintenance medium was prepared by mixing,

MEM with Earle's salts (Sigma, USA)	:	14.2 g
Goat serum	:	20ml
Antibiotic stock solution	:	10ml
Seterlie TGDW	:	1000ml

The pH of the medium was adjusted to 7.6 with sodium bicarbonate (8.5.%) and sterilised by filtering through 0.22μm membrane filter.

3.1.7.4 TRYPSIN, VERSENE, GLUCOSE MIXTURE

Trypsin, Versene and Glucose mixture was prepared by dissolving the following in 100ml of TGDW and sterilised by passing through membrane filters of 0.45μm pore size and stored at 4°C.

Sodium Chloride (AR, CDH)	:	0.8g
Potassium Chloride (AR, Qualigens)	:	0.02g
Disodium hydrogen phosphate (AR, Merck)	:	0.115g
Potassium dihydrogen phosphate (AR, Merck)	:	0.02g
Glucose (AR, Glaxo)	:	0.05g
Sodium bicarbonate (AR, Fischer)	:	0.058g
Trypsin 1:250 (Difco, USA)	:	0.25g
Phenol Red (Sigma, USA)	:	0.001g

3.1.8 MATERIALS REQUIRED FOR RNA EXTRACTION (Chomczynski, 1994)

3.1.8.1 DEPC TREATED TRIPLE GLASS DISTILLED WATER

TGDW was treated with diethylpyrocarbonate (DEPC) (Sigma, USA) overnight at a concentration of 0.01% and autoclaved. DEPC treated water was used in all the RNA extraction work and hybridisation reactions.

3.1.8.2 SOLUTION D

Guanidinium thiocyanate (Sigma, USA)	:	4M
Sodium Citrate pH7	:	25mM
Sarcosyl (Sigma, USA)	:	0.5%
2,Mercapto ethanol (Sigma, USA)	:	0.1M

3.1.8.3 WATER SATURATED PHENOL (Rubert. E. Farrel, 1993)

Phenol (Molecular biology grade) was distilled and stored in aliquots at -20°C. When needed, phenol was melted in a 65°C water bath, mixed with equal volume of DEPC treated water. The mixture was allowed to separate into two liquid phases and the excess water was discarded.

3.1.8.4 CHLOROFORM: ISOAMYL ALCOHOL MIXTURE

Chloroform (AR,BDH)	:	98ml
Isoamylalcohol (AR, BDH)	:	2ml

3.1.8.5 2M SODIUM ACETATE pH4

Dissolved 16.42g of sodium acetate (anhydrous) in 40ml of water and 35ml of glacial acetic acid. Adjusted the solution to pH4 with glacial acetic acid and the final volume to 100 ml with water.

3.1.8.6 10X MOPS BUFFER

MOPS (3,N Morpholino propane sulphonic acid)	:	0.2M
Sodium acetate	:	0.05M
EDTA (Ethylene diamine tetra acetic acid)	:	0.01M

From this 5x Mops buffer was prepared and used for RNA denaturation.

3.1.9 MATERIALS REQUIRED FOR HYBRIDIZATION WITH RADIO - LABELLED PROBE

3.1.9.1 5' DNA TERMINUS LABELLING SYSTEM

5' DNA terminus labelling system obtained from M/s Life technologies inc., USA was used.

3.1.9.2 ISOTOPE $\gamma^{32}P$ - ATP

$\gamma^{32}P$ ATP purchased from BRIT, Hyderabad at a concentration of 1 mCi/ml was used to label the oligonucleotide.

3.1.9.3 OLIGONUCLEOTIDE PROBE

A synthetic 21mer oligonucleotide with the following sequence was obtained from M/S Bangalore Genei, Bangalore and used as the probe sequence.

5' TGT CGC CGG CGT CGA GAC TAT 3'.

3.1.9.4 SALINE SODIUM CITRATE STOCK SOLUTION (20X)

Sodium citrate (AR, BDH)	:	0.3M
Sodium Chloride (AR, BDH)	:	3 M

3.1.9.5 DENHART'S SOLUTION

Ficoll	:	1g
Polyvinyl pyrolidone	:	1g
Bovine serum albumin	:	1g

Volume made upto 100ml

3.1.9.6 HYBRIDIZATION BUFFER

Saline sodium citrate solution	:	5x
Sodium phosphate (Monobasic)	:	20mM
SDS	:	4%
Denhart's solution	:	10x
Denatured salmon sperm DNA	:	80 μg/ml

3.1.9.7 WASHING BUFFERS

A. 3x SSC with 1% SDS

B. 1x SSC with 1% SDS

3.2 METHODS

3.2.1 ISOLATION OF NDV

3.2.1.1 COLLECTION OF SAMPLES

Cloacal swabs were collected from apparently healthy and ND suspected birds brought for vaccination at the Department of clinics, Madras Veterinary College, Madras-7. The samples were transported in phosphate buffered saline and stored at 4°C untill further processing.

3.2.1.2 PREPARATION OF INOCULUM

The Cloacal swabs were squeezed out under sterile conditions into the PBS solution and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was treated with 250 IU/ml of penicillin and 250 μg/ml of streptomycin and used for virus isolation attempts.

3.2.1.3 VIRUS ISOLATION (Allan *et al.*, 1978)

The suspected samples were inoculated into the allantoic cavity of 9 day old embryonated chicken eggs and incubated at 37°C. Candling was done at regular intervals. Death of any embryo within 24 hours was considered as non-specific and the observation was continued for 5 days. The dead embryos were chilled at 4°C overnight to ensure that the blood vessels were empty for the harvest. Embryos which did not die even after 5th day were also chilled at 4°C.

3.2.1.4 HARVESTING AND STORAGE

After chilling, the Amnio Allantoic Fluid (AAF) was collected from each embryo using pasteur pipettes and a spot HA was carried out with 10 per cent washed chicken erythrocytes in a "Gouche" plate. Amnio allantoic fluid collected from several eggs was labelled and stored irrespective of the fact whether they agglutinated chicken erythrocytes or not. All dead embryos were examined for the presence of characteristic NDV lesions.

3.2.1.5 BLIND PASSAGES

The AAF samples which were not agglutinating chicken erythrocytes after first passage were subjected to two more blind passages in embryonated eggs. Spot HA test was carried out at each passage level and the samples which did not show HA activity even after third passage were considered as negative and discarded. The samples which had HA activity were stored at -20°C for subjecting to other confirmatory tests.

3.2.2 IDENTIFICATION OF THE VIRUS

3.2.2.1 LESIONS ON EMBRYOS

Embryos which died after inoculation with the suspected materials were examined for the presence of characteristic gross lesions like haemorrhages on legs, wings, dorsal surface of the body and cranium. (Iyer, 1943; Jungherr *et al.*, 1946).

3.2.2.2 HAEMAGGLUTINATION TEST (HA)

Haemagglutinating activity of the isolates was assessed using freshly collected AAF from dead embryos as per the method of Cunningham (1966) using 0.5 per cent chicken erythrocytes.

3.2.2.3 HAEMAGGLUTINATION -INHIBITION (HI) TEST

Haemagglutination inhibition test was conducted to find out the specificity of the freshly collected AAF using specific NDV antiserum as described by Cunningham (1966).

3.2.2.4 VIRUS NEUTRALIZATION (VN) TEST

Virus neutralization test was conducted as described by Cunningham (1966) making use of known NDV antiserum employing various NDV isolates in nine day old embryonated chicken eggs.

3.2.3 CHARACTERIZATION OF THE VIRAL ISOLATES

3.2.3.1 VIRULENCE CHARACTERS

3.2.3.1.1 MEAN DEATH TIME (MDT) (Allan *et al.*, 1978)

Ten fold serial dilutions of the freshly harvested samples were made in sterile physiological saline from 10^{-1} to 10^{-9}. Three eggs per dilution were inoculated with 0.1ml of the virus material in the morning and marked as 'A' batch and another batch of eggs were inoculated in the evening with the same viral dilution kept at 4°C and marked as 'B'. All the eggs were incubated at 37°C and candled twice daily and the

time of deaths of embryos were recorded. The AAF of dead embryos were tested with chicken erythrocytes by spot test for the presence of HA activity. The observations were continued for seven days and at the end, all the remaining embryos were chilled and spot tested for HA activity. The highest dilution at which all the embryos died in both the batches was taken as Minimum Lethal Dose (MLD) and the arithmetic mean of the time taken for the death of these embryos was taken as MDT.

Embryo lethal dose 50 per cent (ELD 50) for each isolate was calculated making use of the above test as per Reed and Muench (1938) method .

3.2.3.1.2 INTRACEREBRAL PATHOGENICITY INDEX (ICPI) (Allan *et al.*, 1978)

Fifty μl of 1 in 10 dilution of different NDV isolates were inoculated into 10 numbers of day old chicks for each isolate, at the base of the cranium using a 26 gauge, 12 mm long needle. Inoculated chicks were observed for a period of eight days for the development of symptoms and death. A group of 10 chicks inoculated in the same manner with sterile saline were kept as control.

At the end of the observation period, the total number of chicks which were normal or those which showed symptoms of paralysis or death were multiplied by factors 0,1 and 2 respectively. The values were summed up and divided by 80 to give ICPI.

3.2.3.1.3 INTRAVENOUS PATHOGENICITY INDEX (IVPI) (Allan *et al.*, 1978)

Hundred μl of 1 in 10 diluted samples were inoculated intravenously into 10 chicken of six weeks old for each isolate. Ten identical chicks were kept as control. The inoculated chickens were observed daily for a period of eight days. The recordings were made depending upon the condition of the birds as healthy, sick, paralytic or dead.

The IVPI was calculated based upon the weighted values of '0' for normal, one for sick, two for paralytic and three for death over the number of observations made.

3.2.3.2 STRAIN DIFFERENTIATING CHARACTERS

3.2.3.2.1 STABILITY OF HAEMAGGLUTININ AT 56°C (Tanwani, 1974)

One in two dilution of the fresh harvests were made in physiological saline and divided into two halves. One half was kept at 37°C and the other half at 56°C in a water bath. Samples were drawn from both the sets at regular intervals and cooled in ice bath. HA test was performed with the samples as per the method of Cunningham (1966) and the results were expressed as HA values at different time intervals.

3.2.3.2.2 HAEMAGGLUTINATION OF MAMMALIAN ERYTHROCYTES (Winslow *et al.*, 1950)

Haemagglutination test was conducted with the different isolates using one per cent suspension of cattle, goat, horse and human 'O' erythrocytes as per the method of Cunningham (1966).

3.2.3.2.3 ADSORPTION OF HAEMAGGLUTININS BY CHICKEN BRAIN CELLS (*Hanson et al.*, 1967)

A 10 per cent suspension of brain cells was prepared by using 20-30 numbers of 12 day old chicken embryos. Five hundred μl of virus sample was mixed with equal volume of the brain cell suspension and kept at 4°C for 15 minutes for adsorption to take place. The tubes were centrifuged at 5000 rpm at 4°C for 15 minutes and the supernatant fluid containing the unadsorbed virus material was collected. HA test was performed with the original and brain cell adsorbed virus suspensions simultaneously as described by Cunningham, (1966). The difference between the HA titres of the two samples represented the amount of haemagglutinins adsorbed.

3.2.4 ADAPTATION OF NDV ISOLATES TO MDBK CELLS (King, 1993)

Madin Darby Bovine Kidney cells available at the Central Tissue Culture Laboratory of this department was used for virus adaptation. Confluent monolayers of MDBK were prepared using GM in bottles and plates. Six well plastic plates containing confluent monolayers of MDBK cells were infected with 0.5 ml of 1 in 10 diluted allantoic fluid and incubated at 37°C for one hour. After virus adsorption the monolayers were rinsed with MM and fresh MM was added. The plates were incubated at 37°C and observed at 24 hrs interval for six days. All the 11 isolates along with a known lentogenic (RDVF) and velogenic (VP4 - available at the Dept. of Animal Biotechnology) NDV strains were subjected to five serial passages in MDBK cells. After each passage, the cell culture fluids were tested for HA activity using

chicken erythrocytes. Coverslip cultures infected with the isolates were stained with haemotoxylin and eosin and screened for cytopathogenic effects.

3.2.5 CHARACTERIZATION OF NDV BY MONOCLONAL ANTIBODIES (MAb)

The isolates were freeze dried and sent to Dr. D.J. Alexander, Central Veterinary Laboratory, Weybridge, United Kingdom for typing with MAb .

3.2.6 OLIGONUCLEOTIDE PROBE HYBRIDIZATION AND AUTORADIOGRAPHY

3.2.6.1 PROBE PREPARATION

A 21 mer oligonucleotide probe was used for the detection of velogenic NDV isolates. The probe was synthesised by M/s Bangalore Genei Laboratories, Bangalore. The oligos were from the conserved sequences found in most of the velogenic strains of NDV.

3.2.6.2 EXTRACTION OF RNA

As per the method quoted by Chomczynski and Sacchi (1987), RNA was extracted from NDV infected embryonic tissues like brain and spleen. Briefly, 100mg of tissue was taken to which 1ml of solution D was added. The tissue was homogenized in a teflon coated homogenizer over ice. After homogenization, 0.1ml of 2M sodium acetate, 1ml of water saturated phenol and 0.2ml of chloroform - isoamyl alcohol (49:1) were added.

After the addition of each reagent the tube was shaken for thorough mixing. The mixture was kept over ice for 15 min and centrifuged at 14,000 rpm for 20 min at 4°C. The aqueous phase over the phenol layer was collected. To this, two volumes of isopropanol was added and kept at -20°C for 1 hr. Then it was centrifuged at 14,000 rpm for 20min at 4°C. The pellet was resuspended in 0.3ml of solution D and 0.3ml of isopropanol and kept at -20°C for 1 hour. Then it was centrifuged at 14,000 rpm for 20 min at 4°C. The pellet was collected resuspended in 75% ethanol and centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was dissolved in 50μl of DEPC treated water.

3.2.6.3 LABELLING OF PROBE (Jarecki Black and King 1993)

The probe was labelled with 5' DNA terminus labelling kit (Life Technologies Inc., USA) as per manufacturer's instructions in the following manner.

- 18 μl of the oligo diluted to a concentration of 200μg/100μl was taken in an eppendorf tube and denatured at 100°C for 3 min and snap cooled over ice.
- 5 x forward reaction buffer, 5 μl was added to the oligo.
- Ten microlitre of [γ^{32}p] - ATP was added to the above mixture.
- To the above constituents 1μl of T4 polynucleotide kinase enzyme was added.

The constituents were thoroughly mixed and incubated for 30min in a 37°C water bath.

3.2.6.4 SLOT BLOT HYBRIDIZATION (Jarecki Black and King, 1993)

The RNA samples were denatured with denaturing solution (RNA 5μl, 37% formaldehyde 3.3 μl, Formamide 10μl, 5x MOPS 2μl) at 65°C for 3min and snap cooling over ice. In the slot blot manifield (Biorad, USA), Hybond nylon membrane (Gibco, USA) was placed after soaking in 20x SSC. The RNA samples were applied over the slots provided in the manifold and vaccum was applied for the suction of material on to the membrane kept below.

The membrane was rinsed with 10xSSC, air dried and baked at 80°C for 2 hrs. The membrane was prehybridized with Hybridizing buffer for 6 hours at 42°C in a tube. Then the labelled probe was added to the hybridization buffer. The hybridization reaction was allowed to take place overnight at 42°C. The hybridization buffer was removed and washing solution-A (3x SSC, 1% SDS) was added and washed twice at an interval of 30min at 52°C. Then the membrane was washed in solution-B (1x SSC, 1% SDS) twice at 52°C for 15 min.

The membrane was then removed from the tube, air dried, covered with a polythene wraper and autoradiographed using Kodak X-ray films for 48 hrs at -70°C.

After 48 hrs of exposure, the film was removed and developed as per standard procedures.

Results

CHAPTER IV

RESULTS

4.1 VIRUS ISOLATION

4.1.1 SAMPLES SCREENED

A total of 15 cloacal swabs were collected from ailing and apparently healthy desi chicken as detailed in Table-1. Out of the 15 samples screened 11 samples produced characteristic lesions in the embryonated chicken eggs. The passage level at which the isolation was made, characteristic lesions noticed in the embryo and the results of HA activity of AAF with chicken erythrocytes (spot test) are furnished in Table-2. In the present study, the overall percentage rate of isolation was 73.33. Out of the 11 isolates, 7 were obtained in the first passage and 4 in the second passage.

4.1.2 LESIONS IN EMBRYONATED CHICKEN EGGS

Death of the embryos were noticed between 44 and 69.3 hrs. The lesions obtained were occipital haemorrhage, haemorrhages on wings, legs and on the dorsum (Plate-1).

4.1.3 SPOT HA TEST

The Amnio allantoic fluid of those embryos which showed agglutination with 10 per cent suspension of chicken erythrocytes were considered as positive and in this context, all the 11 isolates gave positive HA reaction.

TABLE 1

PARTICULARS OF SAMPLES COLLECTED FROM DESI CHICKEN FOR VIRUS ISOLATION

S.No	Sample No.	Age of the bird	Sample collected	Details of vaccination	Condition of the bird
1.	S1	6 WEEKS	CLOACAL SWAB	NOT VACCINATED	HEALTHY
2.	S2	6 WEEKS	CLOACAL SWAB	NOT VACCINATED	HEALTHY
3.	S3	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
4.	S4	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	HEALTHY
5.	S5	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
6.	S6	6 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
7.	S7	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
8.	S8	6 WEEKS	CLOACAL SWAB	NOT KNOWN	HEALTHY
9.	S9	6 WEEKS	CLOACAL SWAB	NOT KNOWN	HEALTHY
10.	S10	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
11.	S11	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
12.	S12	3 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
13.	S13	6 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS
14.	S14	4 WEEKS	CLOACAL SWAB	NOT VACCINATED	DROWSY
15.	S15	4 WEEKS	CLOACAL SWAB	NOT VACCINATED	ENTERITIS

TABLE 2

ISOLATION DETAILS AND DIFFERENT TESTS EMPLOYED FOR THE IDENTIFICATION

Isolate	Passage level	Effect on embryos		Spot HA with AAF	Whether from ailing or healthy bird
		Dead or alive	Lesions		
D1	1	DEAD	OH, HL	+	HEALTHY
D2	1	DEAD	HW, HL, HB, OH	+	HEALTHY
D3	1	DEAD	HW, HL, HB, OH	+	AILING
D4	1	DEAD	HW, HL, HB, OH	+	HEALTHY
D5	1	DEAD	OH	+	AILING
D6	1	DEAD	HW, HB, OH	+	AILING
D7	2	DEAD	HB	+	AILING
D8	1	DEAD	HB	+	HEALTHY
D9	2	DEAD	HB	+	AILING
D10	2	DEAD	HB	+	AILING
D11	2	DEAD	HB	+	AILING

OH : OCCIPITAL HAEMORRHAGE
HW : HAEMORRHAGE ON WINGS
HL : HAEMORRHAGE ON LEGS
HB : HAEMORRHAGE ON BODY

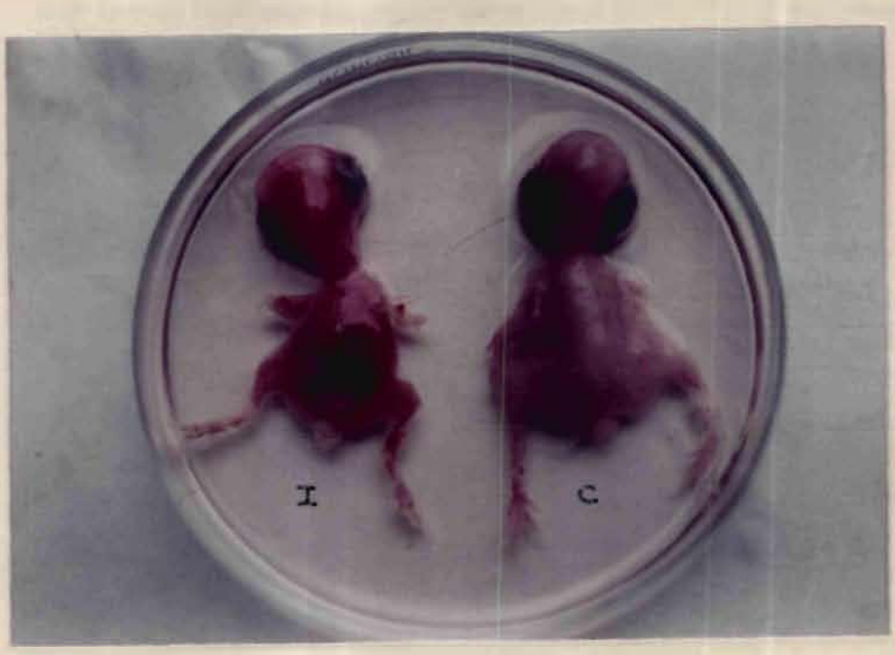

Plate 1. Embryonated chicken egg inoculated with NDV suspected material, showing haemorrhages on head, legs and dorsum.
I : Infected
C : Control

4.2 IDENTIFICATION OF THE ISOLATES

Identification of the viral isolates were based on the results of HA test, HI test with known NDV antiserum and VN test and the results of these tests are presented in Table-3.

4.2.1 HA AND HI TESTS

The HA titres of the different isolates varied from 32 to 512. The HI test was carried out with 4 HA units of the various isolates and known NDV antiserum. The HI titres ranged from 1024 to 2048.

4.2.2 VIRUS NEUTRALIZATION TEST (VNT)

The $\log_{10}$ neutralization index calculated as the difference between the negative log of virus with normal serum titre and the negative log with immune serum titre, was found out. The results of VNT conducted in embryonated eggs making use of varying dilution of isolates and constant amounts of 1 in 4 dilution of the hyper immune serum, are furnished in Table-3. The log neutralization index ranged from 10^{-3} to $10^{-7.2}$.

4.3 CHARACTERIZATION OF NDV ISOLATES BY CONVENTIONAL METHODS

4.3.1 VIRULENCE CHARACTERS

The results of the tests employed for assessing the virulence characters of the NDV isolates are presented in Table-4.

TABLE 3

IDENTIFICATION AND CONFIRMATION OF DIFFERENT ISOLATES AS NDV

Isolate	HA titre reciprocal of virus dilution	HI titre reciprocal of serum dilution	VNNI as log_{10}
D1	256	2048	3.75
D2	256	2048	5.55
D3	64	2048	7.0
D4	32	2048	7.2
D5	512	2048	3.4
D6	256	2048	5.16
D7	256	1024	5.43
D8	128	1024	4.2
D9	512	1024	3.0
D10	512	1024	4.2
D11	128	1024	5.1

TABLE 4

VIRULENCE CHARACTERS OF NDV ISOLATES FROM DESI CHICKEN

Isolate	MDT in hrs.	ELD 50 $\log_{10}$	ICPI	IVPI
D1	45.0	8.7	1.43	2.18
D2	44.0	11.7	1.52	1.8
D3	48.6	12.5	1.61	2.0
D4	56.0	11.7	1.64	2.16
D5	69.3	9.5	0.98	2.16
D6	45.3	11.5	1.71	2.25
D7	56.0	11.5	1.71	1.82
D8	56.0	12.5	1.85	2.07
D9	56.0	10.6	1.81	2.16
D10	52.0	9.2	1.76	2.16
D11	48.0	10.5	1.77	2.23

The MDT of various isolates ranged between 44 hrs and 69.3 hrs. The ELD_{50} titres of the isolates were found to vary from 8.7 to 12.5.

The ICPI experiments were conducted with day old chicks (Plate-2) and the ICPI values of the isolates ranged between 0.98 and 1.85. Similarly the IVPI experiments were conducted in six weeks old susceptible chicks (Plate-3) and the indices varied from 1.77 to 2.25.

4.3.2 STRAIN DIFFERENTIATING CHARACTERS

The results of various strain differentiating characters like stability of haemagglutinins at 56°C, agglutination of mammalian erythrocytes and adsorption of haemagglutinins by chicken brain cells are furnished in Tables-5,6 and 7.

The haemagglutinins of the eleven isolates were found to be stable for periods ranging from 120 min to 210 min (Table-5). At 56°C, the haemagglutinins of NDV D7 were stable for only 120 min where as those of NDV D5, D6 and D9 were stable for 210 min.

All the eleven NDV isolates from desi chicken, agglutinated the erythocytes of cattle, goat and human 'O' and the results are presented in Table-6. However, with regard to equine erythrocytes only two isolates (D5 and D7) agglutinated.

The results of adsorption of haemagglutinins by chicken brain cells are given in Table-7. The percentage of adsorption of haemagglutinins of different NDV isolates ranged between 50 and 87.5.

Plate 2. Results of Intracerebral pathogenicity index - chicks showing paralytic symptom.

Plate 3. Results of Intravenous pathogenicity index - chick showing nervous symptom.

TABLE 5

STABILITY OF HAEMAGGLUTININS OF NDV ISOLATES AT 56°C

Isolate	HA titre at room temp	HA TITRE AFTER EXPOSURE TO 56°C (in min.)										
		5	10	20	30	60	90	120	150	180	210	240
D1	512	512	256	256	128	64	32	16	8	2	NIL	
D2	256	256	128	128	64	32	16	16	8	NIL		
D3	512	512	256	256	128	64	32	16	8	4	NIL	
D4	256	128	128	64	64	64	64	32	32	8	NIL	
D5	256	128	128	64	64	32	16	16	8	4	2	NIL
D6	256	256	128	64	64	64	64	64	32	16	8	NIL
D7	256	256	128	64	64	32	16	8	NIL			
D8	128	128	128	64	32	16	8	2	2	NIL		
D9	512	512	512	256	256	128	64	32	16	8	8	NIL
D10	256	256	128	128	64	32	16	16	8	4	NIL	
D11	256	256	128	128	64	32	32	16	4	2	NIL	

TABLE 6

HA ACTIVITY OF THE NDV ISOLATES WITH MAMMALIAN ERYTHROCYTES

Isolate	HA TITRE WITH ERYTHROCYTES OF			
	Cattle	Goat	Human O	Horse
D1	16	2	128	NIL
D2	16	8	16	NIL
D3	8	2	64	NIL
D4	64	2	128	NIL
D5	128	8	256	16
D6	64	8	256	NIL
D7	64	16	64	32
D8	128	32	64	NIL
D9	8	8	256	NIL
D10	64	8	64	NIL
D11	64	32	8	NIL

TABLE 7

HA TITRE OF NDV ISOLATES AFTER ADSORPTION WITH CHICKEN BRAIN CELLS AND PERCENTAGE OF ADSORPTION

Isolate	HA TITRE		Percentage of adsorption
	Before adsorption	After adsorption	
D1	512	64	87.5
D2	256	64	75.0
D3	512	256	50.0
D4	256	128	50.0
D5	256	64	75.0
D6	256	64	75.0
D7	512	256	50.0
D8	512	128	75.0
D9	512	128	75.0
D10	256	128	50.0
D11	256	64	75.0

4.4 CHARACTERIZATION OF NDV ISOLATES BY MONOCLONAL ANTIBODY TYPING

The results of monoclonal antibody typing of the isolates conducted at Central Veterinary Laboratory, Weybridge, United Kingdom are given in Table-8. It is evident from the results that all the isolates belonged to paramyxovirus (PMV-1). Out of the eleven isolates, four each belonged to group C_1 and E and one to group L. However, isolates D1, D4, and D5 which are classified as lentogenic by monoclonal antibody typing have been reported to have killed the embryonated chicken eggs in 48 hrs. Similarly D11, classified as lentogenic is reported to have high ICPI. MAb typing of isolates D8 and D10 are yet to be completed.

4.5 CHARACTERIZATION OF NDV ISOLATES BY ADAPTING TO MDBK CELLS

All the eleven isolates were passaged five times in MDBK cells along with a known velogenic strain (NDV-VP4) and lentogenic strain (RDVF). Presence of CPE at different passage levels and the HA titres of the cell culture fluid are furnished in Table-9. All the isolates including the control strains produced cytopathic effects in MDBK cells from the first passage onwards. The CPE were observed 48-72 hrs after infection during the initial passages and the intensity was more in velogenic strains.

The CPE produced by the isolates were characterised by rounding and grouping cells, cell fusion, cytoplasmic vaccuolation and the presence of acidophylic intra cytoplasmic inclusion bodies (Plate-4) as compared to the uninfected control (Plate-5).

TABLE 8

MONOCLONAL ANTIBODY TYPING OF NDV ISOLATES

Isolates	Inhibition of PMV-1 polyclonal serum	CVL, Weybridge grouping based on MAb binding	Nature of the virus
D1	+	E	LENTOGENIC*
D2	+	C_1	VELOGENIC
D3	+	C_1	VELOGENIC
D4	+	L	LENTOGENIC*
D5	+	E	LENTOGENIC*
D6	+	C_1	VELOGENIC
D7	+	E	LENTOGENIC
D8	+	NOT DONE	-
D9	+	C_1	VELOGENIC
D10	+	NOT DONE	-
D11	+	E	LENTOGENIC**

* The virus killed the embryos in 48 hrs.
** High ICPI
C_1 Velogenic
E B_1/Lasota
L Water Fowl.

TABLE 9

ADAPTATION OF NDV ISOLATES TO MDBK CELLS

Isolate	Presence of CPE					HA titre of cell culture fluid				
	Passage lelvel					Passage level				
	1	2	3	4	5	1	2	3	4	5
D1	+	+	+	+	+	32	256	64	128	256
D2	+	+	+	+	+	2	8	16	64	64
D3	+	+	+	+	+	2	2	32	64	64
D4	+	+	+	+	+	8	4	32	16	8
D5	+	+	+	+	+	32	64	128	128	64
D6	+	+	+	+	+	2	8	32	128	128
D7	+	+	+	+	-	64	64	128	64	-
D8	+	+	+	+	+	16	4	-	-	-
D9	+	+	+	+	+	32	128	128	256	64
D10	+	+	+	+	+	128	128	64	64	16
D11	+	+	+	+	-	2	32	64	128	-
RDVF	+	+	+	-	-	32	16	4	-	-
NDV VP_4	+	+	+	+	+	256	128	256	256	64

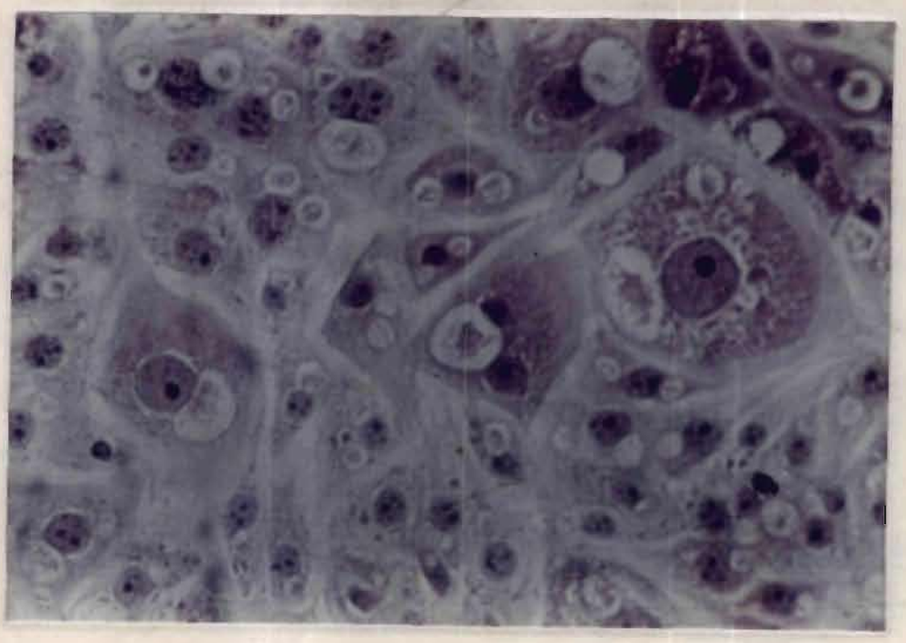

Plate 4 MDBK monolayer - infected with NDV isolate showing acidophilic intracytoplasmic inclusion bodies. Stained with H & E. 200x.

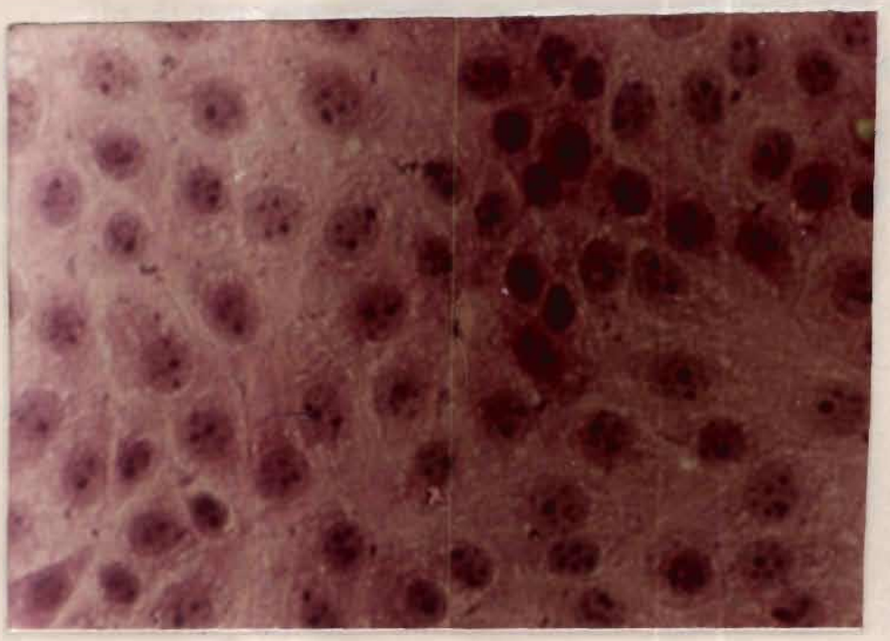

Plate : 5 MDBK monolayer - uninfected, stained with H & E, (200x).

The lentogenic RDVF strain infected cells were rounded with few syncytia formation during the initial passages. However, inclusion bodies were not observed in those cultures. Cytopathogenic effects were induced by all the isolates except for D1 and D11 upto five passages. Similarly the known velogenic isolate induced CPE upto five passages whereas the lentogenic RDVF could not induce appreciable CPE after third passage.

The HA titres of the isolates varied from passage to passage (Table-9) and at end of fifth passage no HA activity was found with regard to isolates D7, D8, D11 and RDVF.

4.6 DIFFERENTIATION OF NDV ISOLATES BY OLIGONUCLEOTIDE PROBE

The 21 mer oligonucleotide probe labelled with γ^{32}P ATP by Terminus labelling method was used to differentiate the NDV isolates. All the eleven isolates and the velogenic control NDV strain gave positive signals in a slot blot hybridization experiment. However the lentogenic RDVF RNA did not give any positive signal with the probe (Plate-6).

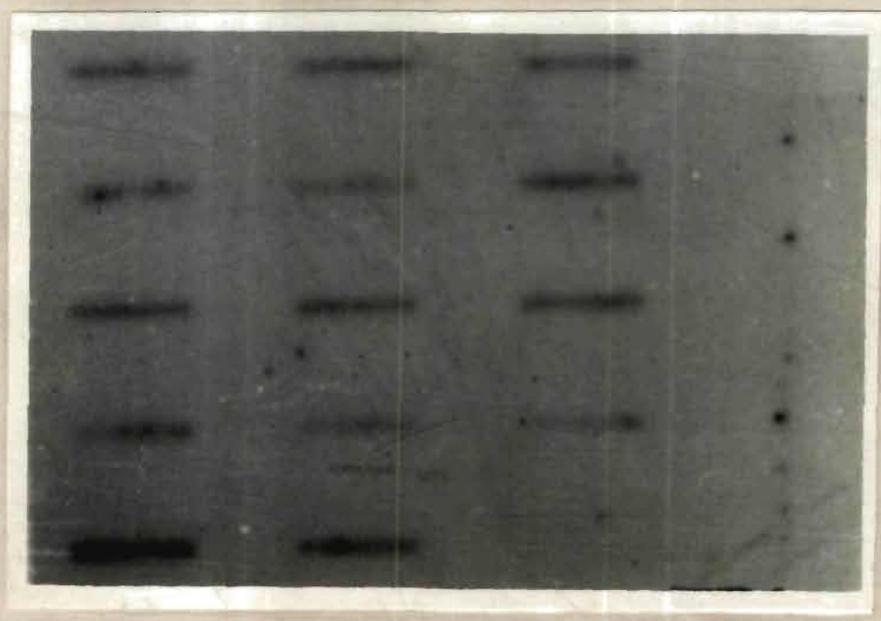

PLATE - 6 SLOT BLOT HYBRIDIZATION WITH VARIOUS NDV ISOLATES USING 21 mer OLIGONUCLEOTIDE PROBE

D_1_____	D_6_____	D_{11}_____
D_2_____	D_7_____	V_c_____
D_3_____	D_8_____	V_c_____
D_4_____	D_9_____	P_c_____
D_5_____	D_{10}_____	N_c_____

V_c - Velogenic control

P_c - Probe control

N_c - Negative control

D_1-D_{11} - NDV isolates

Discussion

CHAPTER V

DISCUSSION

Newcastle disease virus (NDV) is the prototype strain of the genus Paramyxovirus, of the family Paramyxoviridae, and has been designated as Paramyxovirus-1 (PMV-1) (Alexander, 1990). Newcastle disease (ND) is still considered as a dreaded disease in poultry industry owing to its high morbidity and mortality among susceptible population. Since vaccination is the only means of controlling the disease, several live and inactivated vaccines are being used to control the disease. However, the loss due to ND is still on the higher side inspite of regular and systematic vaccination programmes. Although several factors like quality of the vaccine, maintenance of cold chain, vaccination methods, immune status of the birds, etc., are responsible for the success of the vaccination, presence of etiological agent in the viscinity may always pose a severe threat even to the vaccinated population. The role of many wild birds, water fowls and free roaming desi birds as carriers of velogenic NDV strains, could be one of the reasons for the persistance and severity of the disease (Allan *et al.*, 1978; Luthgen, 1981). These birds are reported to carry even velogenic strains of NDV without clinically suffering from the disease. Under the circumstances, it has become necessary to assess the nature of NDV that might be harboured by these birds.

Routine diagnosis of ND usually requires two distinct steps. Firstly, it is necessary to establish that the virus isolated is NDV and not any other avian paramyxoviruses or influenza virus. The second consideration in ND diagnosis is

whether or not, the virus isolated is vaccinal or enzootic (Alexander, 1990). It is in this context that the present study has been undertaken to assess the nature of the virus isolates from desi chicken employing various conventional and recent techniques.

5.1 VIRUS ISOLATION

Out of the fifteen cloacal swabs screened (Table-1), eleven samples ended up with virus isolation. The overall rate of isolation (73.33%) obtained in this study is found to be definitely higher than some of the earlier reports involving commercial birds (Kumanan, 1989). Moreover, the most significant observation in this study is the isolation of four NDV strains from apparently healthy birds. Out of the eleven isolates, seven were obtained in the first passage and four in the second passage (Table-2), since some degree of adaptation to the embryonated eggs is essential when the virus content of the inoculum is low (Beaudette *et al.*, 1948).

The time taken for the isolates to kill the embryos ranged between 44-69.3 hrs. NDV isolates are reported to produce characteristic lesions like occipital haemorrhage, haemorrhage in the wings, legs and body of the embryos (Iyer and Dobson, 1940; Jungherr *et al.*, 1946). The isolates obtained in this study also produced similar lesions like occipital haemorrhage and haemorrhages on the wings and legs (Plate-1). Absence of pock lesions on CAM and curling of toes eliminated the possibility of the occurrence of other viruses like Fowl pox virus (FPV), Infectious laryngo tracheitis virus (ILTV), and Infectious bronchitis virus (IBV). Moreover, the isolates agglutinated 10 per cent chicken erythrocytes during spot HA test which again ruled out the presence of FPV, ILTV and IBV which do not exhibit the property of agglutination of chicken erythrocytes (Lancaster, 1963; Brandly *et al.*, 1946).

5.2 IDENTIFICATION OF THE ISOLATES

The property of HA of chicken erythrocytes and the HI using specific NDV antiserum have been widely used as a confirmatory diagnostic tool in the identification of NDV isolates (Brandly *et al.*, 1946; Hanson, 1972). All the eleven isolates obtained in this study fulfilled these requirements and confirmed themselves as NDV isolates (Table-3).

Similarly, a $\log_{10}$ Neutralization Index (NI) of 2 or greater is considered as positive for NDV and the log NI of the isolates ranged between 3 and 7.2. The results of these tests confirmed the identity of the isolates as Newcastle disease virus (Table-3).

5.3 CHARACTERIZATION OF NDV ISOLATES BY CONVENTIONAL METHODS

The virulence of NDV isolates are usually determined by assessing both the virulence characters and strain differentiating characters (Lancaster and Alexander, 1975; Allan *et al.*, 1978; Kumanan and Venkatesan, 1991). In this study, all the isolates were tested for both the virulence and strain differentiating characters.

5.3.1 VIRULENCE CHARACTERS

Velogenic NDV isolates are reported to kill chicken embryos in 40-60 hrs, whereas mesogenic and lentogenic strains take 60-90 hrs and 90 hrs and more, respectively (Hanson and Brandly, 1955). Except for D5, all the other isolates (Table-4) have their MDT within 60 hrs and could be placed in the velogenic group.

The ICPI of lentogenic strains ranges between 0 and 0.25, whereas the mesogenic strains have an ICPI range of 0.8 to 1.46. However, in case of velogenic isolates, the ICPI was found to be between 1.75 and 2.0 (Hanson, 1956; Singh and Singh, 1970). Based on this test, four isolates are placed in the velogenic group and the remaining seven in the mesogenic group (Table-4).

The IVPI of the eleven isolates ranged between 1.8 and 2.25 (Table-4). Velogenic isolates are reported to have IVPI ranging between 1.7 - 2.8 (Lancaster, 1962; Kumanan, 1989; Vijayarani 1990, Kamaraj, 1993). The results of IVPI test clearly show that all the eleven isolates obtained in this study are of velogenic nature.

MDT, ICPI and IVPI are the generally accepted means of assessing the virulence of NDV isolates (Allan *et al.*, 1978) and the results of this study are in confirmation with the values obtained for velogenic group and accordingly, all the isolates except D5 could be classified as velogenic. With regard to D5, although MDT and ICPI values place it in the mesogenic group, it has got a high IVPI value (Table-4). However, it is of significance to state that none of the three tests could determine the virulence of a particular strain individually. Therefore, it has become necessary to corroborate the results of all the three tests to attribute the virulence of an isolate as it has been done in earlier studies (Martone *et al.*, 1974; Kumanan, 1989).

5.3.2 STRAIN DIFFERENTIATING CHARACTERS

Although the virulence of NDV isolates are determined by virulence characters, some of the strain differentiating characters like stability of haemagglutinins at 56°C

heamagglutination of mammalian erythrocytes and adsorption of haemagglutinins by chicken brain cells are also considered for ascertaining the nature of the isolate.

It has been reported that lentogenic strains are stable at 56°C for only 5-10 min (Hanson *et al.*, 1967). The stability of mesogenic and velogenic isolates were found to be less than 30 min and more than 270 min, respectively (Chandra *et al.*, 1972; Estola, 1974). Keeping this in mind, the present isolates could be classified as velogenic, since all of them were stable for at least 120 min (Table-5). Some of the isolates, namely D5, D6 and D9 were found to be stable for even 210 min. Although this character was reported to be not related to virulence (Kohn and Fuchs, 1969), it was considered along with other virulence attributes to differentiate field and vaccine strains of NDV as lentogenic, mesogenic and velogenic (Chandra *et al.*, 1972).

The results of HA activity of the NDV isolates with mammalian erythrocytes are presented in Table-6. Apart from agglutinating chicken and other avian erythrocytes, NDV has got the property of agglutinating various mammalian erythrocytes like cattle, horse, sheep and pig. This property has been used to distinguish various strains of NDV (Winslow *et al.*, 1950). It has been reported that some of the lentogenic strains like CDF66 and Lasota agglutinate equine erythrocytes. In this study, all the isolates agglutinated the erythrocytes of cattle, goat and human 'O'. However, isolates D5 and D7 only agglutinated equine erythrocytes. Although this property of NDV has been reported as one, which had no relation with the virulence (Kohn and Fuchs, 1969; Janakiram, 1983) like the stability of haemagglutinins at 56°C this property was considered as a criterion to distinguish virulent strains from less virulent strains (Winslow *et al.*, 1967; Hanson *et al.*, 1967).

The avidity of NDV for attachment to chicken brain cells was taken into cognizance for determining the virulence of an isolate (Piraino and Hanson, 1960). It has been reported that few lentogenic NDV strains like Lasota and 'F' were adsorbed to chicken brain cells whereas B_1 was not (Hanson *et al.*, 1967). The haemagglutinins of K strain was also found to be not adsorbed (Tanwani, 1974). However, the avidity has been found to be more in case of velogenic strains. The percentage of adsorption of the haemagglutinins of the different NDV isolates ranged between 50 and 87.5 (Table-7). The results of this study revealed that all the isolates had higher percentage of adsorption which had substantiated to some extent on the virulence of the isolates as reported earlier (Piraino and Hanson, 1960; Kumanan, 1989).

The results of the virulence and strain differentiating characters reveal that except for isolate D5, all the other isolates could be classified as velogenic.

5.4 MONOCLONAL ANTIBODY TYPING OF NDV ISOLATES

The results of monoclonal antibody typing reveal that all the eleven isolates were inhibited by paramyxovirus-1 polyclonal antiserum (Table-8) confirming them to be NDV (Alexander, 1990). However, with regard to the characterization of the isolates, the results obtained are interesting and inconclusive. Out of the eleven isolates, four isolates, namely D2, D3, D6 and D9 are groped as C_1 which represents the velogenic isolates from migratory birds (Alaxander, 1990). Similarly four more isolates, namely D1, D5, D7 and D11 are placed group E which represents lentogenic strains. Similarly D4 is also classified as lentogenic and placed in group L which represents isolates from water fowl. However, details are not avaiable for D8 and

D10. Out of the five isolates (D1, D4, D5, D7 and D11) classified as lentogenic, three isolates namely D1, D4 and D5 are reported to have killed the embryonated chicken eggs in 48 hrs (Ruth Manvell, 1996, Personal communication), which is highly, unlikely, for a lentogenic strain, since the MDT recorded with regard to lentogenic strain was more than 90 hrs (Hanson and Brandly, 1955). Only velogenic strains are reported to kill the embryos in 48 hrs. Similarly the ICPI value of D11 is found to be more than that of a classical lentogenic strain.

Although conventional tests place these isolates, (except D5) in velogenic group, the monoclonal antibody typing has given some inconclusive results with regard to the differentiation of these isolates. This situation has necessitated in recharacterizing atleast some of the isolates with a different set of monoclonal antibodies for confirmatory results.

5.5 CHARACTERIZATION OF NDV ISOLATES BY ADAPTING TO MDBK CELLS

It is reported that MDBK cells could be used as a tool for differentiating velogenic strains of NDV from lentogenic strains (King, 1993) and all strains except those that are classified as lentogenic could be serially propagated in MDBK cells. The eleven isolates along with a known virulent and an avirulent NDV strain were passaged five times in MDBK cells. All the strains produced CPE during initial passages and the intensity of the CPE was more pronounced in the velogenic control strain and most of the isolates made in this study. The isolates produced characteristic CPE, which include rounding and grouping of cells, cell fusion,

cytoplasmic vacuolation and intracytoplasmic acidophylic inclusion bodies (Plate-4) as reported earlier (Kumanan, 1989; King, 1993).

In the present study, except for D7, and D11, all the isolates including the velogenic NDV VP4 induced characteristic CPE upto five passages. However, in case of D7, and D11 the CPE was not appreciated at fifth passage. The lentogenic RDVF could not induce appreciable CPE after third passage, concurring with an earlier report (King, 1993).

The HA titres of the isolates varied from passage to passage and no HA activity was found with regard to isolates D7, D8, D11 and RDVF at the end of fifth passage.

Based on the results of this study, all the isolates except D7, and D11 could be classified as velogenic. In an earlier experiment (King, 1993) the isolates were passaged only three times in MDBK cells. If this is taken as a criteria, isolates D7 and D11 also could be classified as velogenic since they produced CPE upto fourth passage. Although D7 and D11 are grouped as velogenic strains by virulence and strain differentiating characters, the variation obtained in this study could be attributed to the differences in the methodology of MDBK cell passages or differences in the virus strains used in the previous study.

5.6 DIFFERENTIATION OF NDV ISOLATES BY OLIGONUCLEOTIDE PROBE

Although several tests are available to identify and characterize NDV isolates, the diversity of Newcastle disease viruses and the problems encountered in the

existing techniques have prompted the development of newer techniques. Rapid and precise techniques that distinguish velogenic strains from lentogenic and neurotropic from viscerotropic strains will be of great help in determining the course of action during future ND outbreaks. Attempts have been made to differentiate NDV strains based on molecular criteria (Jarecki Black *et al.*, 1992; Jarecki Black and King, 1993).

The envelope glycoproteins of NDV, haemagglutinin neuraminidase (HN) and fusion (F) proteins, play important roles in determining the host immune response and virulence of a particular NDV strain. The virulence of NDV strains have been related to sequences at the cleavage site of Fo. Oligonucleotide probes have been generated from a highly conserved region encoding the fusion gene (Jarecki Black and King, 1993). The probe used in this study was designed to complement a region of the F gene of the Texas GB strain of NDV, called as the cleavage activation site. This region codes for 5 amino acids, two pairs of dibasic amino acids (Arg - Arg - Gln - Lys - Arg) separated by a glutamine residue which is characteristic for velogenic and mesogenic strains. A 21mer probe was designed involving these five aminoacids along with the flanking amino acids namely glycine and phenylalanine.

The 21 mer oligonucleotide probe, labelled with $\gamma^{32}P$ ATP by Terminus labelling method was used to differentiate the NDV isolates. All the eleven isolates along with the known velogenic control gave positive signals in the slot blot Hybridization where as the lentogenic RDVF did not give any positive signal after autoradiography (Plate-6). It is evident from the results that the eleven test isolates are not lentogenic and they could be either velogenic or mesogenic.

The results of this experiment clearly indicates that isolates D1 (MAb typing), D4 (MAb typing), D5 (Agglutination of equine erythrocytes and MAb typing), D7 (Agglutination of equine erythrocytes and adaptability in MDBK cells) and D11 (MAb typing and adaptability in MDBK cells) which tended to behave like lentogenic in some of the tests are definitely not lentogenic. Hence based on the results of all the tests, D5 could be classified as mesogenic and the remaining ten as velogenic.

It could be concluded that the oligonucleotide probe designed from the conserved region of the F gene will be of greater help in differentiating velogenic and mesogenic NDV isolates from lentogenic ones. The most significant and alarming observation made in this study is the role of desi birds as potential carriers of velogenic and mesogenic NDV strains, without clinically suffering from the disease. The presence of such carriers in the surroundings of more susceptible commercial flocks may lead to dangerous consequences. Hence it would be appropriate to always keep in mind, the role of desi birds as carriers of NDV strains while designing and executing the control measures.

Summary

CHAPTER VI

SUMMARY

The present study, undertaken to isolate and characterize Newcastle disease virus (NDV) strains from desi chicken, resulted in eleven NDV isolates with an isolation percentage of 73.33. Out of the eleven isolates, seven were made in the first passage and four in second passage. In embryonated chicken eggs the isolates produced occipital haemorrhage, haemorrhages on wings, legs and body. The Amnio allantoic fluid (AAF) of the embryos were checked for haemagglutination (HA) activity using chicken erythrocytes and the identity of the isolates were confirmed by HA haemagglutination inhibition (HI) test and virus neutralization tests (VNT) in embroynated chicken eggs.

All the eleven isolates were characterized by Mean death time (MDT), Intracerebral pathogenicity index (ICPI), Intravenous pathogenicity index (IVPI), stability of haemagglutinins at 56° C, Agglutination of mammalian erythrocytes and adsorption of haemagglutinins by chicken brain cells. The MDT of the isolates ranged between 44 - 69.3 hrs and Egg lethal dose 50 per cent end point (ELD50) titres were found to be ranging from 8.7 to 12.5. The ICPI and IVPI values of the isolates were found to range between 0.98 to 1.85 and 1.8 to 2.25, respectively. The haemagglutinins of the isolates were stable for periods ranging from 120 min to 210 min. All the isolates agglutinated the erythrocytes of cattle, goat and human 'O' whereas only two isolates agglutinated equine erythrocytes. The percentage of adsorption of Haemagglutinins by chicken brain cells varied between 50-87.5.

The Monoclonal antibody (MAb) typing confirmed that all the isolates belong to paramyxovirus-1 (PMV-1). However, based on the MAb typing results, four, each of the isolates were placed in group C_1 and E and one to group L (Results of MAb typing of two of the isolates were not available).

The NDV isolates were passaged five times in MDBK cells along with a known velogenic and lentogenic strain. Cytopathogenic effect (CPE) were observed in 48-72 hours post infection and infectivity was more in velogenic isolates. The CPE were characterized by cell fusion, cytoplasmic vacuolation and acidophylic intracytoplasmic inclusion bodies. The lentogenic RDVF could not induce appreciable CPE after third passage. The HA titres of the isolates, varied from passage to passage and at the end of fifth passage the HA activity was lost for some of the isolates including the lentogenic RDVF.

Attempts were made to differentiate the isolates by oligonucleotide probe. A 21 mer oligonucleotide probe (complementary to the conserved region of the fusion protein) labelled with γ ^{32}P -ATP by terminus labelling method was used. All the eleven isolates from desi birds and the velogenic control NDV strain gave positive signals in a slot-blot hybridization experiment. However, the lentogenic RDVF did not give any positive signal.

Although minor variations were observed in the different tests employed, it is evident from the results that these isolates are definitely not lentogenic and they could be either velogenic or mesogenic.

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