PHYLOGENETIC ANALYSIS OF FIELD ISOLATES OF NEWCASTLE DISEASE VIRUS

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Certificate

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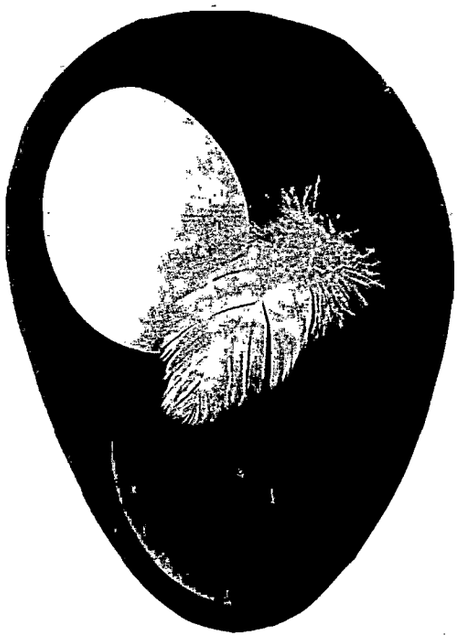
RAJU **SEETHARAMAN GOPINATH**

"DEDICATED TO ALL THOSE EMBRYOS WHICH WERE SACRIFICED FOR THIS ENDEAVOUR . . .



. . . WHICH IN FUTURE MAY SHUN THIS LIVE ANIMAL TESTING FOR EVER".

ABSTRACT

Name and Degree	:	R.S. GOPINATH Ph.D., in Animal Biotechnology
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Phylogenetic analysis of diverse Newcastle Disease Virus (NDV) isolates of India was carried out using Restriction site analysis and Nucleotide sequencing of Fusion and Matrix protein gene regions, to classify them into different clusters and to correlate them epizootiologically.

RT-PCR-RFLP for total of 18 Indian isolates and common vaccine strains of NDV typed by PCR-RFLP of the fusion gene. Hinf I, Bst OI & Rsa I, discriminated 18 different isolates & vaccine strains into 6, 5 & 4 different groups respectively. Hae III, Taq I & Mbo I differentiated 16 isolates & vaccine strains into 5, 3 & 3 groups respectively. Hinf I has been found effective in even discriminating the homogenous monoclonal antibody typed group into different clusters. Homogenous 'C1' grouping of 7 virulent NDV isolates was differentiated into two different clusters. Hinf I : UPGMA analysis was found most discriminative which yielded three different clusters, with vaccine strains & field isolates grouping separately and also differentiated the vaccine strains from field isolates effectively. Two different groups viz., II & IV of earlier classified genotypes were found in India, also the new novel genotype of Taiwan and Indonesia - VII and RI-3 were found with one more fragment.

Direct one-tube RT-PCR for the FPCS 254 bp region with the field tissue sample from love-bird and peacock amplified the expected size and on further nucleotide sequence analysis were typed as velogenic pathotype. Effective pathotype prediction carried out using predicted amino acid sequence of FPCS for the isolates already characterized by Mab, and conventional pathotyping tests, correlated well except for the desi-bird isolate D4 and chicken isolate, LCC9/89, suggesting the isolation of Pigeon variant

NDV from these birds. Also the KPG/98 was found to have FPCS amino acid sequence motif similar to PPMV-1.

Phylogenetic analysis of FPCS & MPNLS sequences classified the NDV isolates from India into two major lineage's containing different clusters/ genotypes of virulent NDV. Further it also differentiated between the vaccine strains and field isolates efficiently. Recent desi bird isolates ('95) were grouped along with early ('87) velogenic chicken isolates suggesting the maintenance of these velogenic virus in the desi bird for an extended time period .

The Indian NDV isolates shared phylogenetic relationship with already existing two major global phylogeny lineage's in a well-balanced pattern with both velo, meso & lentogenic isolates going into both the lineage's. The desi bird velogenic isolates were grouped along with the other exotic bird velogenic isolates viz., parrot, cockatoo and parakeet. The Indian early velogenic isolates grouped along with other velogenic isolates from Texas, Italy and Herts. All the 3 Kerala isolates grouped together, along with R2B Vaccine strain went into the mesogenic cluster of field isolates and vaccine strains. The Indian NDV isolates displayed unique diversity in the global phylogeny map showing conservation among them, compared to other isolates of world and have been successfully assigned in the global Phylogeny map of NDV for the first time in the World .

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LIST OF ABBREVIATIONS

AAF	-	Amnioallantoic fluid
APMV	-	Avian Paramyxovirus
BCL	-	Bootstrap confidence level
bp	-	Base pair
cDNA	-	Complementary DNA
CP	-	Confident property
DEPC	-	Diethyl pyrocarbonate
DNA	-	Deoxy ribonucleic acid
F	-	Fusion protein
FPCS	-	Fusion protein cleavage site
HA	-	Haemagglutination
HI	-	Haemagglutination inhibition
HN	-	Haemagglutinin neuramidase protein
ICPI	-	Intracerebral pathogenecity index
IVPI	-	Intravenous pathogenecity index
JCD	-	Jukes-Cantor distance
K2-PD	-	Kimura 2-Parameter distance
L	-	Large Polymerase protein
M	-	Matrix protein
MAb	-	Monoclonal antibodies
MDT	-	Mean Death Time

MEGA	-	Molecular evolutionary genetic analysis
MPNLS	-	Matrix protein nuclear localisation signal
ND	-	Newcastle disease
NDV	-	Newcastle disease virus
NJ	-	Neighbour - Joining method
NP	-	Nucleocapsid protein
nt	-	Nucleotide.
NVNDV	-	Neurotropic velogenic Newcastle disease virus
OIE	-	Office de Internationale Epizooties
ORF	-	Open reading frame
OTU	-	Operational Taxonomic Unit
P	-	Phosphoprotein
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction.
PHYLIP	-	Phylogeny Inference package
PPMV	-	Pigeon Paramyxovirus
REA	-	Restriction enzyme analysis
RED	-	Restriction enzyme digestion
RFLP	-	Restriction Fragment length polymorphism
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcription - Polymerase chain reaction.
TBE	-	Tris Borate - EDTA
UPGMA	-	Unweighted pair group method with arithmatic means.
VVNDV	-	Viscerotropic velogenic Newcastle disease virus

introduction

CHAPTER - 1

1. INTRODUCTION

Avian paramyxovirus-1 (PMV-1) is the causative agent of a major disease of poultry-the Newcastle disease (ND). Newcastle disease is endemic in India and other tropical countries. Currently the disease has worldwide distribution with a wide host range in which all orders of birds have been reported to be infected by Newcastle Disease Virus (NDV).

Outbreaks of Newcastle disease were first reported in poultry from Java, Indonesia and Newcastle-upon Tyne, England in 1926 (Doyle, 1927). In India, the first reported outbreak of ND with highly virulent NDV occurred as early as 1927 (Edwards, 1928) and spread over the entire subcontinent rapidly. Outbreaks of ND are common in Tamil Nadu causing heavy economic losses among commercial poultry flocks inspite of regular and systematic vaccination (Roy and Venugopalan, 1998). The existence of free-living reservoirs like desi birds, pigeons, crows, sparrows and other psittacine species poses constant threat to domestic/commercial poultry population. Apparently healthy desi birds were found to harbour and shed highly virulent NDV without exhibiting signs of the disease (Kamaraj *et al.*, 1998;Raghavan *et al.*, 1998). The wild birds and psittacine species excreting Viscerotrophic Velogenic NDV (VVNDV) in their faeces without exhibiting clinical signs are potential source of virulent NDV to susceptible birds.

Strain identification is of great importance with endemic diseases like ND in countries like India, where different live virus vaccines are used to establish vaccine identity. Differentiation of NDV isolates has been extremely

helpful in understanding the epizootic of NDV in the earlier panzootics. As far as ND viruses are concerned, all NDV strains are considered to form an antigenically homogeneous group on the basis of conventional serological assay using heterologous polyclonal antisera which are unable to differentiate more or less virulent NDV strains.

Pathogenicity tests viz., Mean death time (MDT), Intracerebral pathogenicity index (ICPI) and Intravenous pathogenicity index (IVPI) are useful markers and guides to the pathogenic nature of the isolate, but give no further information and do not indicate epizootiological links between strains with same virulence. Moreover, these tests are cumbersome, time consuming and unsuitable when a rapid strain differentiation is required or a more detailed tracing of an epizootic is needed (Kumanan *et al.*, 1992). Conventional tests yield very little information about the epizootiology of NDV isolates and in particular whether or not they represent different isolates of the same epizootic virus or more than one different epizootic strain. Other strain classification techniques like plaque formation, rate of elution of chicken erythrocytes, thermostability of haemagglutinins, analysis of structural polypeptides, oligonucleotide fingerprinting, lectin binding have been used to differentiate NDV strains epizootiologically with limited success (Seal *et al.*, 1995).

First epizootiologically meaningful classification of NDV isolates was provided only by monoclonal antibody (Mab) analysis, in which NDV strains of a group shared antigenicity and epizootiological properties (Alexander *et al.*, 1985;1987). Differentiation of NDV strains by Mabs indicated that

strains from different panzootics were distinguishable and strains from same panzootics were not uniform (Lomniczi *et al.,* 1998).

Newcastle disease virus strains available all over the globe are classified on molecular epizootiological approaches namely Restriction site analysis of specific gene fragment (Ballagi-Pordany *et al.*, 1996; Lomniczi *et al.*, 1998), Reverse transcription - polymerase chain reaction (RT-PCR) and sequencing of different gene regions for accurate phylogenetic studies (Heckert *et al.,* 1996; Seal *et al.*, 1996; Yang *et al.*, 1997) and Nucleotide homology analysis of the combination of Fusion protein gene cleavage site and Matrix protein gene nucleolar localization signal site sequences (Seal *et al.*, 1995; 1998).

It is clearly evident from the available literature that there is notable paucity of information on epizootiological classification of Indian NDV isolates. With the available information it is observed that considerable antigenic homogeneity was found in Tamil Nadu isolates. Velogenic isolates of the past ten years (1988-1997), on Mab typing revealed a major homogenous cluster of 'C1' group (Alexander *et al.*, 1987). Other groups recorded were 'E' and 'L', consisting of virulent NDV isolates from healthy free living desi birds (Kumanan *et al.*, 1992). However, Mab typing was not efficient in grouping NDV isolates into different clusters, to correlate them epizootiologically.

Owing to the unsuccessfull classification of Indian NDV isolates epizootiologically by Mab analysis, recent molecular approaches like Restriction site analysis of fusion protein gene fragment were employed for preliminary

epizootiological characterization of NDV isolates of Indian origin. Further classification of NDV isolates by RT-PCR coupled to nucleotide sequencing of the fusion protein gene cleavage site and matrix protein gene nucleolar localization signal site regions and phylogenetic analysis of obtained sequences were applied to Indian NDV isolates.

Development of nucleotide sequence database of Indian strains of NDV would aid in effective pathotype prediction and to differentiate vaccine virus from the epizootic virus. It would also aid in molecular epizootiological analysis of NDV obtained from outbreaks, which in turn would help in effective control of Newcastle disease.

Hence, the present work has been undertaken to carry out phylogenetic analysis of field isolates of NDV with the following objectives :-

(i) To identify pathotypes of Newcastle disease virus isolates using Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and sequencing of the amplified PCR products.

(ii) To study the molecular epidemiology of Newcastle disease virus isolates by phylogenetic analysis of obtained nucleotide sequences.

review of literatures

CHAPTER - 2

REVIEW OF LITERATURE

2.1 NEWCASTLE DISEASE VIRUS GENOME AND ITS IMPORTANCE IN DIAGNOSIS AND EPIZOOTIOLOGY

Newcastle disease virus (NDV) or avian *paramyxovirus* type-1, is a member of paramyxoviridae and has been assigned to the genus *Rubulavirus*, in the subfamily *Paramyxovirinae* (Murphy *et al.*, 1995). De leeuw and Peeters (1999) sequenced the complete genome of NDV strain La Sota. They compared the entire genomic sequence to the other members of Rubula virus, to which Lasota is distantly related. On this evidence they suggested that NDV is not a member of the genus *Rubulavirus*, but should be assigned to a new genus within the subfamily *Paramyxovirinae* (Peeters *et al.*, 1999).

Newcastle disease virus (NDV) is classified as a 'List A' agent, that requires reporting to the Office of International epizootes (OIE Manual,1992). Newcastle disease (ND), is enzootic in India and other tropical countries. In India where dense layer populations, open houses and small distance between farms exist, the eradication of the epizootic is probably not possible. Vaccination programmes combined with strict zoo-sanitary and isolation measures, may help in the eradication of the dangerous forms of ND. Zoo-sanitary safety measures to prevent the importation of the virus into vaccinated populations are of great importance, since the course of the disease is masked and diagnosis is difficult in these populations (Blaha, 1989).

Newcastle disease virus has been extensively studied in India by various research workers (Kumanan *et.al.,* 1992; Vijayarani *et.al.,* 1992; Raghavan *et.al.,* 1998; Swain *et.al.,* 1998a). There are only a few studies on epizootiology of the disease and a big gap exists in the area of epizootiological analysis of ND in India. In this review thrust is given to specific areas of NDV genome and its importance in diagnosis and epizootiology, evolutionary pattern of NDV, global and India's research status of NDV pertaining to molecular epizootiology.

2.1.1 NEWCASTLE DISEASE VIRUS GENOME ORGANIZATION

Newcastle disease virus contains a non-segmented single-stranded RNA genome of negative polarity (Lamb and Kolakofsky, 1996). The RNA is 15,186 nucleotides in size (Krishnamurthy and Samal, 1998; Philips *et al.*, 1998; De leeuw and Peeters,1999) and contains six genes in the order 3'leader-NP-P-M-Fo-HN(HN_o)-L5', which encode the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin neuraminidase (HN) and a large polymerase protein (L) (Millar and Emmerson, 1988). In addition to these gene products, additional proteins viz., `V' and `W' may be produced by an RNA editing event that occurs during transcription of the `P' gene (Steward *et al.*, 1993).

2.1.2 HAEMAGGLUTININ NEURAMINIDASE (HN) PROTEIN GENE

The HN gene have both haemagglutinin (HA) and neuraminidase (NA) activities on a single glycoprotein spike (Scheid *et al.*, 1972). Differences in the size of the HN protein have been detected with avirulent lentogenic viruses encoding a large precursor (Miller *et al.*, 1988; Sakaguchi *et al.*, 1989). The size differences are due to a point mutation in the gene, resulting in conversion of stop codons, thus extending the C-terminus of the protein. In virulent NDV isolates encoding a smaller protein, the C-terminal encoding regions of the HN gene are thought to be analogous to pseudogenes. Preliminary suggestions on the origin of NDV strain were based on HN (HN_0) gene and protein sequences. Sequence analysis of HN gene of 11 different isolates have suggested three possible evolutionary lineages. These linkages have been apparently evolved by accumulation of varying numbers of point mutations and not by recombination (Sakaguchi *et al.*, 1989; Toyoda *et al.*, 1989).

2.1.3 FUSION PROTEIN GENE

The molecular basis for NDV pathogenicity is dependent on the fusion protein cleavage site aminoacid sequence (Nagai *et al.*, 1976a; Chambers *et al.*, 1986a; Lelong *et al.*, 1988; Glickman *et al.*, 1988). The amino acid sequence of the fusion protein cleavage site in lentogenic field isolates was ^{109}SGGGRQGRLIG^{119}, while the mesogenic and velogenic viruses have the sequence ^{109}SGGRRQR (K) RFIG119, containing two diagnostic pairs of dibasic aminoacids, associated with virulence. More virulent isolates have the 'RR' sequence instead of 'GR' at position 112 and 113, as well as 'RR' or 'RK' pair rather than 'GR' sequences at position 115 and 116.

There are fewer basic amino acids in the fusion protein cleavage sites of lentogenic strains than in the proteins of either mesogenic / velogenic strains both of which have similar cleavage site sequences. Efficient pathotyping is possible on generating nucleotide sequence of fusion protein cleavage site. Sequence analysis of the fusion protein gene by Toyoda *et al.* (1989) revealed three possible evolutionary lineages.

2.1.4 MATRIX PROTEIN GENE

The matrix protein has a crucial role in virus assembly, interacting with both the nucleocapsid and viral membrane (Nagai *et al.*, 1976b). Matrix protein gene is highly conserved compared with other viral genes and the sequence is not greatly affected during virus attenuation (Chamber *et al.*, 1986b). Matrix protein gene evolutionary relationships were the same as the relationships based on nucleocapsid protein (NP), eventhough fewer changes occurred in the matrix protein gene. Consequently the matrix protein gene was used to determine phylogenetic relationships among recently isolated virulent North American NDV isolates, as compared with previously reported viruses (Seal *et al.*, 1995; 1996).

2.2 RNA VIRUS PHYLOGENY

In virus phylogeny,the RNA viruses were referred to as "quasi-species" populations, since their genomes replicate in the absence of repair mechanisms and evolve very rapidly with a mutation frequency per nucleotide site in the genome of 10^{-3} to 10^{-5}. A clone of RNA virus will therefore always generate many thousands of different genomes all of which compete during

replication of the clone (Holland *et al.*, 1992). Such population which consists of a master sequence corresponding to the most fit genome sequence with respect to a given environment together with countless competing virus mutants, is labelled as "quasi-species population" or "swarm". The term "quasi-species" (Eigen, 1993) is used to convey that viral genome is not a unique molecular species and that a virus cannot be defined by a single genome sequence (Van Regenmortel *et al.*, 1997).

2.2.1 EVOLUTION OF NEGATIVE STRAND RNA VIRUSES

The negative strand viruses have evolved mechanisms to increase the coding capacity of their genome. RNA editing is observed in NDV, where viruses use more than one initiation to start transcription in two overlapping frames (Steward *et al.*, 1993). Paramyxoviruses insert non-templated 'G' residues during transcription to shift the frame downstream of the insertions (Pelet *et al.*, 1991). RNA virus genome are made up of only a small number of genes, almost all of which are essential. RNA viruses evolve so rapidly that only the most important functional motifs and capacities are retained between widely divergent viruses. RNA viruses cause rapidly moving pandemic that much of the information on the variability and divergence of viruses has been accumulated over a period of time.

2.2.2 EVOLUTIONARY PATTERN OF NEWCASTLE DISEASE VIRUS

Nagai *et al.* (1989) compared HN (HN_o) gene and protein sequences of six different NDV strains viz., Miyadera, Australia Victoria, D26, Ulster, Beaudette C and B1, which revealed high genetic relationship between D26

and Ulster. There is no significant similarity between strains Miyadera and Australia Victoria, as well as between strains B1 and Beaudette C. NDV strains have been differentiated into atleast three distinct genetic subtypes. A preliminary evolutionary tree has been constructed based upon aminoacid sequence of HN protein.

Phylogenetic analyses of HN gene sequence from isolates collected over a period of 50 years revealed several features characteristic of NDV evolution. Existence of atleast three distinct lineages ('A', 'B' and 'C') which must have co-circulated for considerable periods has been suggested (Sakaguchi *et al.*, 1989). The 1951 isolate Miy/51 in group 'C' lineage is most closely related to the isolate Aus/32 in the same group 19 years earlier, but is only distantly related to a 3 years earlier isolate Tex/48 in group 'B' lineage. Similarly D26/76 was isolated 11 years after the appearance of a very similar Que/66 virus. D26/76, Que/66 and Uls/67 were completely asymptomatic and so probably existed and circulated before 1966 without being recognised. Thus multiple evolutionary lineages of NDV might have co-circulated for considerable periods. Remarkable divergence of Chiba/85 and Ibargi/85 isolates in the same lineage 'A' suggests significant accumulation of mutations with time. In addition, these 2 isolates in the same area in Japan, diverge considerably from each other. Thus accumulation of mutations in NDV is found to be non-linear. The evolutionary pattern of NDV is distinct from that of influenza A viruses characterised by linear evolution (Raymond *et al.*, 1986). The strains belonging to group 'B' were all North American isolates while the others were isolated in Europe, Australia or Japan. This suggest that the flight paths of migratory birds play some role in maintaining NDV strain groups.

Group 'A' strains were found to be completely asymptomatic for chicken and nonlethal for chick embryo, whereas group 'C' strains were typically virulent, with a mean death time (MDT) of approximately 50 hours (Nagai *et al.*, 1989). The group 'B' strains are either virulent or avirulent with their MDT varying from 50-120 hours. These lineages correlate to some extent with differences in virulence. However virulence of NDV is primarily determined by susceptibility of fusion glycoprotein to proteolytic cleavage (Toyoda *et al.*, 1987).

2.3 EPIZOOTIOLOGY OF NEWCASTLE DISEASE VIRUS

The epizootic process of NDV is a highly complex nexus between the agent reservoir, transmission vectors/factors and the host population. Psittacine species of the Asiatic, African and South-American jungles are considered to be the NDV reservoirs for viscerotrophic velogenic strains of present time. Wild water fowls may be another virus reservoir, since most lentogenic virus strains have been isolated from wild ducks, geese, herons, cormorants and penguins. Latently infected hens which are not sufficiently immunized or which harbour the virulent field virus in the respiratory tract inspite of humoral antibodies, are the agent reservoirs in domestic poultry (Kamaraj *et al.*, 1998; Raghavan *et al.*, 1998). The movement of latently infected birds is of decisive importance for the spread of the epizootic. International trade of caged birds as well as domestic poultry could spread virus over large distances within a short period of time.

2.3.1 EPIZOOTIOLOGICAL RESEARCH STATUS OF NEWCASTLE DISEASE VIRUS IN THE WORLD

2.3.1.1 PANZOOTICS

Newcastle disease was first observed in Southeast Asia in 1926. From there it went to England in ships and in subsequent years it spread all over the world, with extremely rapid spread within some countries. Panzootics occurred from 1940-48 and from 1962-72. International recording and reporting of ND is carried out by the Food and Agricultural Organisation (FAO) of the United Nations, which formed the basis of several assessments of the geographical distribution of the disease (Lancaster, 1977 ; Spradbrow 1988) has reported that ND is widespread in many countries of Asia, Africa and in America and Europe with the presence of variant virus in pigeons.

Alexander (1988a) considered that three panzootics of ND had occurred since the first recognition of the disease.The initial outbreak occurred in South East Asia. The theory of Panzootic spread of ND had arisen in 1926. Newcastle disease took over 30 years to spread world wide and was enzootic in most countries in early 1960's.

The second panzootic appeared to have begun in Middle East in late 1960's and reached most countries by 1973. Virus responsible for this panzootic appeared to be associated with imported caged Psittacine species.

Initially, pigeons and doves were ignored as a potential source of NDV. However, these birds were primarily affected by the third panzootic, the disease resembled the neurotrophic form in chickens but without respiratory

signs. By 1981 it had reached Europe and then spread rapidly to all parts of the world, largely as a result of contact between birds at races, shows and large international trade of such birds. Spread of the disease to chicken has occurred in several countries including Great Britain where 20 outbreaks in unvaccinated chicken occurred in 1984, as a result of feed that has been contaminated by infected pigeons (Alexander *et al.,* 1985).

2.3.1.2 WORLD WIDE EPIZOOTIOLOGICAL INVESTIGATIONS

Alexander (1995) reported that most countries in Western Europe had a long disease free period during the 1980's. However since the early nineties, ND cases have been reported from these countries in increasing numbers. The source of these infections has remained unknown. Early in 1983, the neurotrophic form of Newcastle disease in poultry was noted which had spread rapidly across Europe during 1981-82. Outbreak of the disease and virus involved in Italy, Belgium and Germany have been subsequently reported. This outbreak lead to the banning of Pigeon races in 1983 to prevent spread to British racing pigeons. However the disease was confirmed in a pigeon loft in Cornwall in July, 1983 and the outbreaks were mainly concentrated in South Wales and Dorset (Alexander *et al.*, 1984a).

In Hungary, the acute form of the disease which was widespread after World War II, was eradicated after all the poultry in the country were vaccinated during a three month period (Benedek and Toth, 1950).

The outbreaks of ND in pheasants in 1996 was the first in Great Britain since 1984 (Alexander *et al.*, 1997). These outbreaks and those in Great Britain in 1984 (Alexander *et al.*, 1985) were shown to have been caused by the variant virus responsible for the panzootic in racing pigeons (PPMV-1) which had reached Great Britain in 1983 (Alexander *et al.*, 1984) and is still detected occasionally in pigeons.

Six outbreaks of ND were recorded in Northern Ireland in 1991 and one in 1996; these two were PPMV-1 infections and occurred as a result of stores of feed having been contaminated by infected feral pigeons. Apart from PPMV-1 infections, no outbreak of ND had been confirmed in poultry in UK since 1978 (Alexander *et al.*, 1998).

Erickson *et al.* (1977) studied the ND viral evolution through bird passage and suggested that Psittacine isolate of viscerotrophic velogenic nature slowly evolved to relatively avirulent strains of NDV by passage in pet birds. However, reversion did not occur during the chicken back-passage studies.

Outbreaks of velogenic Newcastle disease in vaccinated and unvaccinated poultry flocks from commercial and backyard farms in different parts of Nigeria has been reported by Adu *et al.* (1985). A viscerotrophic velogenic isolate from a large broiler operation in Saudi Arabia was recorded by El-zein (1986). Velogenic isolates of NDV from tracheal swabs taken at a Moroccan live poultry market were found to be of viscerotrophic pathotype (Bell, 1986).

Kawamura *et al.* (1987b) isolated velogenic NDV from imported caged birds in South East Asia in 1979-80. Mesogenic and lentogenic NDV were isolated from wild ducks in Japan by kawamura *et al.* (1987c).

Japan NDV isolates from 1930 to 1984, isolated from field outbreaks were characterised by Takehara *et al.* (1987). NDV isolates from imported parrots (*Katakoe sulpurea*) was reported by Hirai *et al.* (1980) and in racing pigeons by Shirai *et al.* (1986).

Buonavoglia *et al.* (1991) have isolated velogenic NDV strains from pigeons in Italy. On plaque cloning of the viruses, each NDV strain was found to contain different clones of genetically mixed viral populations. The pigeon NDV isolates were typed as lentogenic, using MDT while ICPI was the same as that of velogenic NDV strains.

Velogenic viscerotrophic Newcastle disease was diagnosed in domestic psittacines in different states of USA. The disease assumed outbreak proportions in the Illinois, Indiana, Michigan and Texas. The affected psittacine birds were Amazon parrots, cockatiles and conures. VVNDV was also isolated from quarentined birds viz., parakeets, mynah, partridges and groups tested positive were denied entry into the Untied States (Johnson, 1992). Panigrahy *et al.* (1993) have also suggested illegally imported birds not tested are a potential source of VVNDV and a threat to domestic poultry.

Avian paramyxovirus - 1 was isolated from posted spinal cord samples from imported frozen pekin duck carcasses into Germany from Thailand in 1990-91. Transmission of NDV through frozen carcasses has been found to be a potential threat for introduction of new NDV strains into a country

Koh-Yeo *et al.* (1996) isolated lentogenic and velogenic ND viruses from captive birds imported from Indonesia, Vietnam, Belgium and Netherlands into Singapore, during 1992-1995.

Velogenic NDV isolates were recovered from dead and healthy roaming free-birds viz., ducks and pigeons in Nigeria (Echeonwu *et al.*, 1993). Heckert *et al.* (1996) compared NDV isolates from cormorants in Canada and USA and suggested that 1990 and 1992 outbreaks were caused by the same epizootic virus.

NDV field isolates from Puerto Rico, Georgia, Alabama, Mississippi and Texas were characterized by Marin *et al.* (1996) and the minor genetic hetrogenecity among lentogenic field isolates of NDV was revealed.

Gribanov *et al.* (1999) suggested a replacement of NDV populations in Russia and a rapid evolution of the virus based on sequence determination of the fusion gene of isolates and origin of the pathogenic NDV strains which have been circulating currently is still not found.

Neurotrophic velogenic NDV (NVNDV) was isolated from Juvenile double-crested cormorants simultaneously from Minnesota, North Dakota, South Dakota and Nebraska of United States in 1992. The NVNDV was identified as the cause of neurologic disease in a North Dakota Turkey flock in 1992. Risk to susceptible populations of both wild avian species and domestic poultry made early recognition and confirmation of NVND in wildbirds a priority (Meteyer *et al.,* 1997).

Yang *et al.* (1999) on analysing NDV isolates of three major outbreaks ian Taiwan in 1969, 1984 and 1995 together with the 1998 outbreak, suggested that the outbreaks were distinct from the third panzootic caused by PPMV-1 viruses and suspected these outbreaks constituted a fourth panzootic of ND.

The epidemiology of NDV outbreak in free-living pheasants on the island of Faeno in Denmark in 1996 was described by Jorgensen *et al.* (1999) and it is believed that the virus was transmitted to the pheasants by feral birds.

Recurrence of 1992 epizootic of ND in three western Canadian provinces were described by Glaser *et al.* (1999). The simultaneous onset of the epizootics in Juvenile birds over a wide geographic area implied that the virus acquired by adults prior to the migration and carried back to nest sites exposing susceptible nestlings. They also suggested that NDV has established in double crested cormorants owing to the repeated epizootics in cormorants since 1990.

Kuiken *et al.* (1998) described the epidemics of pathogenic NDV in double-nested cormorants in Canada and United States and its occurrence several times in recent years (Wobeser *et al.*, 1993; Meteyer *et al.*, 1997).

The majority of NDV strains isolated from water fowls viz., ducks and geese are lentogenic, causing no obvious disease. Takakuwa *et al.* (1998) have provided information on how NDV is maintained in duck population in their breeding places. Potentially virulent strains are found circulating among migratory water fowl populations which may be implicated in outbreaks of ND in domestic poultry. ND outbreaks were observed in south Marmara region of Turkey from December 1992 to April 1993. Velogenic isolates were the cause of the epizootic recorded in domestic pigeons and chickens (Oncel *et al.*, 1997). Viscerotrophic velogenic NDV isolates caused outbreak of ND in California from 1972-1974. Seal *et al.* (1998) have suggested that VVNDV have entered the United States via importation of exotic birds. Werner *et al.* (1999) have reported that velogenic NDV isolates were responsible for the epizootic from 1993 to 1995 in small poultry flocks, which was also isolated from pigeons and exotic birds. It is suggested that the PPMV-1 viruses are enzootic in pigeons in Germany.

2.3.2 EPIZOOTIOLOGICAL RESEARCH STATUS OF NEWCASTLE DISEASE VIRUS IN INDIA

Isolation of a mesogenic strain of NDV, different from the Mukteswar vaccine strain (R_2B) based on mean death time and intracerebral pathogenicity index was reported by Maiti *et al.* (1984). Isolation of a naturally occurring mesogenic strain of NDV could be of epidemiological importance, as no outbreak involving a mesogenic strain has been reported until 1984 in India.

Characterisation of field isolates of NDV from India by conventional strain differentiating techniques viz., stability of haemagglutinins, haemagglutination activity with mammalian erythrocytes, stability of infectivity at pH 3.0 was employed with limited success (Chandra *et al.*, 1972; Sulochana and Mathew, 1991; Kumanan *et al.*, 1992; Vijayarani *et al.*, 1992; Raghavan *et al.*, 1998).

Sulochana *et al.* (1981) studied the epizootiology of ND in Indian house crows using experimental infection and isolation of NDV from live and dead crows suggested the carrier status of the birds. It was also concluded that crows excrete the virus only for a short period and act as carriers for short duration. However, crows can play an important role in transmitting NDV due to their eating habits. An NDV isolate from Indian house crow was characterized by monoclonal antibody typing (Roy *et al.*, 1998a). The isolate was grouped as 'E' type which encompasses B1 or La Sota-like viruses and was classified as a mesogenic strain.

Viscerotrophic velogenic and mesogenic strains of NDV have been isolated from pigeons in India by Sulochana and Mathew (1991) and Gurkipal Singh (1993) and Gurkipal Singh *et al.* (1989) respectively. On Mab typing the viscerotrophic velogenic isolate was placed under group 'B' and was typical of a viscerotrophic velogenic strain which was originally isolated in the 1930. The strain was found highly pathogenic to chickens (Sulochana and Mathew, 1991).

Occurrence of NDV concurrently with *E.coli* and Infectious bursal disease virus in broilers were reported by Mukherjee and Khanapurkar (1994).

An outbreak of ND in Japanese quails in Tamil Nadu was recorded by Kumanan *et al.* (1990). Isolation and characterisation of the NDV from Japanese quails suggested that the isolate was velogenic and highly pathogenic to chickens (Kumanan and Venkatesan, 1991; Srithar et.al., 1994). Three NDV isolates from local outbreaks were characterised as velogenic by conventional pathogenicity tests (Vijayarani et.al., 1992).

Field isolates of NDV obtained from broilers, layers and Japanese quails in Tamilnadu, India were characterized by pathogenicity tests and monoclonal antibody typing for distinguishing between different strains (Kumanan *et al.*, 1992). On analysis using a panel of 28 Mabs (Russel and Alexander, 1983) none of the isolates inhibited panels specific for the current panzootic in pigeons or for vaccine strains like La Sota and F, suggesting considerable antigenic homogeneity. All the isolates were placed under 'C1' group and were of velogenic characteristic.

Raghavan *et al.* (1998) characterised NDV isolates from apparently healthy and ailing desi chickens using conventional pathotyping, Mab typing and oligonucleotide hybridization. All the isolates on hybridization with a 21 - mer probe from the conserved region of the fusion gene of Texas GB strain of NDV. (Black and King, 1993) gave positive signals indicating the velogenic / mesogenic characteristic of the isolates. The Mab typing yielded 'E' & 'L' groups apart from the major 'C1' group. In another study, five NDV isolates from desi birds were characterised as velogenic using conventional techniques (Kamaraj *et al.*, 1998).

Differences in correlation between pathotyping and Mab typing of field isolates of NDV was observed by Roy *et al.* (1998b). The pathotyping tests suggested that isolates were of velogenic / mesogenic type, but on Mab typing, the isolates were of mesogenic or Lentogenic category. Possibility of lentogenic and mesogenic vaccine strains gaining virulence due to continuous passage in the field is suggested (Roy *et al.*, 1998b).

Swain *et al.* (1998a) raised mouse monoclonal antibodies against Indian velogenic NDV isolate and used for characterizing field isolates from chicken, guineafowl and pigeons. No significant differences were observed on Mab analysis of those isolates.

Velogenic NDV isolates from apparently healthy captive wild birds were characterized by pathotyping and Mab typing. All the isolates tested were velogenic type on pathotyping. Three isolates typed by Mab panels placed them under 'C1' group and have been found exclusively velogenic for chickens (Roy *et al.*, 1998b).

2.4 CONVENTIONAL TECHNIQUES FOR CHARACTERIZING NDV ISOLATES

Preliminary characterization of NDV isolates by pathogenicity tests for assessment of virulence is mandatory prior to molecular characterization. Pathogenicity tests like MDT (Hanson and Brandly, 1955) to classify the isolates on the basis of the mean time for the minimum lethal dose to kill the chicken embryos viz., Velogenic < 60 hours, Mesogenic 60 to 90 hours and

lentogenic > 90 hours. It is considered imprecise for NDV isolates from hosts other than chicken (Alexander, 1988b). The Intracerebral pathogenicity index (ICPI) in day old chicks (Allan *et al.,* 1978) which is not very sensitive in distinguishing between virulent viruses. Moderately virulent strains were also found to give high ICPI values, as in the case of Komarov and Mukteswar strains. The other test used for strain differentiation is the Intravenous pathogenicity index in six-week-old chickens (IVPI), in which most virulent viruses give IVPI values approaching 2.0, while lentogenic and mesogenic viruses give values of 0.0 (Alexander, 1989).

Other techniques for strain differentiation like thermostability of HA activity (Hanson *et al .,* 1949), plaque formation in cell culture (Schhloer and Hanson, 1968), rate of elution of chicken RBC's agglutinated by the virus (Spalatin *et al.,* 1970) and variations of structural polypeptides (Moore and Burke, 1974) have also been tried in the past with limited success. Virus neutralization (VN) or agar gel diffusion technique (Pennington, 1978) has been followed in different combinations of the above tests by various research workers for characterising the field isolates of NDV. Lectin binding profiles (McMillan *et al .,* 1986) has also been tried in the past with limited success.

2.5 MOLECULAR EPIZOOTIOLOGY OF NEWCASTLE DISEASE VIRUS

The deployment of molecular tools for unravelling the complex epizootiological process in Newcastle disease, has led to generation of data confirming the hypothesis of outbreaks and spread of the disease. The use of

nucleic acid technology to disease diagnosis and for real-time epidemiological studies in identifying the pathotype and specific genotypes for correlation of epizootiological data has been carried out for various avian pathogens including Newcastle disease virus (Cavanagh *et al.*, 1997). The other area is the monoclonal antibody (Mab) technology which led to development of highly specific antibodies and enabled both grouping and differentiation of NDV isolates from various epizootics (Alexander, 1990).

2.5.1 MONOCLONAL ANTIBODY TYPING

It is important to classify the NDV isolates as whether they are vaccinal or enzootic for subjecting legislation that may be in force for epizootic viruses (Alexander, 1990). Mabs against highly conserved epitopes of NDV have been reported by several workers(Russel and Alexander, 1983; Ishida *et al.*, 1985; Meulemans *et al.*, 1987; Faaberg and Peeples, 1988; Lana *et al.*, 1988; Panshin *et al.*, 1995, 1997; Swain *et al.*, 1998a).

Monoclonal antibodies specific for one or more of the commonly used live vaccine strains (Erdei *et al.*, 1987; Meulemans *et al.*, 1987) have enabled rapid identification of such viruses during epizootiological investigations.

Alexander *et al.* (1985) used a Mab that inhibited the vast majority of NDV isolates and strains, but not the variant virus responsible for the pigeon panzootic, to allow rapid identification of the pigeon virus.

A monoclonal antibody against the pigeon variant gave high haemagglutination inhibition titres to the isolates of that variant, but not to any other PMV - 1 virus, but had a cross reactivity with some PMV-3 viruses (Collins *et al.*, 1989).

Use of panels of Mabs to distinguish different isolates and place them into meaningful groups in terms of biological, epizootiological properties have been carried out by several groups. (Abenes *et al.*, 1983; Hoshi *et al.*, 1983; Russel and Alexander, 1983; Ishida *et al.*, 1985; Srinivasappa *et al.*, 1986; Meulemans *et al.*, 1987; Lana *et al.*, 1988). They also confirmed that considerable variation exists between viruses placed in the APMV-1 serogroup using polyclonal antibodies. The usefulness of such diversity in the diagnosis and epizootiology of ND outbreaks depend largely on the relationships of such antigenic diversity with biological properties of the virus and maintenance of the detectable antigenicity during an epizootic (Alexander *et al.*, 1997). Panels of Mabs have been found useful in distinguishing between epizootic and vaccinal viruses and for tracing the spread of virus in an epizootic (Hoshi *et al.*, 1983; Nishikawa *et al.*, 1983 ; Russell and Alexander, 1983; Meulemans *et al.*, 1987; Lana *et al.*, 1988; Jestin *et al.*, 1989; Collins *et al.*, 1994; Heckert *et al.*, 1996; Alexander *et al.*, 1997; King and Seal, 1997; Oncel *et al.*, 1997; Werner *et al.*, 1999).

Alexander *et al.* (1997) on cluster analysis using an extended panel of 26 Mabs with a 9 MAbs group suggested that clustering based on Mab binding patterns does not neccessarily have any phylogenetic significance since a single nucleotide difference leads to change in epitope recognized by MAbs.

Russel *et al.* (1990) and Collins *et al.*(1996) have reported that grouping produced by phylogenetic analysis of ND strains are not inconsistent with those found by MAb analysis.

Ballagi-Pordany *et al.*(1996) and Lomniczi *et al.*(1998) grouped ND viruses by restriction site analysis of a region from the F gene and emphasized the similarity between their groupings and those determined by the 9 MAb panel used by Alexander *et al.*(1997).

Newcastle disease viruses emerged in Europe in 1991 showed distinct MAb binding (Northern Europe group, pattern 37) which enabled the spread of this virus to be traced through eight countries during the next three years. It has also allowed distinction between that virus and viruses responsible for the outbreaks during that period, as in portugal (Alexander, 1995) thus aiding to find the epizootiological links between outbreaks caused by different viruses.

Lomniczi *et al.* (1998) suggested that MAb binding group NE (Alexander *et al.*, 1997) is equivalent to the genetic group VII based on F gene restriction site grouping/analysis. Further it is indicated that more information about the antigenic and genetic inter-relationships of NDV strains required a systematic comparison.

Kawamura *et al.* (1987a) on MAb analysis of the NDV isolates from caged birds from south east Asia, suggested that most of the isolates were distinguishable from more classical NDV viz., B1 and Miyadera strains. Four distinct antigenic groups of NDV isolates from wild ducks in Japan were reported by Kawamura *et al.* (1987c) and were antigenically distinct from B1 and Miyadeta strains.

Antipeptide antibodies have been evaluated to predict characteristics of the cleavage motifs of the fusion protein precursors (F_0) (Hoddar *et al.*, 1994) of 25 isolates of NDV and viruses with varying virulences were grouped into 12 sets according to their MAb reactivities (Scanlon *et al.*, 1999)

King and Seal (1997) on analysis of a pheasant isolate, found the MAb binding profile to be typical of other US NDV lentogens, but differed in matrix gene sequence which was similar to Ulster and Queensland V4 viruses, originally isolated in Northern Ireland and Australia respectively.

Avian Paramyxovirus-1 strains isolated in Germany during 1992 to 1996 were examined with a panel of MAbs (Werner *et al.*, 1999). It has been found that all the lentogenic isolates reacted with two MAbs specific for La Sota-like viruses, but none reacted with MAbs 39 or 161/167, which are specific to PPMV-1. Isolates of high virulence were the epizootic virus which caused multiple outbreaks of ND in Germany. The binding patterns with both MAb panels produced by the viruses isolated in 1993 to 1994 differed from those isolated in 1995. The MAb binding pattern obtained on analysis using a panel of 26 Mabs for the 1993 to early 1994 isolates was the same as that for viruses isolated from other ND outbreaks in Western European countries (Alexander, 1995; Alexander *et al.*, 1997). The isolates from late 1994 showed more differences in antigenicity and gave usual binding pattern using the panel of 26 MAbs, which was distinct from the NE group viruses (Werner *et al.*, 1999).

2.5.2 RESTRICTION SITE ANALYSIS

Reverse transcription coupled to Polymerase Chain Reaction (RT-PCR) for selective amplification of a short segment of the viral genomic RNA and restriction site analysis of the amplified fragment has been used for differentiation of vaccine and wild-type viruses (Jestin and Jestin, 1991). A 238 bp fragment of the fusion gene including the fusion protein cleavage site has been analysed with three restriction enzymes namely, Hae III, Nar I and Mbo II. Analysis of restriction sites of the amplicon from La Sota clone 30 strain yielded two fragments of 131 bp and 141 bp with Hae III and with a group of fragments at positions between 184-192 bp and 80-89 bp with Nar I. Mbo I did not yield any fragments on analysis of 30 APMV-1 strains.It has been observed that two strains from feral ducks appeared to have a deletion of 25 bp inside the amplified region, as detected by smaller length of the amplified product and the detection of Nar I site (Jestin and Jestin, 1991).

Stauber *et al.* (1995) analysed a segment of the fusion protein gene from vaccine strains Hitchner B1, LaSota and inactivated vaccine P3G with restriction enzymes Nar I and Hae III. Two fragments of 131 and 144 bp were observed with all three strains using Hae III. Digestion with Nar I did not cleave P3G strain, but digested La Sota and Hitchner B1 strains.

Restriction site analysis of a partial matrix gene amplicon of LaSota and B1 vaccine strains of different companies with Mbo I and Hinf I, generated strain specific characteristic cleavage maps for both (Wehmann *et al.*, 1997). These results suggested that vaccine strain differentiation is indispensable for identification of vaccine seeds and lentogenic field isolates derived from respiratory conditions of poultry.

Restriction site analysis was carried for lentogenic field isolates derived from regions where exclusively or predominantly only one type of vaccine (B1 or Lasota) was employed in Canada and Hungary. On analysis of restriction sites of the matrix gene amplicon, the viruses collected in Hungary were typed as Lasota-like viruses. The Canadian isolates were predominantly B1 type and corresponded to vaccine strains used in the region (Wehmann *et al.*, 1999). The lentogenic vaccine strains classified into genotype I and II (Ballagi-Pordani *et al.*, 1996) were analysed and suggested the increase in genotype I strains, where such vaccine strains are used (Wehmann *et al.*, 1999).

Restriction site analysis of fusion gene fragment of NDV strains representing most of the countries generated six distinct groups of NDV isolates and unique fingerprints of vaccine strains (Ballagi - Pordany *et al.*, 1996). The genetic groups classified were based on the presence and/or absence of a unique restriction site on the fusion gene amplicon, using enzymes Hinf I, Bst OI and Rsa I. RT-PCR Restriction site analysis for strain comparison was found useful for grouping NDV strains into distinct categories in which, strains shared epizootiological relationships or possibly common descent (Table.1) It also aided unambiguous identification of individual strain namely, vaccine strains (Ballagi-Pordany *et al.*,1996).

Lomniczi *et al.* (1998) studied the fusion gene restriction sites of different NDV strains from various host species and countries. They indicated that the first and second panzootics in poultry were composed of etiologically different streams of infection each caused by genetically distinct viruses. They

Table - 1 : Genetic grouping of NDV strains by restricition analysis of fusion gene
(Ballagi-pordany *et al.*,1996, Lominiczi *et al.*, 1998*)

GROUP	STRAINS
I	Lentogenic strains
II	Old(1960's)north american isolates lentogenic and mesogenic strains
III	Early isolates from far east
IV	Early european strains of first panzootic (1920') & their descendants with modifications
V	Psittacine isolates & epizootics of chickens at the early 1970's
VI	Middle east(1960's) & later isolates from asia and europe
VII*	Recent european NDV strains - novel genotype
RI-3*	Far-east isolates-Indonesia and Taiwan

suggested that atleast three different genotypes (Group II - IV) were responsible for the epizootics during the first panzootic, each showing geographical restrictions. Two new genotypes of NDV emerged during the second panzootic. The strains were believed to be transmitted by imported psittacine birds, typed as group V and were responsible for outbreaks in England and other European countries as well as in California in 1970-71. It differed from those caused epizootics in the Middle East and Greece during the late 1960s belonging to group VI. Pigeon PMV-1 viruses formed a separate cluster with the group VI. Viruses related to the psittacine transmitted group (V) have recently been implicated in isolated cases of ND in colonies of cormorts and in turkeys in United States (Seal *et al.*, 1995; 1996).

2.5.3 NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSIS

First step towards the molecular epidemiology and evolution of the paramyxovirus, PMV-1 the Newcastle disease virus was made by Sakaguchi *et al.* (1989) and Toyoda *et al.* (1989), by comparing the nucleotide sequence of HN gene and fusion gene respectively. The HN gene sequence analysis of 13 strains isolated over a period of 50 years demonstrated the existence of atleast three distinct lineages, which must have co-circulated for considerable periods (Sakaguchi *et al.*, 1989). Toyoda *et al.* (1989) suggested three distinct evolutionary lineages correlating with virulence as expressed by MDT for chick embryo.

Collins *et al.* (1993) characterised NDV strains covering ten antigenic groups for the variation of amino acid sequence at fusion protein cleavage site. All the virulent viruses and the mesogenic komarov strain showed the amino acid sequence ^{112} R/K-R-Q-K/R/R^{116} for the 'C' terminus of the F2 protein and phenyl alanine at the 'N' terminus of the F1 protein, residue 117. They suggested that the mesogenic isolate of the antigenic variant NDV responsible for the recent panzootic in racing pigeons - PPMV-1, had the sequence ^{112} G-R-Q-K-R-F^{117}.

The analysis of aminoacid sequence of PPMV-1 strains of NDV showed close antigenic identity, determined by MAb typing, but had considerable variation in their pathogenicity for chickens. The high virulence of some of the viruses examined, confirmed that a double pair of basic aminoacids in the region of the F2/F1 cleavage site is not necessary for the full expression of virulence (Collins et.al., 1994).

Seal *et al.* (1995) have analysed the fusion protein cleavage site (FPCS), and matrix protein nuclear localization signal (MPNLS) region of a wide variety of NDV isolates and strains. They used a degenerated oligonucleotide primer pair for amplifying the FPCS and MPNLS fragment. On sequencing and analysis of amino acid sequence at the FPCS, the virulent and mesogenic isolates had the sequence ^{109} SGGRRQR(K)RFIG^{119}, while the lentogenic field isolates had the sequence ^{109}SGGGRQGRLIG^{119}. The two diagnostic pairs of dibasic aminoacids 'RR' associated with virulence was found in more virulent isolates. The matrix protein region gave no specific distinguishing sequence variations among lentogenic field isolates in comparison with the mesogenic and velogenic pathotypes, but could observe sequence differences among the psittacine and cormorant - derived isolates.

By phylogenetic analysis of the FPCS and MPNLS sequences, the NDV strains were classified into two major groups, one branch contained neurotrophic velogenic isolate Texas / GB, mesogenic kimber and the lentogenic vaccine strains B1 and Lasota. The second branch included the viscerotrophic velogenic Herts / 33 and neurotrophic velogenic Australia / Victoria isolates, with only one mesogenic isolate in this group. Phylogenetic analysis was able to efficiently determine viral origins apart from pathotype prediction. The NDV isolate from Fontana, obtained from chickens during 1970's ND outbreak in Southern California was genotypically related to the psittacines type isolates. The finding supported epizootiological information implicating pet birds as the cause of the outbreaks. It has been concluded that two major phylogenetic groups one with ND viruses common worldwide including North American and the second with viruses from psittacines and isolates considered exotic to United States, are in existence (Seal *et.al.*, 1995 Fig. 1).

Analysis of a major region of the matrix protein gene sequences, which revealed nucleotide differences between individual isolates indicating two groups of NDV isolates. First group encompassed isolates Texas/GB, VGGA, Kimber and vaccine strain B1, Beaudette C and Lasota, which shared 95% homology at nucleotide sequence level compared to B1 as reference. The second group on similar sequence comparison had a 91% homology and included isolates Largo, turkey/ND, Fontana, Australia / Victoria, Herts / 33, D26, Ulster and Queensland / V4. The avirulent pathogens could be separated from their respective mesogenic and velogenic counterparts within their respective groups. The phylogenetic analysis suggested that psittacine-type viruses may be circulating in wild and pet bird populations or, are being introduced from sources outside the United States (Seal, 1996).

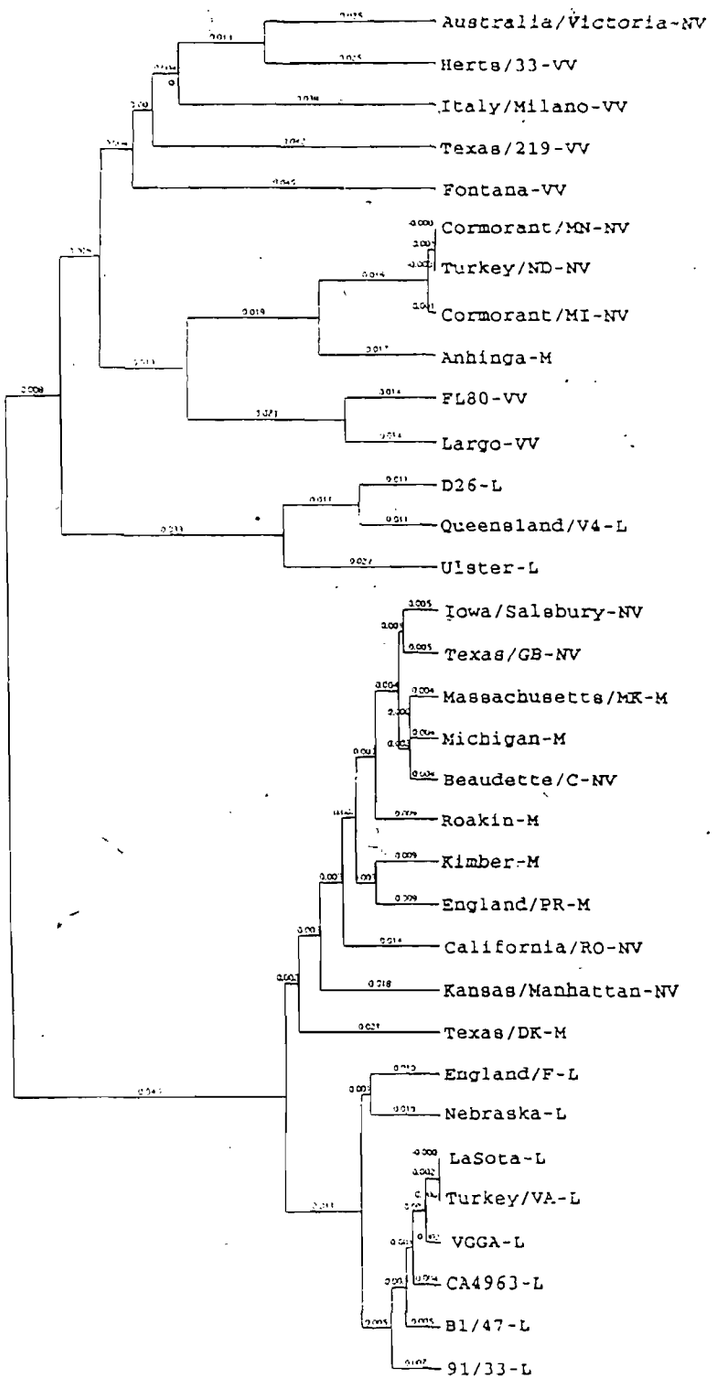


Fig. 1 : Phylogenetic analysis of contiguous nucleotide sequences from the amplified products of NDV fusion and matrix protein genes. A Phylogenetic tree was generated by UPGMA following alignment of all sequences. Number represent the percent chance of a nucleoti .de substitution at one site. L-lentogenic,M-mesogenic,VV-Viscerotrophic velogen, and NV-Neurotrophic velogen (Seal *et al.*, 1995).

Collins *et al.* (1996) analysed the entire fusion gene sequence of pigeon PMV-1 variant of NDV and compared with published sequences of other NDV strains and isolates. Phylogenetic analysis of the PPMV-1 fusion gene sequences showed that the PPMV-1 viruses formed a new fourth lineage, but is closely related to strain Warwick with which it was presumed to share a common origin.

Phylogenetic relationships among highly virulent NDV isolates obtained from exotic birds and poultry in USA from 1989 to 1996 were analysed by Seal *et al.* (1998) as studied earlier (Seal *et al.*, 1995). The isolates were categorized with other highly virulent NDV strains that caused outbreaks in Southern California during 1990 and 1992. These isolates were related to NDV that might have the APMV-1 strain chicken / Australia / AV/ 32 or a related virus as a possible progenitor. Recent virulent NDV isolates and those recorded during disease outbreaks since the 1970s are phylogenetically distinct from current vaccine viruses and standard challenge strains (Seal *et al.*, 1998). (Fig. 2)

NDV isolates obtained from cormorants, turkey, pelican and a gull in Canada and the USA in 1975, 1990 and 1992 were analysed for the fusion gene signal region (Heckert *et al.*, 1996). The data obtained indicated that the 1990 and 1992 outbreaks were caused by the same epizootic virus and further that the population of NDV in these wild birds may be very stable.

Marin *et al.* (1996) characterised the FPCS sequence of NDV isolates obtained from Puerto Rico, Georgia, Alabama and Mississippi. It is found that all the isolates had a predicted fusion cleavage sequence comparable to

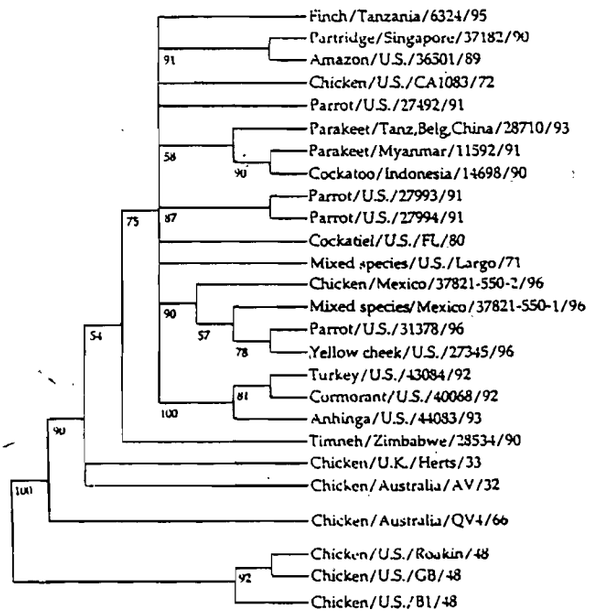


Fig. 2 : Phylogenetic analysis of contiguous nucleotide sequences from the amplified products of NDV fusion and matrix protein genes. A Phylogenetic tree was generated by parsimony analysis of the aligned contiguous nucleotide sequences. Numbers represent the bootstrap confidence levels following 1,000 replications **(Seal *et al.*, 1998).**

lentogenic NDV strains. Phylogenetically the viruses were grouped with the B1 NDV strain, indicating occurrence of minor heterogenecity among lentogenic field isolates.

Phylogenetic analysis of the fusion gene of NDV isolates obtained from waterfowls in Alaska and Siberia during 1991 to 1996, revealed that potentially virulent NDV strains are maintained in migratory waterfowl populations in nature (Takakuwa *et al.*, 1998). Moreover these NDV isolates were closely related to virulent strains and possessed the virulent type fusion gene cleavage site.

Lomniczi *et al.* (1998) furthered the restriction site analysis genotype groups by 'F' gene sequencing and suggested that group VI caused repeated epizootics by molecular variants. These slightly diverged variants emerge in endemic foci of unknown locations before being introduced into susceptible population. They suggested a new genotype VII / European strains which had genetic similarity with Indonesian ND viruses. The NDV strains responsible for epizootics between 1992 and 1996 belonged to two distinct genotypes, genotype VI with strains from Denmark, Sweden, Switzerland and Austria and genotype VII with strains from Netherlands, Spain and Italy.

King and Seal (1998) analysed sequences of FPCS and MPNLS regions of mesogenic Annhinga isolate and lentogenic field isolates. The Annhinga isolate was closely related to cormorant isolate responsible for 1992 outbreak. The parrot isolates showed similarity with other United States lentogens rather than showing homology to exotic ND viruses.

NDV isolates from Israel were analysed phylogenetically by Stram *et al.* (1998). They were able to resolve the ambiguous results of the group nature of a isolate which differed only by 6% to the other Israeli velogenic isolate, by comparing with published nucleotide sequences on phylogenetic analysis.

An outbreak of ND was studied in pheasants using sequence analysis of an RT-PCR amplified segment of Fo viral protein. The sequences were similar to virulent APMV-1 strains and suggested that the virus was transmitted to the pheasants by feral birds (Jorgensen *et al.*, 1999).

NDV isolates from three major outbreaks in Taiwan viz., 1969, 1984 and 1995 along with 1998 isolates were phylogenetically analysed with their fusion gene amplicon sequences (Yang *et al.*, 1999). They found that the 1969 isolate was similar to genotype III viruses. The isolates from 1984, 1995 and 1998 were grouped as genotype VII viruses. The results of the study suggested that the 1969 outbreak was caused by genotype III virus and 1984 and 1995 outbreaks by genotype VII viruses. A 'fourth panzootic' of ND caused by genotype VII viruses, which is distinct from PPMV-1 has been proposed.

NDV isolates obtained during 1992 to 1996 from different host species were analysed after obtaining the fusion gene cleavage site sequences, along with other pathotyping tests. Velogenic isolates from chickens were responsible for the epizootic in 1993 to 1995 in many small flocks and was obtained from pigeons and exotic birds. All the velogenic epizootic isolates sequenced showed the same sequence at the cleavage site, regardless of the year of isolation and

the antigenic reaction. The PPMV-1 mesogenic isolates sequences appeared to be more variable with many possessing a new motif with five basic aminoacid residues at the proteolytic cleavage site. The presence of Lysine, K, at the residue position 114 which was not recorded previously, is reported (Werner *et al.*, 1999).

Gribanov *et al.* (1999) studied Russian NDV isolates and vaccine strains by sequencing fusion gene cleavage site including several hypervariable regions. They suggested a replacement of NDV populations in Russia and a rapid evolution of the virus, though could not describe the origin of the circulating pathogenic NDV strains.

Phylogenetic analysis of vaccine strains FPCS region (Stauber *et al.*, 1995; Seal *et al.*, 1996) and HN gene (Seal *et al.*, 1996) were carried out to find the stability of the vaccine strains. The sequences obtained correlated with their respective parent viruses.

2.5.4 PHYLOGENETIC ANALYSIS AND CONSTRUCTION OF TREES

Phylogenetic analysis of viral genomic and protein sequences is a common method used currently to determine origins and evolutionary relationships among isolates. A phylogenetic tree based on a gene (nucleotide or aminoacid sequences) is called as a 'gene tree'. Gene trees are studied to know the evolutionary relationships of genes belonging to a multi gene family or of polymorphic alleles within and between species (Kumar *et al.*, 1993).

2.5.4.1 PHYLOGENETIC TREE BUILDING METHODS

Tree-building methods from molecular data (Felsenstein, 1993) could be classified into distance methods and discreet character methods. In distance methods, a pairwise evolutionary distance is computed for all species, whereas in discreet character methods data with discreet character states viz., nucleotide states in DNA sequences are used and a tree is constructed by considering the evolutionary relationships of DNA sequences at each character or nucleotide position.

2.5.4.2 PHYLOGENETIC ANALYSIS SOFTWARES

Nucleotide sequence editing, analysis and prediction of amino acid sequences and alignments have been conducted using Intelligenetics Gene works 2.4 software (Seal *et al.,* 1995; 1996; 1998; King and Seal, 1998). Lomniczi *et al.* (1998) used Megalign program in the Laser gene package using clustal multiple alignment algorithm (Higgins and Sharp, 1989) for nucleotide sequence alignment.'Pile up' program of the Genetic computing Group (GCG) package was used for sequence alignment by Collins *et al.* (1996); Stram *et al.* (1998) and Yang *et al.* (1999) before subjecting the data to phylogenetic analysis software.

Phylogenetic analysis of aligned sequences were carried out using softwares, viz., Intelligenetics Gene works 2.4 using the unweighted - pair group method with arithmatic means (UPGMA) (Nei, 1991) which is based on pairwise comparisons of most similar sequences (Seal, 1996; Seal *et al.*, 1995;

King and Seal, 1998). Additionally, phylogenetic trees were constructed by use of phylogenetic analysis using Parsimony (PAUP) (Swofford, 1993) Software with a heuristic search and 1000 bootstrap replicates (Seal *et al.,* 1995; 1998, Yang *et al.,* 1999).

The Molecular Evolutionary Genetic Analysis (MEGA) software (Kumar *et al.,* 1993) was employed by using UPGMA for phylogenetic analysis of NDV sequences (Seal *et al.,* 1995; Seal., 1996).

The Phylogeny Inference Package (PHYLIP) (Felsenstein, 1993) software's seqboot was used to produce bootstrap replicates and programmes DNAPARS and DNADIST (Kimura '2-parameter' model, K) were used to calculate distance matrices from each replicate data set. Finally DNAML programme was used to derive trees using distance matrices - Neighbour joining (NT) (Saitou and Nei, 1987) and UPGMA (Sneath and Sokal, 1973). The program DRAWTREE was used to produce graphics output of the phylogenetic tree of NDV sequences (Collins *et al.,* 1996; Yang *et al.,* 1999).

Lomniczi *et al.* (1998) used the software TREECON for windows (Van de Peer and Watcher, 1994) for establishing phylogenetic relationship between different NDV isolates. The NDV sequences from waterfowls were phylogenetically analysed using the UPGMA method with the software ODEN by Takakuwa *et al.* (1998).

2.5.5 OTHER MOLECULAR TECHNIQUES

RNA oligonucleotide finger printing has been employed for NDV strains (McMillan and Hanson, 1980), differentiation of exotic NDV strains (McMillan and Hanson, 1982), identifying genetic relationships among

lentogenic strains (Palmieri, 1989; Palmieri and Perdue, 1989) and comparison of velogenic NDV strains (Palmieri and Mitchell, 1991). This method was found to identify differences in the RNA fingerprints of viscerotrophic velogenic strains compared to neutrophic velogenic strains.

DNA oligonucleotide probe complementing the cleavage activation site of the fusion gene of velogenic NDV isolate was used in a slotblot hybridization technique to differentiate lentogenic from the velogenic and mesogenic NDV isolates (Jarecki-Black *et al.*, 1992; Jarecki-Black and King, 1993; Kalaimathi, 1998; Raghavan *et al.*, 1998). In situe hybridization using an anti-sense digoxigenin-labelled ribo probe corresponding to the sequence of gene encoding for matrix protein was used for identifying the different pathotypes in histopathological tissues (Brown *et al.*, 1999).

Hybridization of RT-PCR products of the fusion gene cleavage site with oligonucleotide designed to match the recent circulating pathotypes has led to successful pathotyping of more than 90% of nearly 200 German NDV isolates (Oberdorfer and Werner, 1998). It has been suggested that during NDV epidemic, sequence information on current strains has to be obtained and primers have to be modelled to match respective pathotype specific sequences around the proteolytic cleavage site.

Characterisation of pathotype specific epitopes of NDV fusion glycoproteins, from NDV variants by Matrix-assisted laser desorption / Ionisation (MALDI) time-of-flight (TOF) mass spectrometry (MS) by post-source decay sequencing has been carried out by Lopaticki *et al.* (1998).

materials & methods

CHAPTER - 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 BIOLOGICALS

3.1.1.1 Newcastle disease virus isolates

Twelve NDV strains isolated over a period of twelve years, maintained in the repository at the Department of Animal Biotechnology, Madras Veterinary College, Chennai, were used in this study (Table 2). Three field isolates of NDV were obtained from Avian Diseases Division, Indian Veterinary Research Institute, Izatnagar.Three NDV field isolates were obtained from Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Kerala.

3.1.1.2 Newcastle disease virus vaccine strains

Mukteswar (R_2B) vaccine strain was acquired from Biological products Division, Indian Veterinary Research Institute, Izatnagar. The vaccine strain La Sota (Hoechst, India) was obtained from a commercial outlet.

3.1.1.3 Hyperimmune serum

Hyperimmune serum against NDV was raised as per standard procedures in chicks maintained at Department of Animal Biotechnology, MVC, Chennai.

Table - 2: History of Newcastle disease virus isolates

ISOLATE/ STRAIN	Year	Host	Place	Pathotyping			Antigenic grouping by Mabs	Pathotype
				MDT	ICPI	IVPI		
JQC6/87/V	1987	Japenese Quail	Chennai	56.5	1.65	2.22	C1	Velogenic
LCN2/87/V	1987	Layer chicken	Namakkal	48.0	1.85	2.51	C1	Velogenic
LCN3/87/V	1987	Layer chicken	Namakkal	48.5	1.80	2.75	C1	Velogenic
BCC4/87/V	1987	BC	Chennai, VYD	51.5	1.76	2.46	C1	Velogenic
LCC5/89/V	1987	Layer chicken	Chennai, VLK	54.0	1.66	2.47	C1	Velogenic
LCC9/89/V	1989	Layer chicken	Chennai, RH	46.0	1.62	2.61	C1	Velogenic
LCC15/89/V	1989	Layer chicken	Chennai, MPK	52.5	1.86	2.51	C1	Velogenic
D1/95/L	1995	Desi bird	Chennai	45.0	1.43	2.18	E	Lentogenic
D2/95/V	1995	Desi bird	Chennai	44.0	1.52	1.80	C1	Velogenic
D3/95/V	1995	Desi bird	Chennai	48.6	1.61	2.0	C1	Velogenic
D4/95/L	1995	Desi bird	Chennai	56.0	1.64	2.16	L	Lentogenic
D5/95/M	1995	Desi bird	Chennai	69.3	0.98	2.16	L	Mesoogenic
KNBB/96/L	1996	Chicken	Karnataka	96.0	0.03	-	-	Lentogenic

3.1.2 BIRDS AND EMBRYONATED EGGS

3.1.2.1 Day-old chicks

Day old White Leghorn chicks were obtained from Poultry Research Station, Nandanam, Chennai and was used for conducting ICPI tests.

3.1.2.2 Embryonated chicken eggs

Seven day old embryonated chicken eggs were obtained from poultry Research station, Nandanam, Chennai and used for conducting MDT test, isolation and propagation of NDV isolates.

3.1.3 CHEMICALS

All the chemicals used for preparing various reagents were of AR/GR grade.

3.1.4 REAGENTS

3.1.4.1 For Virus identification and propagation

Alsever's solution

Glucose (BDH, India)	-	2.5g
Tri Sodium citrate (BDH, INDIA)	-	0.8g
Sodium Chloride (E-Merck)	-	0.42g
Citric acid (BDH, India)	-	0.055g

Distilled water to make 100 ml. Sterilized by autoclaving at 110°C for 10 min and stored at 4°C until used.

Phosphate Buffered Saline (PBS)

Sodium chloride (E-Merck)	-	8.00g
Disodium hydrogen phosphate (BDH, India)	-	1.15g
Potassium Chloride (BDH, India)	-	0.20g
Potassium dihydrogen phosphate (BDH, India)	-	0.20g

Distilled water to make 1000ml.

Sterilized at 121^{oC} for 15 min and stored at 4°C until used.

3.1.4.2 For virus purification

Di-ethyl Pyro-carbonate - Phosphate buffered saline (DEPC-PBS)

Phosphate buffered saline was treated with 0.1% Di-ethyl pyro-carbonate (Sigma, USA) and was used for dissolving the crude virus pellet after ultracentrifugation of clarified allantoic fluid.

Sucrose Solution

36 per cent and 55 per cent sucrose solutions were prepared by dissolving 7.2 and 11.0 gram of sucrose in 10ml of distilled water and made up to 20ml with distilled water. Both the solution were sterilized by filtration with 0.22µm filter units (Millipore, India).

3.1.4.3 For RNA Isolation and Electrophoresis

Chloroform : Isoamyl alcohol

Chloroform (SRL, India)	:	49 ml
Isoamyl alcohol (E-Merck, India)	:	1 ml

Distilled water treated with DEPC

Triple Glass distilled water (TGDW) was treated with 0.1% Diethyl pyrocarbonate and was either stirred for 6 hours or left at 37°C incubator overnight and sterilized at 121°C for 15 min after dispensing in sterile containers.

Solution - D

Guanidine thiocyanate (USB, USA)	:	4 M
Sodium citrate (pH 7.0) (Sigma, USA)	:	25 MM
N-Lauryl sarcosine (Sigma, USA)	:	0.5%
2 - Mercapto ethanol (GIBCO-BRL, USA)	:	0.1 M

Solution-D was prepared by dissolving 250g of Guanidine thiocyanate in 293 ml of DEPC-treated distilled water and by adding 17.6ml of 0.75M Sodium citrate pH 7.0, 26.4ml of 10% Sarcosyl at 65°C and 0.36ml of 2-Mercaptoethanol.

70% Ethanol

30ml of DEPC-treated distilled water was added to 70ml of Ethyl alcohol (99.9%) to make 100ml of 70% ethanol.

Formaldehyde gel running buffer (10x)

MOPS (pH 7.0)	:	0.2M
Sodium acetate	:	80mM
EDTA (pH 8.0)	:	10mM

To 20.9g MOPS, 800ml of DEPC treated distilled 50mM Sodium acetate was added. The pH of the solution was adjusted to 7.0 with 2N NaOH, and 10ml of DEPC - treated 0.5M EDTA (pH 8.0) was added. The volume of the solution was adjusted to 1 litre with DEPC treated distilled water. The solution was finally filtered through 0.2µm membrane and stored at RT in dark storage bottle.

Sodium acetate (2M)

To 13.608 g of Sodium acetate, 30ml DEPC treated distilled water was added and stirred to mix. The pH was adjusted to 4.0 with Glacial acetic acid, and then made upto 50ml volume with DEPC treated distilled water.

Water saturated phenol

Melted phenol was equilibrated with equal volume of DEPC treated distilled water and water was extracted from phenol phase after stirring vigorously. This procedure was repeated until the phenol phase reached pH 4.0. The saturated phenol was stored at 4°C and was used for RNA extraction.

Gel loading buffer (6 x)

Glycerol	:	50%
EDTA (pH 8.6)	:	1mM
Bromophenol Blue	:	0.25%
Xylene cyanol FF	:	0.25%

0.24 - 9.5 Kb RNA ladder Gibco BRL, NY, USA

RNA ladder was used for Total RNA gel electrophoresis to identify the size of any RNA bands obtained.

3.1.4.4 For Complementary DNA (cDNA) Synthesis

- ™II RNase H⁻ Reverse Transcriptase - GIBCO BRL, NY,USA
- 0.1M Dithiothreitol - GIBCO-BRL, NY, USA
- 5 x first strand buffer - GIBCO BRL, NY, USA
  [Tris HCl (pH 8.3) 250mM, KCl 375mM, $Mgcl_2$ 15mM)
- Placental Ribonuclease inhibitor - GIBCO BRL, NY, USA
- 10mM 2-deoxynucleoside 5 - triphosphate mix - GIBCO BRL, NY, USA
- Random Primers (9 OD_{260} units/100µl) - GIBCO BRL, NY, USA
- Ribonuclease H (3.2 u/µl) - GIBCO BRL, NY, USA

3.1.4.5 For Polymerase Chain Reaction

Consensus primer for a 1349 nucleotide long region of the Fusion gene

Primer 1 (sense) :

5' - TGA CTC TAT CCG TAG GAT ACA AGA GTC TG - 3'

Primer 2 (antisense) :

5' - GAT CTA GGG TAT TAT TCC CAA GCC A-3'

Degenerate oligonucleotide primer pairs for the FPCS and MPNLS regions

- Fusion protein cleavage site (FPCS) region

Primer 1 (sense) :

5' - CCT TGG TGA ITC TAT CCG IAG - 3'

Primer 2 (antisense) :

5' - CTG CCA CTG CTA GTT GIG ATA ATC C-3'

- Matrix Protein Nuclear localization signal (MPNLS) region

Primer 1 (sense) :

5' - TCG AGI CTG TAC AAT CTT GC- 3'

Primer 2 (antisense) :

5' - GTC CGA GCA CAT CAC TGA GC - 3'

I = Inosine.

- Platinum™ Taq DNA polymerase - GIBCO BRL, NY, USA
- 10 x PCR Buffer, Minus Mg (200mM Tris-Hcl (ph 8.4), - GIBCO BRL, NY, USA
- 500 mM Kcl - GIBCO BRL, NY, USA
- 50mM Magnesium Chloride - GIBCO BRL, NY, USA
- 10mM dNTP mix - GIBCO BRL, NY, USA

3.1.4.6 For Restriction Endonuclease Analysis

- **Restriction Enzymes**

Restriction enzymes like Bst OI, Rsa I and Hinf I were obtained from M/s Promega corporation, USA and used. Taq I, Hae III, Mbo I, Alu I and Msp I were obtained from M/s GIBCO-BRL, USA and used in this study.

- **50x TAE Buffer**

Tris Base	:	242.0g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100.0 ml

Distilled water to make 1000ml

Working concentration (1x) was 40mM Tris acetate and 1mM EDTA.

3.1.4.7 For Manual Nucleotide sequencing

Purification of PCR Products

Wizard™ PCR product purification kit - Promega Corpn., WI, USA.

Nuclease - Free water - Promega Corpn., WI, USA.

Radioisotope

PLC : Alpha 33p - deoxy Adenosine Triphosphate (α^{33} p-dATP) triethyl ammonium salt in aqueous solution - 250 µC ; 370 mBq/ml (10 mCi/ml) specific activity (approx.) 110 TBq/m mole (3000 Ci/mmole) obtained from JONAKI (BRIT), CCMB, Hyderabad was used.

Sequencing Kits

- Sequenase version 2.0 DNA sequencing Kit - United States - Biochemical, OH, USA
- Omni Base™ DNA cycle sequencing System and Omni Base™ sequencing Enzyme mix - Promega Corpn., WI, USA.

Polyacrylamide gel electrophoresis
40% Acrylamide stock solution

Acrylamide	:	38.0 g
N, N' - Methylene bis acrylamide	:	2.0 g
Distilled water to make upto 100 ml		

Preparation of Gel solutions of various concentration

	6%	8%
40% Acrylamide stock (in ml)	14.5	20
Urea 7M (gms)	42	42
10x TBE (ml)	10	10
Distilled water (ml)	40	35

Dissolved the urea in the solution and made upto to 100 ml with distilled water.

Gel recipes for urea-formamide gel (100 ml)

	6%	8%
Acrylamide	5.7 g	7.6 g
Bis-acrylamide	0.3 g	0.4 g
Urea (7M)	42.0g	42.0g
10 x TBE	10.0 ml	10.0 ml
Formamide	30.0 ml	30.0 ml

After dissolving urea, the solution was made up to 100 ml with distilled water.

10 X TBE Buffer

Tris Base	:	108 g
Boric acid	:	55 g
Na_2 EDTA H_2O	:	9.3 g

Distilled water to make 1,000 ml and filtered with whatman 1 filter.

3.1.4.8 For Automated Nucleotide sequencing

Sequencing kit

Thermosequenase dye terminator cycle sequencing core kit obtained from M/s Amersham Life Sciences, USA was used.

Polyacrylamide Gel electrophoresis

Gel Recipe (5%)

Urea (6 M)	:	18.0 g
Deionised water	:	21.5 ml
10X TBE	:	5.0 ml
50% Long Ranger™ Premix solution (FMC, Inc., USA)	:	5.0 ml
Distilled water to make upto 50 ml		

The gel mixture was stirred until urea was completely dissolved. Filtered the mixture by attaching a Nalgene™ fitter apparatus with 0.2µM filter to a vacuum source to pull the liquid through the filter and degased the solution.

3.1.5 SOFTWARES

3.1.5.1 Restriction Endonuclease Analysis Digest

Version 1.0 by Ramin C. Nakisa, available at Web site ftp. ebi.ac.uk/pub/software/Dos/ was used for scanning DNA sequence files for restriction sites.

3.1.5.2 Kodak 1D Image Analysis software -

KODAK Digital Science, USA was used to determine molecular *weight of restricted DNA fragment from gel images.*

3.1.5.3 Automated Sequencing

- ABI PRISM ™ sequence Data collection software PE Corpn, applied Biosystems Div., CA, USA.
- ABI PRISM ™ sequence Analysis software
- ABI PRISM ™ Edit view software.

For sequence data collection, analysis and printing using Macintosh system and colour Inkjet printer.

3.1.5.4 Nucleotide and Protein sequence Alignment

- **Clustal W** version 1.6 - Multiple sequence alignment program. (Thompson *et al.*, 1994) available at website ftp/.ebi.ac.uk/pub/software/Dos was used.

- **ProMSED 2** Protein Multiple Sequence Editor - 2, for Windows 3.11/95 was used for protein and DNA sequence alignment, editing, comparison and analysis, available at web site: ftp.ebi.ac.uk/pub/software/Dos (Anatoly Frolov and Alexey Eroshkin (1995),

3.1.5.5 Nucleotide sequence translation to protein sequence

SEQAID II

SEQAID II package version 3.81 (Rhoads and Roufa, 1991), available at ftp.ebi.ac.uk/pub/software/DOS was used for finding the open reading frames and conceptual protein sequence determination by translation of nucleotide sequence.

3.1.5.6 Phylogenetic Analysis Softwares

MEGA [Molecular Evolutionary Genetic Analysis]

MEGA Version 1.02 was used for estimating evolutionary distances and construction of phylogenetic trees. The Software is available from Sudhir Kumar, Institute of Molecular Evolutionary Genetics, 328 Mueller laboratory, The Pennsylvania State University, University Park, PA 16802, USA, email: imeg@psuvm.psu.edu (Kumar *et al.*, 1993)

PHYLIP [Phylogeny Inference Package]

Version 3.5C for Pentium Windows System by Joe Felsentein, Department of Genetics, University of Washington, Seattle, Washington 98195, USA (email:joe@genetics.washington.edu), available at web site: http: //evolution.genetics.washington.edu was used.

This package contains several programs for phylogenetic analysis using different methods. This also has programs for the phylogenetic tree output like DRAWGRAM - for plotting phenograms etc., and DRAWTREE for plotting unrooted tree diagrams with many options for tree manipulation and previewing. These programs were used in this study.

3.2 METHODS

3.2.1 STERILIZATION OF PLASTICWARES AND SOLUTIONS

All plasticwares used were fresh ones for RNA isolation techniques. Fresh Microcentrifuge tubes and PCR tubes (0.2ml) were dispensed in sterile containers using gloved hands, wrapped with autoclavable plastic covers and autoclaved for 15 min at 121°C. Care was taken not to touch or bring the plasticware into contact with bare hands or other materials.

All solutions and buffers prepared with 0.1% DEPC - treated distilled water were autoclaved for 30 mins at 121°C for 15 min, to remove DEPC.

3.2.2 NEWCASTLE DISEASE VIRUS ISOLATION AND IDENTIFICATION

3.2.2.1 Preparation of Inoculum

Tissues and organ samples (Brain, Spleen) collected from birds suspected for ND were transported in 50% Glycerol Saline on ice in sterile containers. The samples were triturated after washing with sterile PBS and the suspension was centrifuged for 15 minutes at 5,000 rpm (4°C). The supernatant was collected in a sterile vial and treated with 250 IU/ml of Penicillin and 250 µg/ml of streptomycin and incubated at 37°C for 30 min. The supernatant was used for virus isolation.

3.2.2.2 Virus Isolation (Allan *et al.*, 1978)

The prepared inoculum was injected into the allantoic cavity of 9 day old embryonated chicken eggs at the rate of 0.1ml per egg, and incubated at 37°C. The inoculated embryos were candled every 12 hours and dead embryo's within 24 hours after inoculation were discarded. The dead embryo's found on candling after 24 hours of inoculation were chilled at 4°C for 4 hours. The Amnio-allantoic fluid (AAF) was harvested and checked for Haemagglutination activity using chicken erythrocytes.

3.2.2.3 Virus Identification

Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests were carried out with freshly collected Amnioallantoic fluid (AAF) as per Allan and Gough, (1974).

3.2.3 CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES BY CONVENTIONAL TECHNIQUES

Newcastle disease virus isolates were characterised by Mean Death Time (MDT) and Intracerebral Pathogenicity index (ICPI) using standard techniques (Allan *et.al.,* 1978).

3.2.4 PROPAGATION AND PURIFICATION OF NEWCASTLE DISEASE VIRUS ISOLATES

Newcastle disease virus isolates were propagated in nine-day old embryonated chicken eggs (Allan et.al., 1978). The incubated embryo's were candled twice daily and dead embryos were chilled at 4°C for atleast 4 hrs prior to harvesting the AAF. The harvested AAF was stored at - 30°C freezer before subjecting to purification of virus. The frozen AAF was thawed and clarified by centrifugation at 5,000 rpm for 15 min at 4°C. The clarified AAF was subjected to ultracentrifugation using Ti_{70} rotor at 35,000 rpm for 1 hour 40 min at 4°C. The crude virus pellet from all the 8 tubes were suspended in 2 ml of DEPC-treated PBS and aliquoted into 500µl each in four 2 ml freezing vials. The vials were stored at - 80°C until used. Further purification of the crude virus pellet was carried out by discontinuous sucrose gradient ultracentrifugation using 5 ml of 36% and 2 ml of 55% sucrose solution. The gradient was centrifuged at 65,000 rpm in a Beckman Ti_{60} rotor using 7ml ultracentrifugation tubes for 12 hours at 4°C. The virus band was collected and stored at -80°C until used.

3.2.5 PREPARATION OF RNA

3.2.5.1 Total RNA isolation from embryo/organ samples

Total RNA isolation from embryo/organ samples was carried out as per Chomezynski and Sacchi (1987) with minor modifications. Briefly 100mg tissue was ultra frozen in the (homogenizer) mortar by pouring liquid nitrogen directly onto the tissue. The frozen tissue was crushed into powder immediately with the pestle and 1ml of solution D was added to the powdered tissue. The sample was homogenized thoroughly and sequential extraction precipitation and isolation of RNA was carried out as per standard protocol of Chomczynski and Sacchi (1987).

3.2.5.2 Viral Genomic RNA isolation

The viral genomic RNA was isolated from purified virus by using Trizol Reagent (GIBCO-BRL,USA), a monophasic solution of phenol and guanidine isothiocynate in an improved protocol to the single step RNA isolation method described by Chomczynski and Sacchi (1987) according to manufacturers instructions. Briefly, to 100µl of purified virus/virus crude pellet, one ml of trizol was added and homogenized thoroughly by repeated pipetting. After a 5 min incubation at 15 to 30°C, 200µl of chloroform was added, extracted vigorously by shaking the tubes for 15 sec. Incubated for 3 minutes at 15 to 30°C and centrifuged at 12,000 xg for 15 mins at 2 to 8°C. The aqueous supernatant containing RNA was precipitated with 500µl of isopropanol and centrifuged at 12,000 xg for 10 min at 2 to 8°C. The RNA pellet was washed

with 75% ethanol once and vacuum dried in speed vac (Savant, USA), without centrifugation. The RNA was dissolved in 20µl of DEPC - treated distilled water and used for cDNA synthesis.

3.2.6 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR) FOR SAMPLE SCREENING

A single tube RT-PCR was carried out with the total RNA isolated from ND suspected samples, using Access RT-PCR system, according to manufacturer's instructions. Briefly, 10µl of total RNA was added in a single tube containing AMV-RTase TfL NDA polymerase, AMV/TfL Reaction Buffer, dNTP mix, specific upstream and down stream FPCS primers and $MgSO_4$. First strand cDNA was synthesized at 48°C for 45 minutes followed by PCR with initial denaturation of template at 94°C for 2 mins. The second strand cDNA synthesis and amplification of FPCS was carried out in a 40 cycle PCR with the following conditions:

Denaturation	94°C for 1 min
Annealing	50°C for 40 secs
Extension	68°C for 1 min

A final extension was carried out at 68°C for 7 min.

3.2.7 COMPLEMENTARY DNA (cDNA) SYNTHESIS

Complementary DNA was synthesized with the viral genomic RNA isolated from purified virus using superscript II™ reverse transcriptase and random hexamers according to manufactures instructions. Briefly RNA was denatured with random hexamer at 70°C for 10 min and snap cooled in ice for

5 min. The reaction mixture was then assembled containing RT Buffer, Diothiothreitol, dNTP mix, Placental RNase inhibitor and super script II™ Reverse transcriptase in a 20µl reaction. The reaction mix was incubated at 25°C for 10 minutes before adding Reverse transcriptase and then at 42°C for 60 min. The first strand cDNA synthesized was treated with 1 unit RNase H at 37°C for 30 min, to remove the RNA template strand from the cDNA-RNA hybrid. The cDNA was used in PCR for the fusion gene (1349 nt region), FPCS and MPNLS regions.

3.2.8 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction for the fusion gene (1349 nt region), FPCS and MPNLS region of the NDV isolates were carried out in a 100 µl reaction using platinum ™ Taq DNA polymerase. The reaction mix consisted of 2.5 µl of cDNA (synthesized in a 20µl reaction) and 1.5 mM $MgCl_2$ in all the three different sets of reactions for amplification of Fusion gene, FPCS and MPNLS regions.

PCR mix for 100µl volume

10x PCR Buffer, (Minus Mg)	:	10.0 µl
50 mM $MgCl_2$	:	3.0 µl
10 mM dNTP mix	:	2.0 µl
Upstream Primer (50 pmol)	:	1.0 µl
Downstream Primer (50 pmol)	:	1.0 µl
Platinum Taq DNA polymerase (2.5U)	:	0.5 µl
cDNA template	:	2.5 µl
Nuclease-free water (to make 100µl)	:	80.0 µl

3.2.8.1 PCR for the 1349 nucleotide long region of the fusion gene (Ballagi - Pordany *et al.*, 1996)

The amplification was carried out with an initial denaturation at 94°C for 2 min followed by two different annealing temperature cycling conditions as given below:

94°C for 45 secs	
55°C for 30 secs	5 cycles
72°C for 1 min	
94°C for 45 secs	
48°C for 1 min	30 cycles
72°C for 3 min	

A final extension hold at 72°C for 7 mins was given. Five µl of the amplified product was checked in a 1.5% agarose gel with DNA molecular weight standards.

3.2.8.2 PCR for fusion protein cleavage site (FPCS) and Matrix protein nuclear localization signal (MPNLS) regions

The PCR amplification conditions for both FPCS and MPNL regions were similar with the following cycling profile after a initial denaturation at 94°C for 2 min.

94°C for 30 Sec	
50°C for 40-Secs	30 cycles
72°C for 1 min	

A final extension period of 10 min at 72°C was given to extend the truncated products to full length. The amplified products were checked on a 2% agarose gel along with DNA.

3.2.9 RESTRICTION ENDONUCLEASE ANALYSIS (REA) OF PCR AMPLICONS.

3.2.9.1 Restriction enzyme Digestion (RED)

Restriction enzyme digestion reaction was set up for each enzyme with specific buffers recommended by the manufacturers. A typical RED reaction was of 20 µl volume, and was assembled in the following order:

Sterile distilled water	:	1.8µl
RE 10x Buffer	:	2.0µl
Acetylated BSA, 10 µg/µl	:	0.2µl
PCR product DNA	:	15.0µl

The above reagents were mixed well by gentle pipetting and 1µl of restriction enzyme containing 10U/µl was added.

The reaction contents were mixed gently by flicking the tubes and centrifuged to spin down the contents. The mixture was incubated at recommended, optimum temperature for each restriction enzyme used.

3.2.9.2 Agarose gel electrophoresis and photography

The restriction enzyme digested PCR products were electrophoresed in 2.5% agarose gels with ethidium bromide in 1X TAE Buffer. The run was carried out at 100 Volts for 12 hrs and 30 min. The samples were run along with DNA molecular weight standard at the first and last lanes of the gel to estimate and correlate the molecular weight of digested fragments.

The agarose gels after electrophoresis were viewed under UV transilluminator and photographed with a polaroid MP-4 Land camera using polaroid (pos/neg) instant films.

3.2.9.3 Restriction endonuclease analysis (REA) of Fusion gene, (1349 bp) amplicon

Restriction endonuclease analysis of 1349 bp fusion gene region amplicon was carried out with restriction enzymes Bst OI, RsaI, Hinf I, Taq I, Hae III and MboI.

Newcastle Disease Virus Fusion gene fingerprint analysis

Molecular Weight estimation

The molecular weights of the obtained fragments were estimated using Kodak 1D Image Analysis software with scanned images of agarose gel photographs.

Distance Matrix calculation

The molecular weights of the RED fragments estimated were used for finding similar or different banding patterns for each analysed enzyme.

The distance measures of the bands obtained for different isolates were calculated by Jaccard's Distance coefficient (Millemann *et al.*, 1995) using the formula:

$$D = \frac{1\text{-}C}{2N\text{-}C}$$

where; C is the number of bands in common, N is the total number of different bands of each isolate/strain.

Phylogenetic analysis

The obtained distance measures of the restriction fingerprint patterns were written onto a file in the Molecular Evolutionary Genetic Analysis (MEGA) software, input format as upper right triangular matrix. The distance measures patterns obtained for each analyzed enzyme were separately analysed using the software MEGA. The Unweighted Pair Group method based on Arithmatic Mean (UPGMA) was used for generating phylogenetic trees.

Restriction endonuclease Analysis 'In silico'

REA 'In silico' of the LaSota nucleotide sequence was carried out using the software Digest as per the software protocol.

The restriction fragment data obtained for La Sota vaccine strain was used for comparison with the field isolates REA fusion gene fingerprints.

3.2.9.4 Restriction Endonuclease Analysis of FPCS and MPNLS amplicons

Restriction endonuclease analysis of FPCS amplicons of different isolates were carried out with Alu I and Hinf I. The MPNL amplicons were subjected to REA with Alu I and Msp I.

3.2.10 DIRECT DOUBLE STRANDED NUCLEOTIDE SEQUENCING OF THE FUSION PROTEIN CLEAVAGE SITE AND MATRIX PROTEIN NUCLEOLAR LOCALISATION SIGNAL REGION AMPLICONS

3.2.10.1 Purification of RT-PCR-FPCS and MPNLS amplicons

The FPCS and MPNLS regions were amplified in a total of 300µl reaction from all the isolates. The total 300µl PCR product from each isolate was purified using Wizard ™ PCR production purification Kit (Promega, USA) according to manufacturer's protocol. The final Elute was in a 50µl volume which was aliquoted into two tubes equally and stored at -20°C. The elute was checked in agarose gel for approx estimating the concentration, and evaluating the purity.

3.2.10.2 Manual Radioactive Nucleotide Sequencing

Manual Nucleotide sequencing of the purified PCR product was carried out by Sanger's dideoxy chain termination method. Sequenase™ Version 2.0 DNA sequencing kit (USB, USA) was used with α^{33}P-dATP for sequencing using both forward and reverse primers as per manufacturers instructions.

Cycle sequencing was carried out with omnibase™ DNA cycle sequencing system with α^{33} P -dATP incorporation with specific upstream and downstream primers of the purified PCR amplicon; according to manufacturers protocol. The sequencing reaction conditions employed were essentially same as that of the PCR but for the number of cycles reduced to 25.

The sequencing reaction samples were then loaded onto either 6% or 8%, 0.4mm thick denaturing polyacrylamide gel containing, 7M urea and formamide.

The running conditions were, prerun at 2000 V for 15 min and run at 2500V, 50W constant power for 1 hr 30 minutes, at 50°C

The terminated samples were loaded in the following order 'GATC GTAC' to allow better comparison and sequence read. After the run, the gels were fixed in 10% acetic acid until the tracking dye changed colourless. The gel, wrapped with plastic film and backed with Whatmann filter paper No.1 was dried under vacuum at 70°C for 1 hr. The dried gel was then exposed to Amershom UK, Hyperfilm™, for 36 hours at - 70°C. The Autoradiography film was developed and dried as per standard methods.

The sequence data was manually read from the autoradiogram as per standard method of calling base from bottom of the run to the top and recording their nucleotides from the four lanes in the 5' to 3' direction corresponding to the primer used.

The sequence data obtained using upstream and downstream primers were loaded onto separate text files on the computer system. The sequence data obtained with downstream primer was reverse complemented using the software seqaid V II, V 3.81. The sequence data obtained with upstream primer and reverse complemented sequence obtained with downstream primer were aligned with the software CLUSTAL W (Thomson, *et al.*, 1994) to generate the entire sequence of the PCR amplicon sequenced.

3.2.10.3 Automated Nucleotide Sequencing

Direct sequencing of purified PCR amplicons were carried out by cycle sequencing using thermo sequenase dye terminator cycle sequencing core kit with fluorescent labelled dye-terminators incorporation method as per manufacturers protocol. The sequencing samples were analysed by gel electrophoresis run on a AB1 377 Prism ™ fluorescent sequencing instrument.

Sequencing reaction set up

The PCR product template of approximately 150 ng (2µl) was taken for each set of reaction, diluted to a total volume of 11µl with deionised distilled water.

A sequencing reagent premix, for a single reaction was assembled as given below:

Reaction buffer	1µl
dNTP mix (10mM)	2µl
ddATP (dye-labeled)	1µl
ddCTP (dye-labeled)	1µl
ddGTP (dye-labeled)	1µl
ddTTP (dye-labeled)	1µl
Thermo sequence DNA polymerase (20u/µl)	1µl
	8µl

A master mix was prepared for multiple samples sequencing. The sequencing Reaction was setup with the diluted PCR product template, primer (either upstream or downstream) and the sequencing reagent pre-mix, as given below:

Sequencing reagent pre-mix.	8µl
Primer (10µM)	1µl
DNA template	11µl
	20µl

Cycle Sequencing Conditions

The cycling conditions employed were an initial denaturation step of 96°C for 1 min followed by 30 cycles of:

96°C for 30 Secs
50°C for 15 Secs
60°C for 4 Mins

The sequencing reaction was set in heated block at 96°C of the Perkin Elmer Gene Amp 9600 Thermal Cycler.

Preparation of the Samples for gel loading

The extended products after cycle sequencing were purified and prepared for gel loading as given below:

To the 20µl sequencing termination, 7µl of 7.5M ammonium acetate was added and mixed by inverting the tubes. Then, 2.5 volumes (approximately 68µl of 100%) cold (-20°C) ethanol was added and centrifuged at 12,000 rpm for 10 mins at 4°C. The supernatant was aspirated and the pellet was vacuum-dried for 5 minutes. Finally the DNA pellet was resuspended with 4 µl of loading dye with repeated pipetting for dissolving the pellet completely. Also the tube was vortexed for 20 secs to ensure complete resuspension. The tubes

were centrifuged to spin down the contents. The samples were heated at 95°C for 5 min to denature and snap cooled on ice. The samples were kept on ice until loading 2.5µl of each reaction onto the Sequencing gel of ABI 377 prism sequencer.

Sequencing gel electrophoresis

The denaturing sequencing gel (5%) of 02.mm thickness, was cast using the gel cassette and plates as per manufacturers instructions in PE ABI 377 Prism DNA sequencer gel casting accessory.

The gel was loaded onto the equipment and a plate check was run using the ABI 377 PRISM software. After cancellation of plate check, 1x TBE buffer was added to both upper and lower chambers and the front heat plate was set in position.

A prerun was started for 30 min and the following electrophoresis conditions were used.

Voltage(KV) - 2.40

Current(MA) - 50.0

Power (W) - 200

Temperature - 51°C

After a 30 min pre run, the first set of samples were loaded onto the gel in odd numbered lanes. The pre-run was resumed for 10 minutes and again paused for loading the second set of samples onto the gel in even numbered

lanes. The pre-run was cancelled and the run was started with essentially same electrophoretic conditions with a Laser power of 40.00. The gel run was continued for ten hours.

Sequence Data Collection and Analysis

The sequencing gel run data was collected as a 'Gelfile' by the ABI 377 PRISM Data collection software V 1.1 using matrix SN1089. The gel image was taken for analysis of each sample lane using AVI 377 PRISM sequence data analysis software, V 3.0, the Base caller for each sample.

The electropherogram viewed was analysed after evaluating and setting the Base call start, Base Call end and Primer Peak location. The sequence data was retrieved after re-analysis. The electropherogram was displayed with peaks of fluorescent labelled dye-terminators (ddATP-Green, ddCTP-Blue, ddGTP-Black, ddTTP-Red) on the 'Y'-axis with respective bases on top of each peak on the X-axis. The nucleotide (N) which could not be assigned to a specific base by the matrix used were arrived at by checking the Reverse Primer generated sequence of the same sample.

Sequence retrieval

The analysed sequences were directly stored onto Text files separately for each sample. The reverse primer generated sequence was reverse complemented using the software seqaid II V3.81 and aligned with forward Primer generated sequence using software CLUSTAL'W' V1.6, to generate the complete sequence of the PCR amplicon sequenced.

3.2.11 NUCLEOTIDE SEQUENCE ANALYSIS

The nucleotide sequence of all the samples were analysed using various softwares for evaluation of open reading frame and conceptual translation of nucleotide sequence to protein sequence.

Open Reading frame (ORF) and protein sequence estimation

SEQAID II package V 3.81 was used to find out the open reading frames (ORF's) of the nucleotide sequence data obtained in all three reading frames. The reading frame without any stop codons was conceptually translated into protein sequence using the same software package. The graphic output of ORF's were written to separate files for all the obtained sequences. Also the nucleotide sequence aligned with protein sequence data was written to separate text files.

Nucleotide and protein sequence alignment

The FPCS nucleotide sequence data of all the isolates were written onto a single file in FASTA format which was used as input file for the sequence alignment software CLUSTAL W. The sequences were aligned and written as an alignment file. The MPNLS nucleotide sequence data was similarly processed to align all the sequences.

The conceptually translated aminoacid sequence of the FPCS and MPNLS regions were aligned essentially as described above.

The FPCS and MPNLS nucleotide sequences were written continuously one after another as a contiguous PCR amplicons sequence in FASTA format and aligned using CLUSTAL W software.

Nucleotide and aminoacid sequence variation estimation

The MEGA V 1.02 Molecular Evolutionary Genetic Analysis software was used to estimate the nucleotide site variations in the aligned sequences. The distances measure was estimated between all the sequences with standard errors and written to textfiles for printing.

The software ProMSEd2 - Protein Multiple Sequence Editor 2 for windows, V 3.11/95 was used for aminoacid sequence alignment and estimation of variation between the sequences analysed. The aminoacid sequence variation was written as bitmap file for printing in multicolour indicating aminoacid variations.

3.2.12 PHYLOGENETIC ANALYSIS OF OBTAINED NUCLEOTIDE SEQUENCES OF FUSION PROTEIN CLEAVAGE SITE REGION AND MATRIX PROTEIN NUCLEAR LOCALISATION SIGNAL REGION AMPLICONS

The software's CLUSTAL W V1-6, MEGA V1.02, PHYLIP package V 3.5c were used for phylogenetic analysis of obtained nucleotide sequence data. All the three software were run sequentially in a windows IBM PC for generating phylogentic trees.

3.2.12.1 CLUSTAL W

CLUSTAL W V 1.6, was used to generate input files in PHYLIP format called 'Tree Files' for the software's DRAWGRAM/ DRAWTREE to output graphics of trees estimated. CLUSTAL W V 1.6, used the Neighbour - Joining method of Saitou and Nei(1987), based on the matrix of 'distances' between all sequences for estimating aligned nucleotide sequences to generate tree files. The tree output files were written for FPCS, MPNLS, FPCS combined with MPNLS contiguous sequence alignments as per the software's manual.

3.2.12.2 PHYLIP

PHYLIP package V 3.5c containing software-DRAWTREE and DRAWGRAM ware used for generating graphic output of trees evolved by CLUSTAL software.

DRAWGRAM was used to interactively plot phenogram-like rooted tree diagrams with many options including orientation of tree and branches, style of tree, label sizes and angles, tree depth and margin sizes, stem lengths and placement of nodes in the tree; with previewing in the monitor for the best tree. After finding the best tree the tree was written to a plotfile, which was copied to the Laserjet printer with the following command.

Copy / B PLOTFILE PRN:

The trees were printed as Graphics by the Laser jet printer.

DRAWTREE program was used to interactively plot unrooted tree diagram, with many options including orientation of tree and branches. The trees were generated essentially in a similar method followed in the case of DRAWGRAM.

3.2.12.3 MEGA

Molecular Evolution and Genetic Analysis (MEGA) software V 1.02 was used to estimate distances and construct phylogenetic trees using two different distance estimation methods.

3.2.12.3.1 Jukes-Cantor Distance (JCD) (Jukes and Cantor, 1969)

Jukes-Cantor distance assumes that the rate of nucleotide substitution is the same for all pairs of the four nucleotide A,T,C and G and it gives a maximum likelihood estimate of the number of nucleotide substitutions(d) between two sequences. It is given by:

$$\hat{d} = -3/4 \log_e (1 - 4/3P)$$

Where P is computed by equation

$$P = \frac{nd}{n}$$

Where P = proportion of nucleotide sites at which the two sequences compared are different

nd = number of nucleotide differences

n = total number of nucleotide compared.

3.2.12.3.2 Kimura 2-Parameter distance (K2-PD) (Kimura, 1980)

K2-PD estimate transitional and transversional substitutions in the nucleotide sequence data were analysed. K2-PD gives a maximum likelihood estimate of d between two sequences.

$$\hat{d} = -\tfrac{1}{2} \log_e (1-2P-Q) - \tfrac{1}{4} \log_e (1-2Q)$$

3.2.12.3.3 Phylogenetic inference & Tree building method

Gene trees based on the contiguous FPCS and MPNLS region amplicon sequences were constructed using the most efficient distance method in recovering the correct topology, the Neighbor-Joining method proposed by Saitou and Nei (1987). This method was used to produce an unrooted tree and the outgroup Operational Taxonomic Unit (OTU) was used to find the root.

3.2.12.3.4 Statistical tests of obtained phylogenetic tree

Standard Error Test

The standard error test of NJ trees was conducted following Rzhetsky and Nei (1994) method. After the NJ tree was obtained by the saitou -Nei algorithm, the branch lengths of the tree were re-estimated by using the ordinary least squares method and standard errors of the estimate were computed. The statistical significance of 'b' (estimate of an interior branch length) from 'O' was tested by '*t*'-test with degrees of freedom ∞.

$$t = \frac{b}{s(b)}$$

where s(b) is the standard error of b. the confidence probability (CP) - which is complement of probability (1-α) was computed (α; significance level). The reliability of a branch is high when CP is high and the branch length is considered statistically significant when CP is ≥0.95 or 0.99.

Bootstrap test

Bootstrap test randomly resamples with replacement from the original sequence data, the same number of nucleotide as the actual number used for constructing the NJ tree. An NJ was produced from this set of resample nucleotide data after 2000 Bootstrap replicates. The Bootstrap confidence level (BCL) was computed assuming an identity value 1, if the interior branch of NJ tree gives the same partition as that of the bootstrap tree and other interior branches were given value 'O'. The process repeated 2000 times and the percentage of times each interior branch of the NJ tree received identify value 1 was computed.

The phylogenetic Gene Trees obtained by various method and the tests for reliability of the trees obtained by those methods were compared for conservation of the Branching between different isolates. Different clusters formed by those methods were also compared for conservation/similarity.

results

CHAPTER - 4

RESULTS

The present study describes the epizootiological classification of NDV isolates from different regions carried out by PCR-RFLP of the fusion gene (1349bp), Nucleotide sequencing of the Fusion protein cleavage site region (254bp) and Matrix protein nuclear localization signal region (232bp). Phylogenetic analysis of obtained PCR-RFLP fingerprint and Nucleotide sequence data, were carried out and compared with conventional pathotyping, and Mab typing of the NDV isolates.

4.1 NEWCASTLE DISEASE VIRUS ISOLATES

The three NDV isolates from Kerala namely KQA-ALW/98, KQL-CHE/98 and KPG/98 and the three isolates from Avian Disease Division, IVRI, Izatnagar, namely BRL-58/93, WB-198/94 and NPL-5/93 were revived in embryonated chicken eggs and checked by HA and HI tests (Table.3).

4.2 CONVENTIONAL CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES

Mean death time (MDT) and Intracerebral pathogenicity index (ICPI) tests were carried out for NDV isolates - BRLY-58/93, NPL-5/93, WB-198/94, KQA-ALW/98, KQC-CHE/98 and KPG/98. The MDT varied from 42.0 to 48.0 hours in virulent field isolates and one isolate - NPL-5/93 had a MDT of 66.0 hours (Table.3).

Table - 3: Haemagglutination (HA) ,Haemagglutination inhibition (HI) and Mean death time (MDT), Intracerebral pathogenicity index (ICPI)tests of Newcastle Disease Virus isolates

ISOLATE/ STRAIN	Year	Host	Place			Pathotyping	
				HA titre	HI with 4 HAU	MDT	ICPI
BRLY-58/V	1993	Chicken	Bareilly, UP	4096	+	46.5	1.60
WB-198/94/V	1994	Chicken	West Bengal	1024	+	42.0	1.60
NPL-5/93/V	1993	Chicken	Nepal	2048	+	66.0	1.92
KQA-ALW/98	1998	Japenese Quail	Kerala	512	+	44.0	1.95
KQC-CHE/98	1998	Japenese Quail	Kerala	512	+	46.0	1.80
KPG/98/V	1998	Pigeon	Kerala	1024	+	48.0	1.85
PCKC/98	1998	Peacock	Chennai	–	–	–	–
LBC/99	1999	Lovebird	Chennai	–	–	–	–
MUKTESWAR/46/M	1946	Chicken	Ranikhet, UP	1024	+	46.5	1.40

The R_2B vaccine strain was also subjected to MDT & ICPI tests and yielded values similar to the published report (Table.3).

4.3 PROPAGATION AND PURIFICATION OF NEWCASTLE DISEASE VIRUS ISOLATES

The bulk propagated amnioallantoic fluid (AAF) of each NDV isolate on ultracentrifugation resulted in a good virus crude pellet.

4.4 ISOLATION OF RNA

4.4.1 Total RNA isolation from Newcastle disease suspected organ samples

The total RNA isolated from organ samples suspected for ND when subjected to Formaldehyde agarose gel electrophoresis yielded two distinct bands of 28S and 18S rRNA (Plate 1).

4.4.2 Viral genomic RNA isolation

Isolation of viral genomic RNA yielded very small pellet, which was dissolved and directly used for cDNA synthesis.

4.5 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR) FOR NEWCASTLE DISEASE SUSPECTED SAMPLE SCREENING

RT-PCR using FPCS primers, with the total RNA isolated from ND suspected organ samples of Peacock (PCKC/98) and Love-Bird (LBC/99) amplified the expected 254bp sized product (Plate 2). While using MPNLS primer the samples did not amplify the expected product- -in case of viruses like pigeon NDV and avian paramyxovirus 2 and 3.

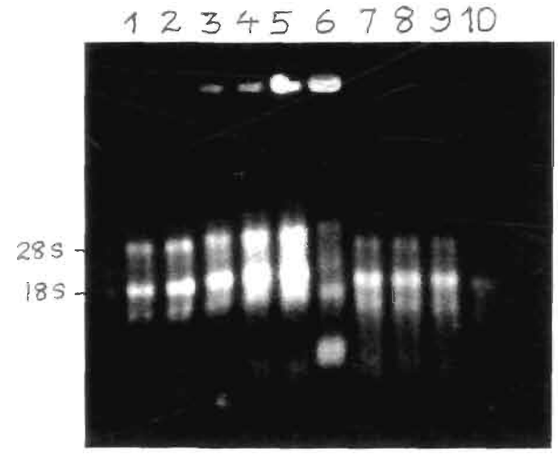


Plate - 1 : RNA gel electrophoresis in formaldehde gel: Total RNA from ND suspected peacock and Lovebird organ samples

Lane 1 - 5 & 7 - 10 : RNA samples

6 : 0.24-0.95 Kb RNA ladder

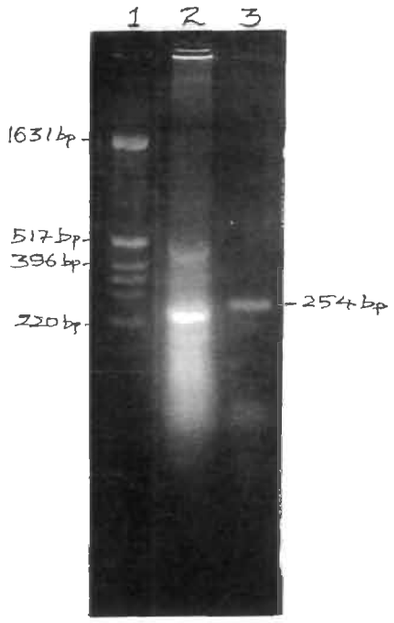


Plate - 2 : Access RT-PCR of Fusion protein cleavage site, 254 bp amplicons from ND suspected peacock and Lovebird organ samples, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

Lane 1 : pBR322/ Hinf I digest

2 : PCKC/98/V

3 : LBC/99/V

4.6 COMPLEMENTARY DNA SYNTHESIS

The cDNA synthesized with viral genomic RNA and random hexamers used in all the three different PCRs for Fusion gene (1349bp), FPCS (254bp) and MPNLS (232bp) yielded expected PCR products (Plate 3).

4.7 POLYMERASE CHAIN REACTION

4.7.1 Fusion protein gene 1349 nucleotide region

The 1349bp PCR product amplification using viral genomic RNA-synthesized cDNA representing different pathotypes of NDV were produced using a consensus primer pair for the fusion protein gene (Plate 3).

4.7.2 Fusion protein cleavage site (FPCS) 254 nucleotide region

The amplification of coding sequence surrounding FPCS using degenerate oligonucleotide primers from three different pathotypes of NDV, resulted in a 254bp amplicon (Plate 4).

4.7.3 Matrix protein nuclear localization signal (MPNLS) 232 nucleotide region

Amplification of 232bp region surrounding nucleotide sequence encoding MPNLS from three different pathotypes of NDV was produced using degenerate oligonucleotide primers. The MPNLS amplification was obtained from all the isolates, except NPL-5/93, PCKC/98, LBC/99 with the protocol used (Plate 5).

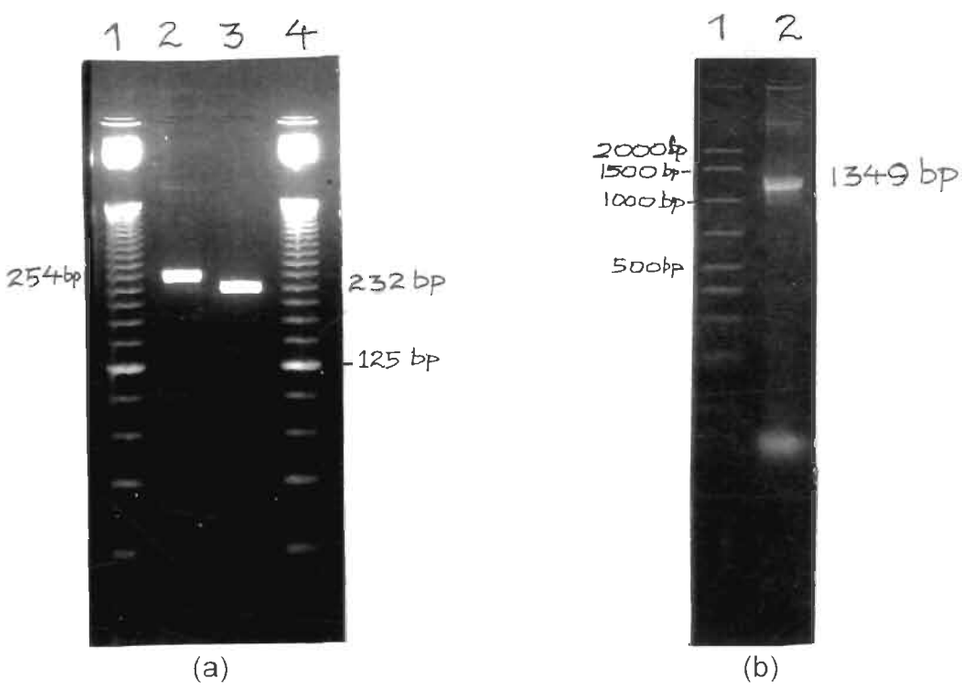


Plate - 3 : RT-PCR of (a)Fusion protein cleavage site, 254 bp, & Matrix protein Nuclear localization signal region and (b) Fusion protein gene(1349 bp) amplicon from NDV isolates electrophoresed in (a) 4.0 % and (b) 2.5% agarose gel and stained with Ethidium bromide

(a)Lane 1&4 : 25 bp DNA ladder (b) Lane 1 : pBR322/ Hinf I digest
2 : FPCS, 254 bp amplicons 2 : Fusion protein(1349 bp)gene
3 : MPNLS,232 bp amplicon amplicon

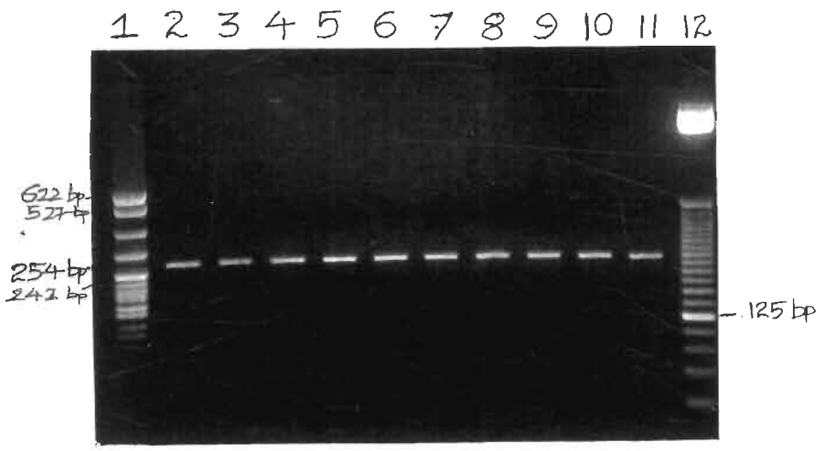


Plate - 4 : RT-PCR of Fusion protein cleavage site, 254 bp amplicons from NDV isolates electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

Lane 1 : pBR322/ Msp I digest

2-11 : FPCS, 254 bp amplicons of representative strains

12 : 25 bp DNA ladder

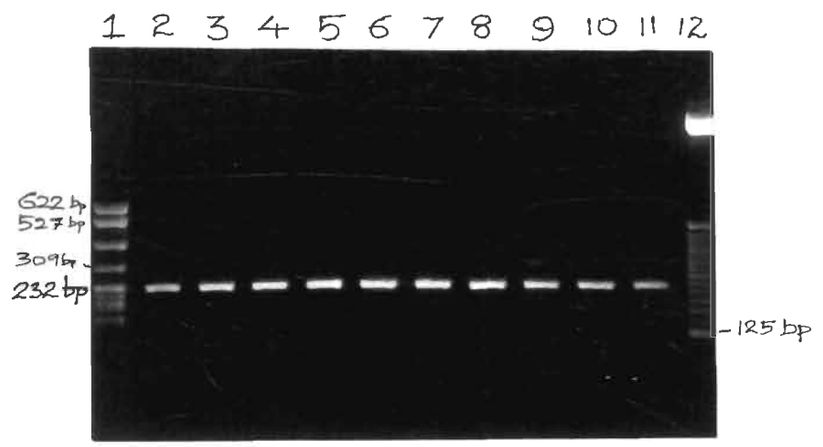


Plate - 5 : RT-PCR of Matrix protein gene Nuclear localization signal region, 232 bp amplicons from NDV isolates, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

Lane 1 : pBR322/ Msp I digest

2-11 : MPNLS, 232 bp amplicons of representative strains

12 : 25 bp DNA ladder

4.8 RESTRICTION ENDONUCLEASE ANALYSIS (REA) OF FUSION GENE (1349BP) AMPLICON

Restriction Endonuclease Analysis of Fusion gene (1349bp) amplicon was carried out with restriction enzymes Hinf I, Bst OI, RsaI, Hae III, Mbo I and Taq I.

4.8.1 Hinf I Restriction fragment profiles

Hinf I generated 6 different fingerprint patterns/groups of the 18 NDV isolates analysed (Plate 6). The group H 1 and H 2 consisted of 7 NDV isolates. while group H 3 consisted of 4 NDV isolates. The vaccine strains La Sota and 'B1' went into two different groups H 4 and H 5, while 'F' and 'K were grouped together in H 6 (Table 5a).

4.8.2 Bst OI Restriction fragment profile

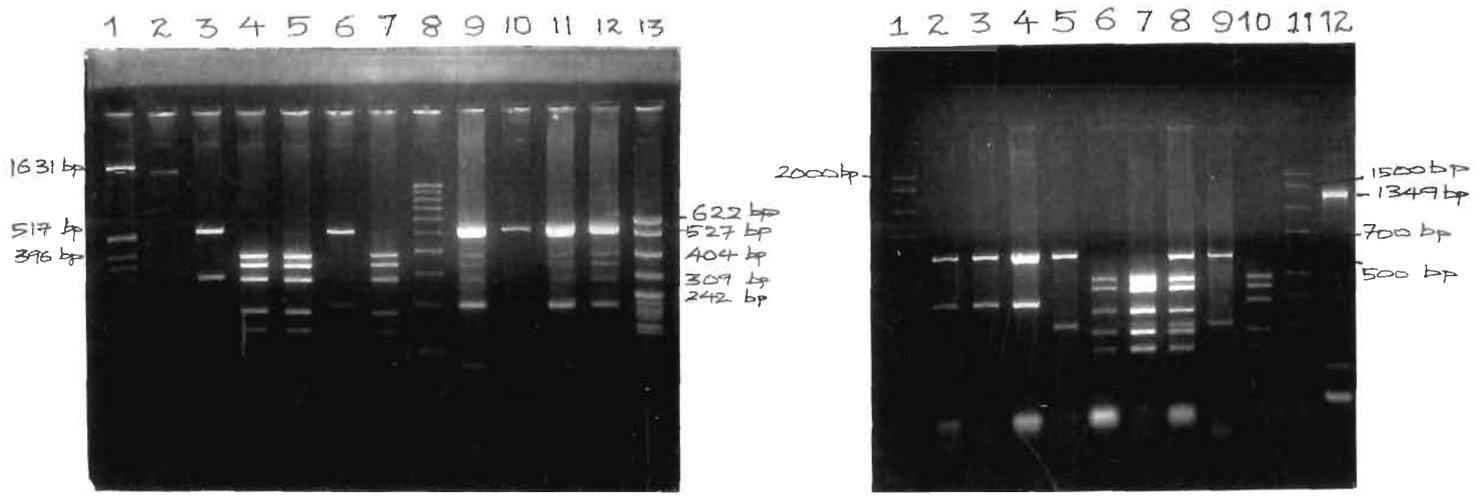
Five different fingerprints patterns were generated with Bst OI enzyme (Plate 7). The group B1 consisted of 8 NDV isolates, including the vaccine strain B2. The group B 2 and B 3 consisted of two NDV isolates each, while group B 4 comprised of 3 NDV isolates and 4 vaccine strains. Group B 5 had 3 NDV isolates from desi birds (Table 5a).

4.8.3 Rsa I Restriction fragment profile

Four different fingerprint patterns were generated with Rsa I digestion (Plate 8). The group R1 consisted of 7 NDV isolates and 2 vaccine strains La Sota and 'B1'. The group R 2 had 3 NDV isolates while group R 3

Table-4 : Restriction Site grouping of NDV Isolates based on Fusion gene (1349bp) amplicon restriction endonuclease analysis

ISOLATE/ STRAIN	Year	Host	Place	Genetic groups - Fusion gene(1349Bp) Hinf I,Bst OI and Rsa I restriction sites
JQC6/87/V	1987	Japanese Quail	Chennai	**VI**
LCN2/87/V	1987	Layer	Namakkal	**VI / RI-3**
LCN3/87/V	1987	Layer	Namakkal	**IV**
BCC4/87/V	1987	Broiler	Chennai, VYD	**VI/ RI-3/ V**
LCC5/89/V	1987	Layer	Chennai, VLK	**II**
LCC9/89/V	1989	Layer	Chennai, RH	**II**
LCC15/89/V	1989	Layer	Chennai, MPK	**II**
BRLY-58/93/V	1993	Chicken	Bareilly, UP	**VI**
WB-198/94/V	1994	Chicken	West Bengal	**II**
D1/95/L	1995	Desi bird	Chennai	**II**
D2/95/V	1995	Desi bird	Chennai	**VI / V**
D3/95/V	1995	Desi bird	Chennai	**VI / V**
D4/95/L	1995	Desi bird	Chennai	**VI / V**
D5/95/M	1995	Desi bird	Chennai	**II**
KQA-ALW/98	1998	Japanese Quail	Kerala	**II**
KQC-CHE/98	1998	Japanese Quail	Kerala	**II**
KPG/98/V	1998	Pigeon	Kerala	**II**
MUKTESWAR/46/M	1946	Chicken	Ranikhet, UP	**II**

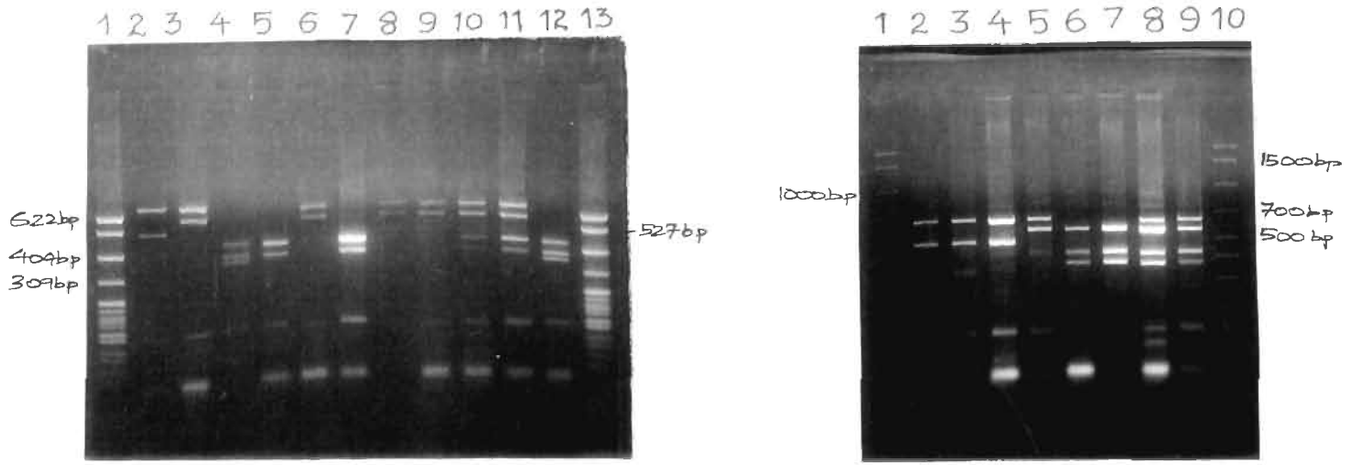


(A) (B)

Plate 6: Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Hinf I, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane 1 - pBR322/Hinf I digest
2 - Uncut-fusion gene 1349bp amplicon
3 - MUKTESWAR(R_2B) /B/M
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - 100 bp ladder
8 - BCC4/87/C1/
9 - LCC5/89/C1/
10 - LCC9/89/C1/
11 - LCC15/89/C1/
12 - WB-198/94/
13 - pBR322/Msp I digest

B : Lane 1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - BRLY-58/93/
11 - 50-2000 bp ladder
12 - U/c Fusion 1349bp amplicon

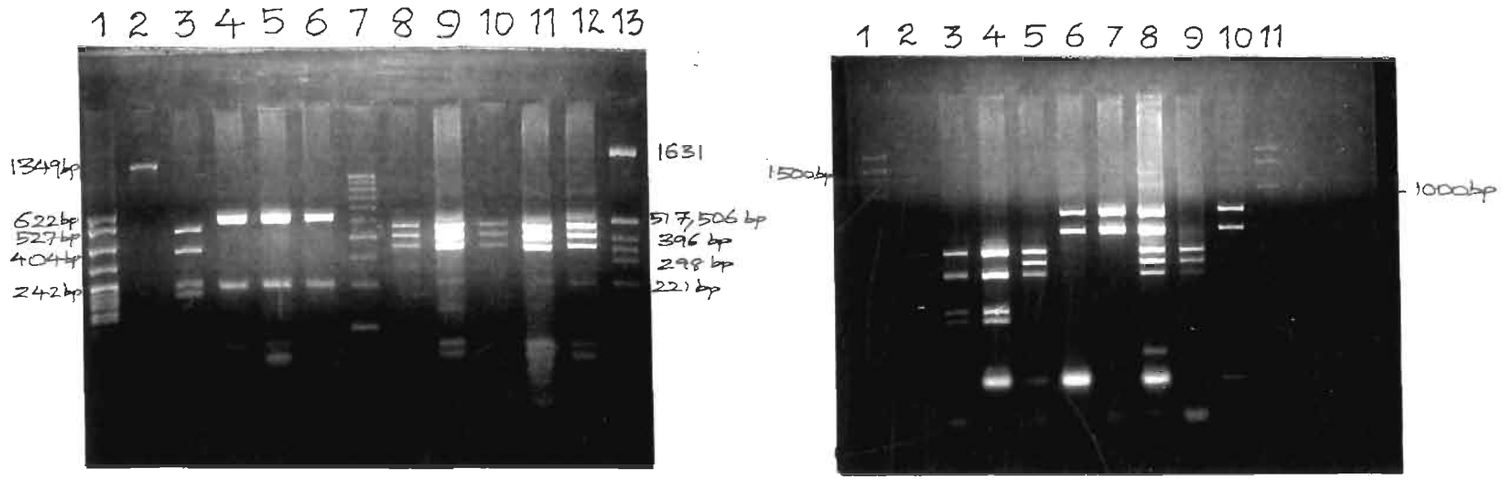


(A) (B)

Plate - 7 : Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Bst OI, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane
1 - pBR322/ Msp I digest
2 - MUKTESWAR(R_2B) /B/M
3 - LaSota
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - BCC4/87/C1/
8 - LCC5/89/C1/
9 - LCC9/89/C1/
10 - LCC15/89/C1/
11 - WB-198/94/
12 - BRLY-58/93/
13 - pBR322/ Msp I digest

B : Lane
1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - 50-2000 bp ladder



(A) (B)

Plate - 8 : Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Rsa I, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane 1 - pBR322/ Msp I digest
2 - Uncut-fusion gene 1349bp amplicon
3 - MUKTESWAR(R_2B) /B/M
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - 100 bp ladder
8 - BCC4/87/C1/
9 - LCC5/89/C1/
10 - LCC9/89/C1/
11 - LCC15/89/C1/
12 - WB-198/94/
13 - pBR322/ Hinf I digest

B : Lane 1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - BRLY-58/93/
11 - 50-2000 bp ladder

comprised of 3 NDV isolates along with 3 vaccine strains viz., R_2B, F and K. The final group R 4 consisted of 4 NDV isolates, of which 3 are from Desi birds (Table 5a).

4.8.4 Hae III Restriction fragment profile

Hae III generated five different fingerprint patterns from 18 NDV isolates analysed (Plate 9). Group A1 consisted of 6 NDV isolates, while group A2 had 5 NDV isolates, while group A3 comprised of 3 NDV isolates from Kerala and one vaccine strain B_2B. The group A4 had only one NDV isolate JQC6/87. while group A5 consisted of two vaccine strains viz., La Sota and B1 (Table 5b).

4.8.5 Mbo I Restriction fragment profile

Mbo I generated only 3 different fingerprint patterns from the 18 NDV isolates analysed (Plate 10). The group M1 consisted of ten NDV isolates and two vaccine strains R_2B and B1. Group M2 consisted of 6 NDV isolates 3 each from chicken and desi birds. Group M3 had the vaccine strain La Sota grouped alone, separately from other NDV isolates (Table 5b).

4.8.6 Taq I Restriction fragment profile

Taq I generated only three different fingerprint patterns (Plate 11) with two major group T 1 and T 2. Group T 1 consisted of 6 NDV isolates and one vaccine strain R_2B. Group T 2 had 8 NDV isolates while Group T 3 had the two vaccine strains La Sota and B1 (Table 5b).

Table - 5(a): Molecular weight of the Fusion gene(1349) bp amplicon restriction site analysis with different restriction enzymes: Hinf I, Bst OI and Rsa I

Hinf I	Isolates	Molecular Size
H1	JQC6/87, LCN2/87, BCC4/87, D2/95,D3/95,D4/95, BRLY-58/93	380,336,281,181, 139,21
H2	LCN3/87, LCC5/89,LCC9/89, LCC15/89, WB-198/94, D1/95, D5/95	519,517,203,79
H3	KQA-ALW/98, KQC-CHE/98, KPG/98, MUKTESWAR/46	519,517,282
H4	LaSota	542,525,203,79
H5	B1	542,517,203,79
H6	F, K	542,517,282

Bst OI		
B1	LCN3/87, LCC5/89, LCC9/89, LCC15/89 WB-198/94, D1/95, D5/95, B1	646,566,137
B2	JQC6/87,BRLY-58/93	419,364,341,144,8
B3	LCN2/87,BCC4/87	422,419,364,144
B4	KQA-ALW/98,KQC-CHE/98,KPG/98, MUKTESWAR/46, LASOTA, F AND K	646,485,137,81
B5	D2/95,D3/95,D4/95	566,419,364

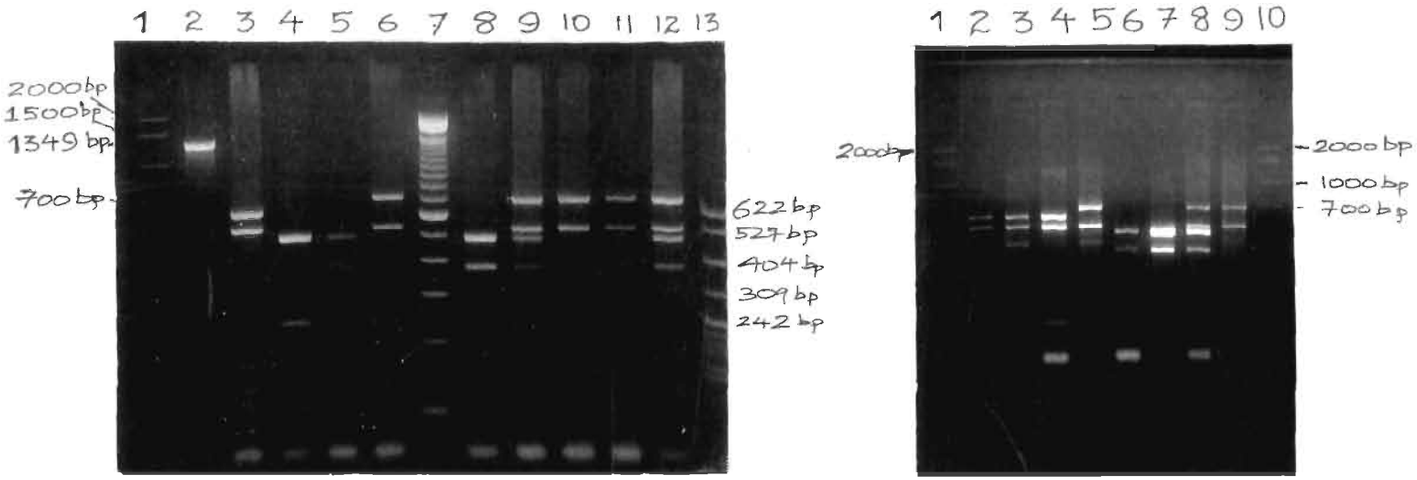
RsaI		
R1	JQC6/87, LCN2/87,LCN3/87,	539,538,215,57
R2	BCC4/89,LCC5/89, LCC9/89, LCC15/89 WB-198/94, D1/95, D5/95,	465,404,350,73,57
R3	KQA-ALW/98, KQC-CHE/98, KPG/98, MUKTESWAR/46, F, K	465,350,215,189, 73,57
R4	D2/95, D3/95,D4/95, BRLY-58/93	754,595

Table - 5(b): Molecular weight of the Fusion gene(1349 bp) amplicon restriction site analysis with restriction enzymes, Hae III, Taq I and Mbo I

HaeIII	Isolates	Molecular Size
A1	LCN3/87,LCC5/89,LCC15/89 WB-198/94, D1/95, D5/95,	704,525,120
A2	LCN2/87, BCC4/87, D2/95, D3/95,D4/95	485,485 , 379
A3	KQA-ALW/98, KQC-CHE/98, KPG/98,MUKTESWAR/46	618,538,120,73
A4	JQC6/87	485,485,235,147
A5	LaSota, B1	693,531,124

Taq I		
T1	LCN3/87, LCC5/89, LCC9/89,LCC15/89 WB-198/94, D4/95, MUKTESWAR/46/M	1019, 220,110,1030,231,98
T2	KQA-ALW/98, KQC-CHE/98, KPG/98, D1/95, D5/95, JQC6/87, LCN2/87, BCC4/87,	709,380,260
T3	LaSota, B1	1031,219,98

Mbo I		
M1	LCN3/87, LCC5/89, LCC9/89,LCC15/89 WB-198/94, KQA-ALW/98, KQC-CHE/98, KPG/98, MUKTESWAR/46/M, B1	1139,210,1131,214
M2	JQC6/87, LCN2/87, BCC4/87,D2/95, D3/95, D5/95,	625,505,219
M3	LaSota,	823,308,214,4

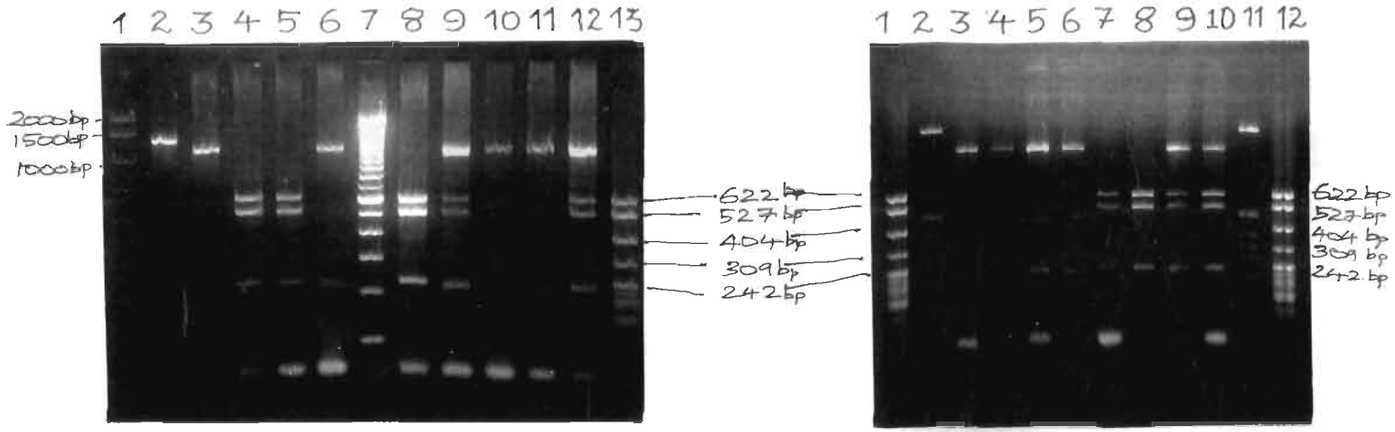


(A) (B)

Plate - 9 : Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Hae III, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane 1 - 50-2000 bp ladder
2 - Uncut-fusion gene 1349bp amplicon
3 - MUKTESWAR(R_2B) /B/M
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - 100 bp ladder
8 - BCC4/87/C1/
9 - LCC5/89/C1/
10 - LCC9/89/C1/
11 - LCC15/89/C1/
12 - WB-198/94/
13 - pBR322/Msp I digest

B: Lane 1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - 50-2000 bp ladder

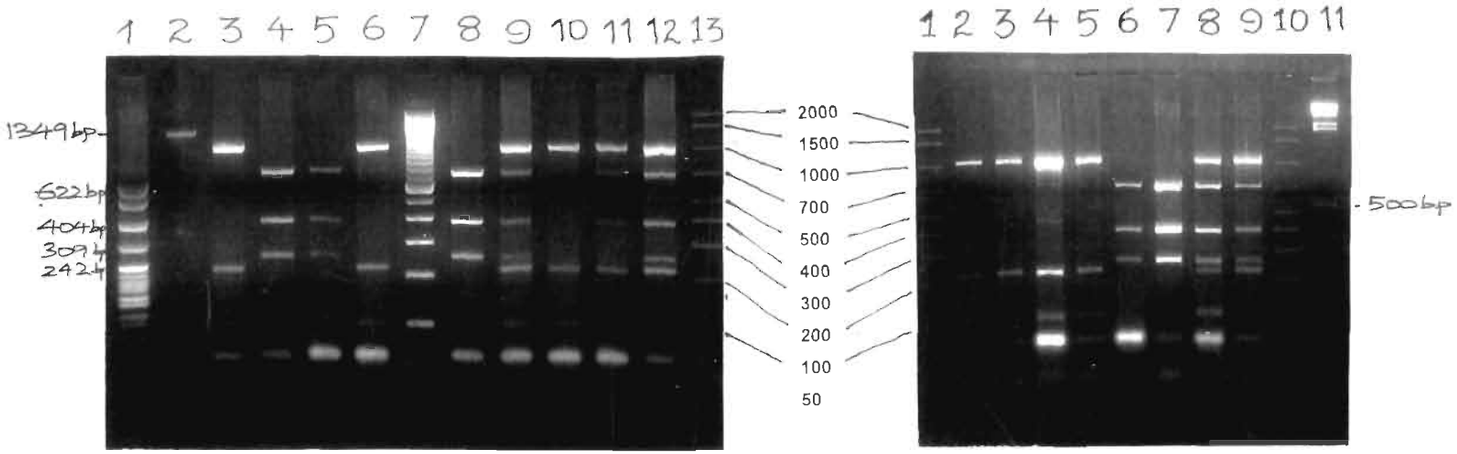


(A) (B)

Plate 10 : Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Mbo I, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane 1 - 50-2000 bp ladder
2 - Uncut-fusion gene 1349bp amplicon
3 - MUKTESWAR(R_2B) /B/M
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - 100 bp ladder
8 - BCC4/87/C1/
9 - LCC5/89/C1/
10 - LCC9/89/C1/
11 - LCC15/89/C1/
12 - WB-198/94/
13 - pBR322/Msp I digest

B : Lane 1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - BRLY-58/93/
11 - pBR322/Hinf I digest
12 - pBR322/Msp I digest



(A) (B)

Plate - 11: Restriction fragment profile of the Fusion protein gene,1349 bp amplicon of NDV isolates cut with Taq I, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

A: Lane 1 - pBR322/Msp I digest
2 - Uncut-fusion gene 1349bp amplicon
3 - MUKTESWAR(R_2B) /B/M
4 - JQC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - 100 bp ladder
8 - BCC4/87/C1/
9 - LCC5/89/C1/
10 - LCC9/89/C1/
11 - LCC15/89/C1/
12 - WB-198/94/
13 - 50-2000 bp ladder

B : Lane 1 - 50-2000 bp ladder
2 - KQA-ALW/98/V
3 - KQC-CHE/98/V
4 - KPG/98/V
5 - D1/95/E/L
6 - D2/95/C1/
7 - D3/95/C1/V
8 - D4/95/L/L
9 - D5/95/E/L
10 - λ/Hind III digest

4.8.7 Analysis of fusion gene (1349bp) fingerprints of NDV isolates

The molecular weight of obtained fragments using different restriction enzymes were estimated using Kodak 1 D Image Analysis software for all NDV isolates tested (Table 5a&b)The Jaccard Distance coefficient measures for restriction enzyme profiles was estimated and the distances of Hinf I, Bst OI and Rsa I profiles were taken as input for phylogenetic analysis, chosen on the basis of having more variation in fingerprint patterns (Table-4&5a&b).

4.8.8 Phylogenetic analysis of NDV isolates based on Fusion gene (1349bp) fingerprint Distances

The obtained fusion gene fingerprint distances of Hinf I, Bst O1 and Rsa 1 profiles on analysis using MEGA-software with the Unweighted Pair Group based on Arithmetic mean (UPGMA) method yielded phylogenetic trees grouping NDV isolates and vaccine strains.

4.8.8.1 Hinf I : UPGMA Phylogenetic grouping

The Hinf I phylogenetic grouping classified the vcaccine strains BI and La Sota in a single cluster. The NDV isolates JQC6/87, LCN2/87 and BCC4/87 formed the major ancester of the vaccine strains cluster and other field isolates cluster. The vaccine strains "F" and "K" were closely branched to other vaccine strains but grouped along with vaccine strain R2B, KQA-ALW/98, KQC-CHE/98, KPG/98 AND LCN 3/87, LCC 5/89, LCC 9/89, LCC 15/89, WB-198/94, D1/95 and D5/95 cluster (Fig.3).

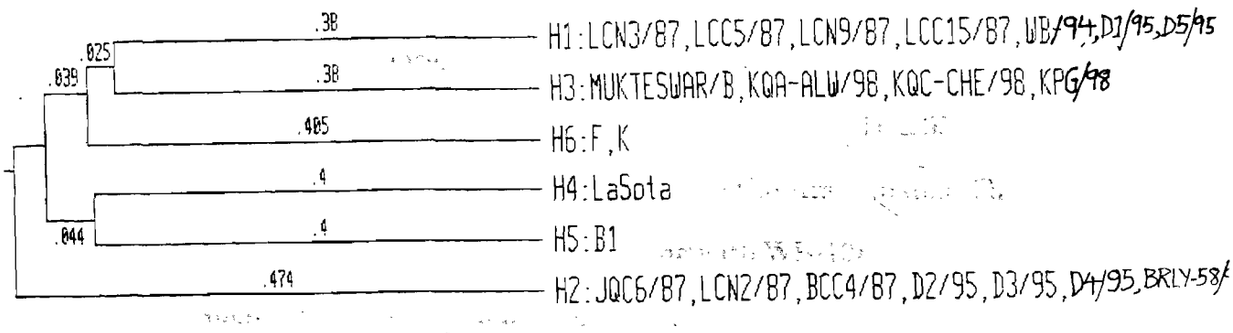


Fig. 3 : Hinf - UPGMA tree, using MEGA based on Jaccard's distance coefficient measures of Fusion gene(1349 bp) fingerprints.
Scale: each - is approximately equal to the distance of 0.013941

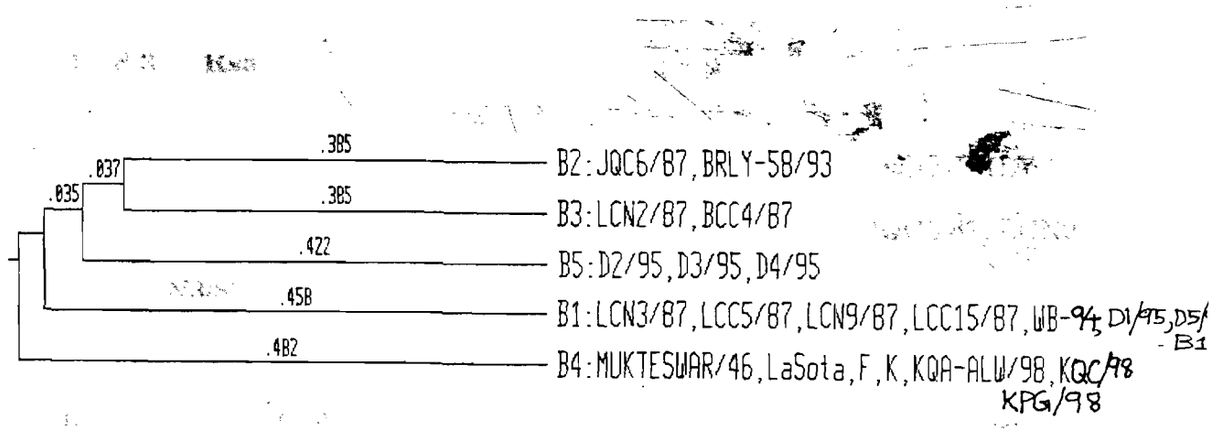


Fig. 4 : Bst OI - UPGMA tree, using MEGA based on Jaccard's distance coefficient measures of Fusion gene(1349 bp) fingerprints.
Scale: each - is approximately equal to the distance of 0.014191

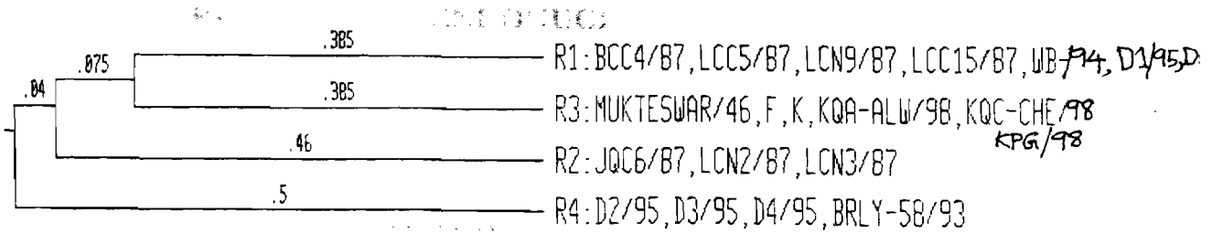


Fig. 5 : Rsa I - UPGMA tree, using MEGA based on Jaccard's distance Coefficient measures of Fusion gene(1349 bp) fingerprints.
Scale: each - is approximately equal to the distance of 0.014706

4.8.8.2 Bst OI : UPGMA Phylogenetic grouping

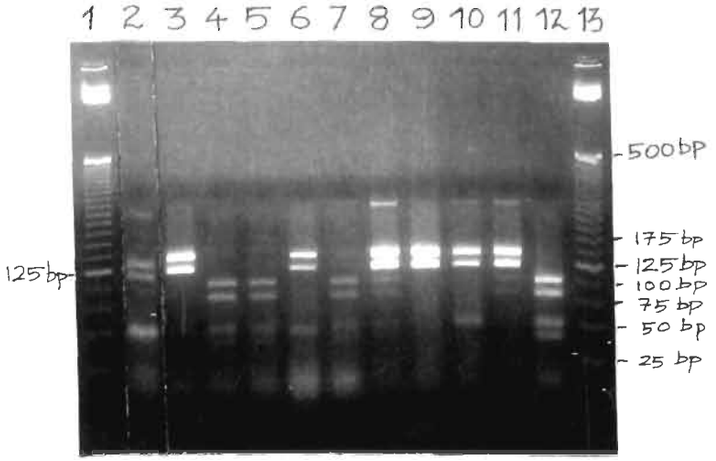
The NDV isolates JQC 6/87, BRL-58/93, LCN 2/87 and BCC 4/87 together formed a single cluster. The desi bird isolates D 2/95, D 3/95 and D4/95 were the next branching group related to the first cluster. The isolates LCN3/87, LCC5/87, LCC9/89, LCC15/89 along with WB-198/94 formed the next major branching to the previous cluster. The isolates KQA-ALW/98, KQC-CHE/98 and KPG/98 along with vaccine strains R_2B, La Sota, F and K formed the major ancestor of all the previous branching (Fig.4).

4.8.8.3 Rsa I : UPGMA Phylogenetic grouping

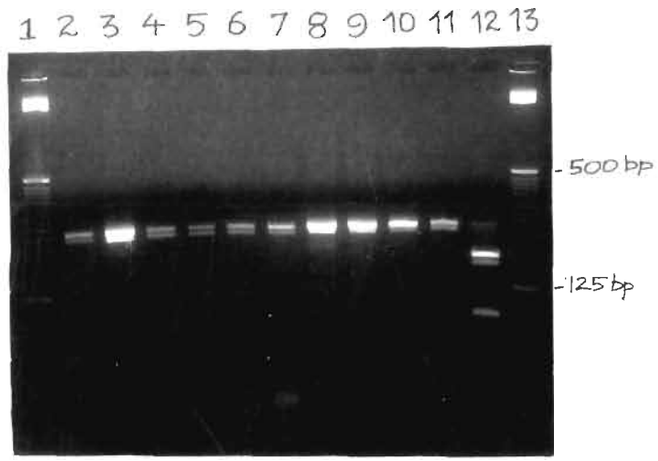
Isolates from desi birds D2/95, D3/95, D4/95 along with BRLY-58/93 formed the first cluster with the velogenic chicken isolates JQC6/87, LCN2/87 and LCN3/87. The next major clustering was of the group with isolates KQA-ALW/98, KQC-CHE/98, KPG/98 along with vaccine strains R_2B_1 'F' and 'K'. The major ancestor branching of the cluster was formed by the isolates BCC4/87, LCC5/87, LCC9/89. LCC15/89 and WB-198/94 along with vaccine strains La Sota and B1 (Fig.5a&5b)

4.9 RESTRICTION ENDONUCLEASE ANALYSIS (REA) OF FUSION PROTEIN CLEVEAGE SITE (FPCS) AMPLICON

Restriction endonuclease analysis (REA) of FPCS of representative NDV isolates with Alu I, revealed two different patterns (Plate 12) while Msp I digestion yielded a homologous pattern with all isolates except isolate BRLY-58/93 which produced a unique pattern (Plate 13).



(12)



(13)

Plate - 12 & 13: Restriction fragment profile of the Fusion protein gene cleavage site,254 bp amplicon of NDV isolates cut with Alu I, (12) and Msp I (13) electrophoresed in 4.0% agarose gel and stained with Ethidium bromide

12: Lane 1 - 25 bp DNA ladder
2 - MUKTESWAR(R_2B) /B/M
3 - LaSota
4 - QC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - BCC4/87/C1/
8 - LCC5/89/C1/
9 - LCC9/89/C1/
10 - LCC15/89/C1/
11 - WB-198/94/
12 - BRLY-58/93/
13 - 25 bp DNA ladder

13: Lane 1 - 25 bp DNA ladder
2 - MUKTESWAR(R2B)/B/M
3 - La Sota
4 - QC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - BCC/4/87/C1/
8 - LCC5/89/C1/
9 - LCC9/89/C1/
10 - LCC15/89/C1/
11 - WB-198/94/
12 - BRLY-58/93/
13 - 25 bp DNA ladder

4.10 RESTRICTION ENDONUCLEASE ANALYSIS (REA) OF MATRIX PROTEIN NUCLEAR LOCALIZATION SIGNAL (MPNLS) REGION AMPLICON

Restriction endonuclease analysis (REA) of MPNLS amplicon of representative NDV isolates with Alu I generated only two different patterns (Plate 14), while Hinf I generated three different patterns (Plate 15).

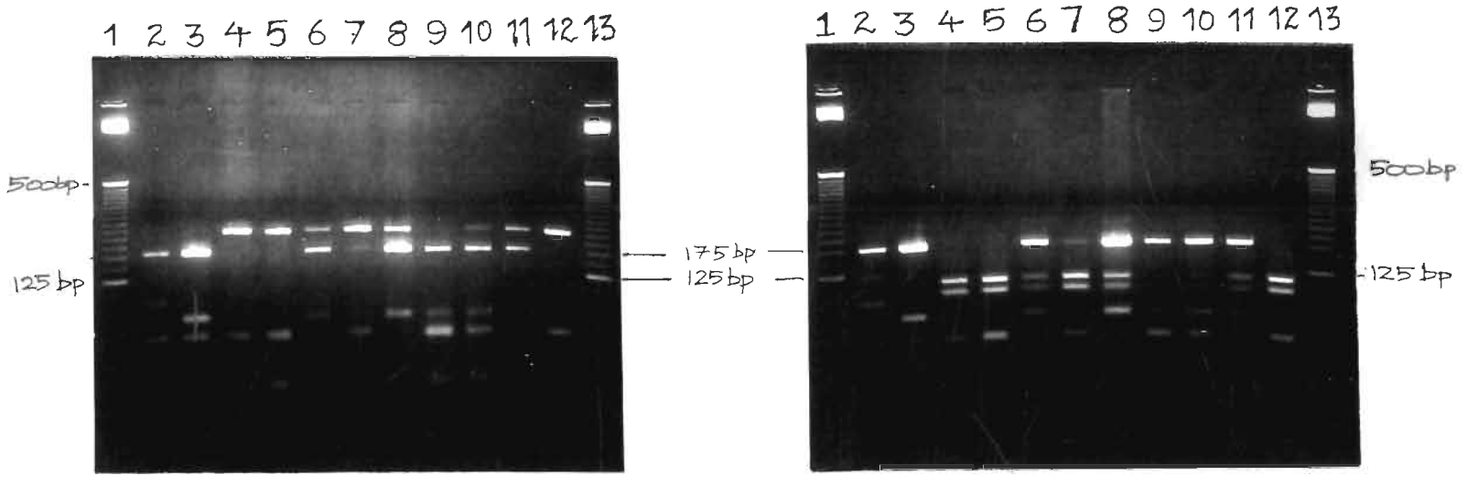
4.11 PURIFICATION OF FPCS AND MPNLS AMPLICONS

The PCR products from all NDV isolates on purification using Wizard in PCR purification columns yielded PCR product free of primers and primer dimer complexes (Plate 16).

4.12 DIRECT DOUBLE STRANDED NUCLEOTIDE SEQUENCING OF THE FPCS AND MPNLS AMPLICONS

4.12.1 Manual Radioactive nucleotide sequencing

Manual radioactive nucleotide sequencing was standardized with control M13 DNA (Plate 17). The sequencing of MPNLS region of R_2B vaccine strain was carried out with both forward and reverse primers using urea + formamide sequencing gel which helped to resolve compression faced with urea alone sequencing gels (Plate 18). The sequencing of MPNLS region of JQC6/87 was carried out with forward and reverse primers using urea alone gel (Plate 19). The autoradiograms were manually read and the sequence was later compared to automated sequencing results, which was found to have good correlation.



(14) (15)

Plate - 14 & 15: Restriction fragment profile of the Matrix protein gene Nuclear Localisation signal region , 232 bp amplicon of NDV isolates cut with Alu I, (14) and Hinf I (15) electrophoresed in 4.0% agarose gel and stained with Ethidium bromide

14: Lane 1. - 25 bp DNA ladder
2 - MUKTESWAR(R_2B) /B/M
3 - LaSota
4 - QC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - BCC4/87/C1/
8 - LCC5/89/C1/
9 - LCC9/89/C1/
10 - LCC15/89/C1/
11 - WB-198/94/
12 - BRLY-58/93/
13 - 25 bp DNA ladder

15: Lane 1 - 25 bp DNA ladder
2 - MUKTESWAR(R2B)/B/M
3 - La Sota
4 - QC6/87/C1/V
5 - LCN2/87/C1/V
6 - LCN3/87/C1/
7 - BCC/4/87/C1/
8 - LCC5/89/C1/
9 - LCC9/89/C1/
10 - LCC15/89/C1/
11 - WB-198/94/
12 - BRLY-58/93/
13 - 25 bp DNA ladder

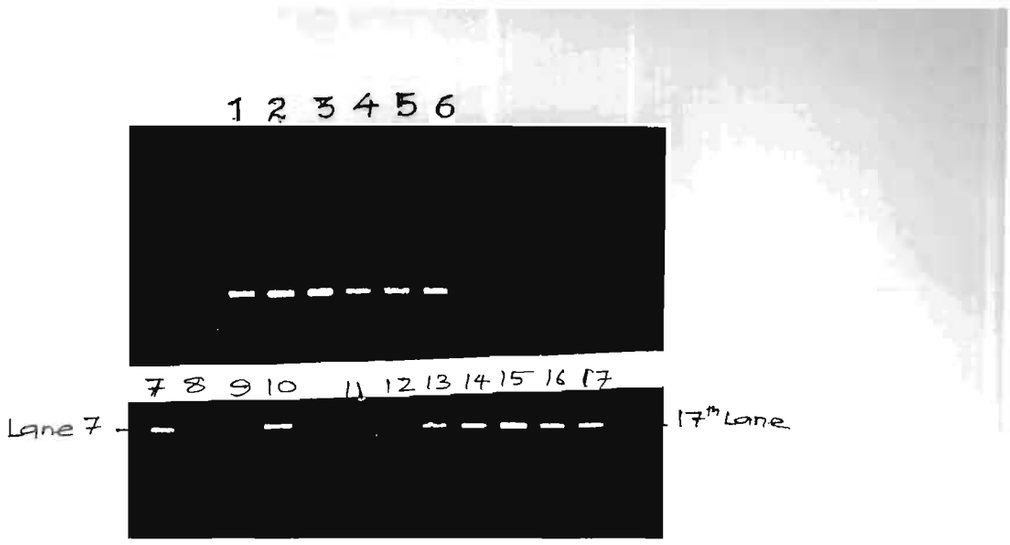


Plate 16 : Purification of RT-PCR amplicons, electrophoresed in 2.5% agarose gel and stained with Ethidium bromide

Lanes 1-6 - Unpurified RT-PCR amplicons (UPPER LANES)
Lanes 7-17 - Purified RT-PCR amplicons (LOWER LANES)

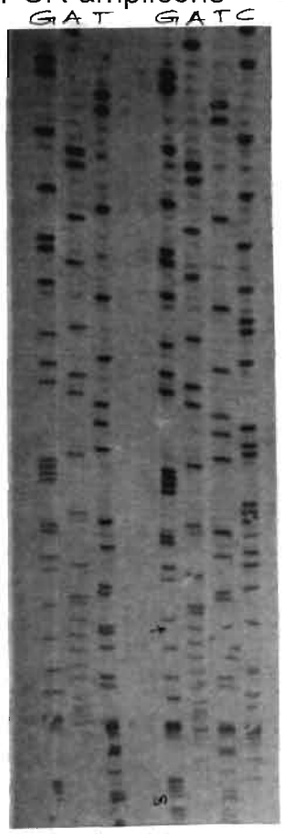


Plate 17 : Manual radioactive nucleotide sequencing - Control, M13 DNA

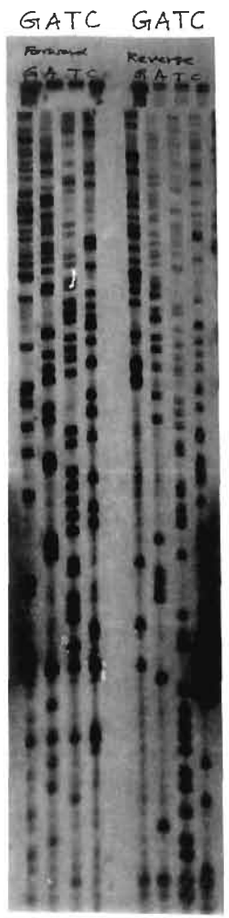


Plate 18 : Manual radioactive nucleotide sequencing - MUKTESWAR/46

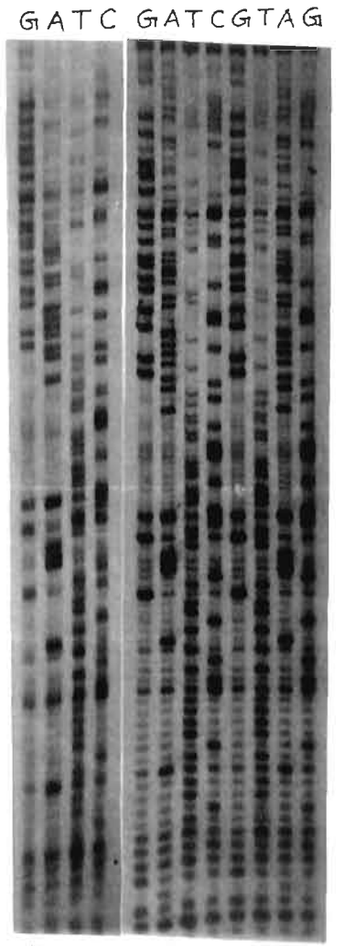


Plate 19 : Manual radioactive nucleotide sequencing JQC6/87

4.12.2 Automated Nucleotide sequencing

Automated nucleotide sequencing of the purified FPCS PCR amplicons of selected NDV isolates viz., JQC6/87, LCN2/87, LCN3/87, BCC4/87, LCC9/89, BRL-58/93, WB-198/94, D1/95, D2/95, D3/95, D4/95, KNBB/96, KQA-ALW/98, KQC-CHE/98, KPG/98, Mukteswar/46, NPL-5/93 along with direct RT-PCR positive PCKC/98, LBC/99 and one cell cultured adapted strain CCA/93 was carried out.

The MPNLS amplicon of choosen isolates viz., JQC6/87, LCN2/87, LCN3/87, BCC4/87, LCC9/89, BRL-58/93, WB-198/93, D1/95, D3/95, D4/95, KQA-ALW/98, KQC-CHE/98, KPG/98 and Mukteswar/46 were sequenced by automated nucleotide sequencing. The gel file of the automated sequencing (Fig.6) was subjected to analysis of each sample lanes using sequence Data analysis software,to obtain sequence of each isolate as electrophoregrams. The electrophoregram of FPCS and MPNLS amplicon sequence of the analysed isolates are presented (Fig.7-26). The individual sequences of FPCS and MPNL loaded as separate text files in Fasta format was used for further sequence analysis.

4.13 NUCLEOTIDE SEQUENCE ANALYSIS

The raw nucleotide sequence data was subjected to open reading frame prediction, conceptual translation into aminoacid sequence and nucleotide and aminoacid sequence alignment.

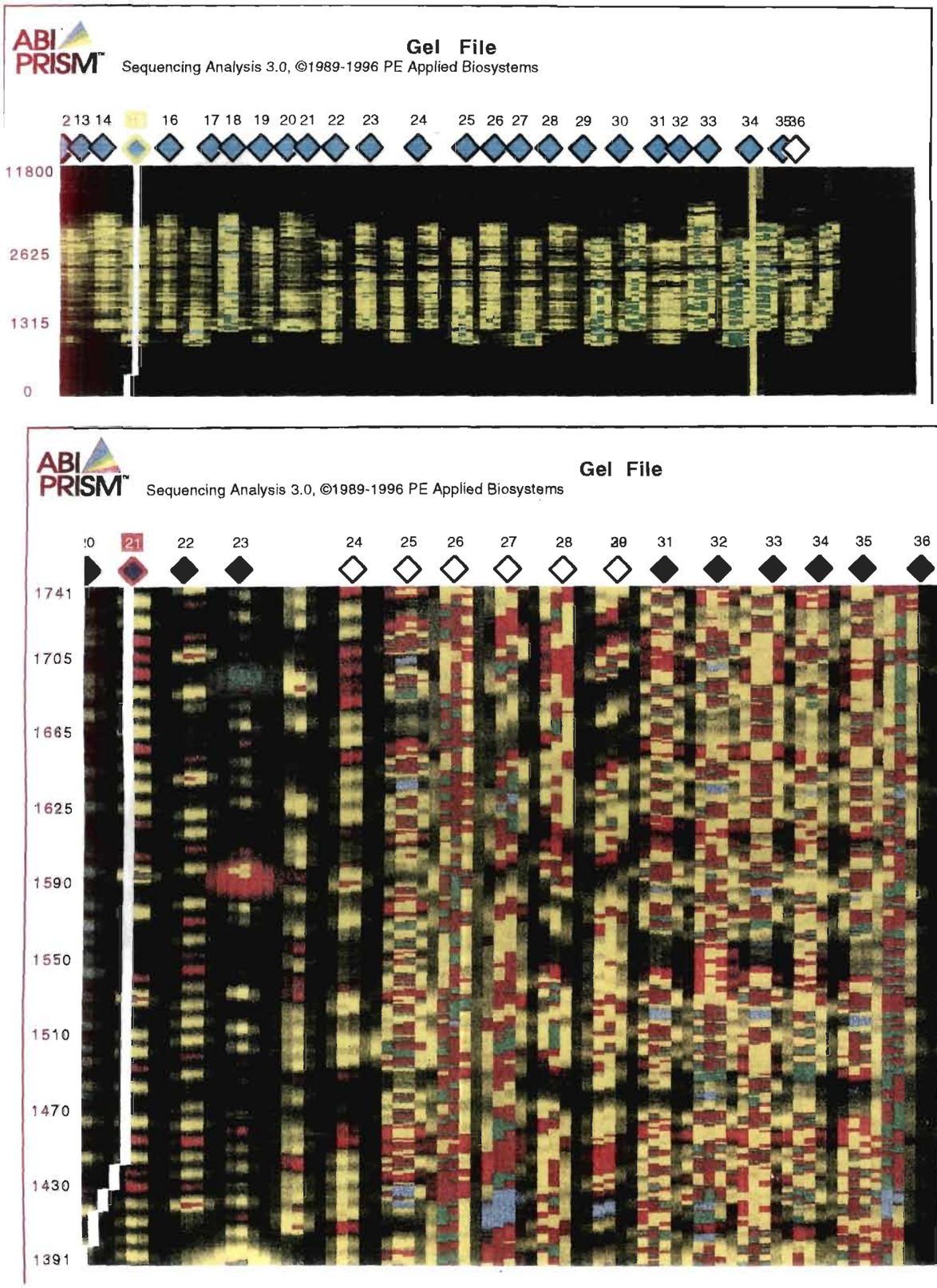


Fig. 6: Gel File : Automated sequencing of NDV Isolates - Analysis of the samples lanes

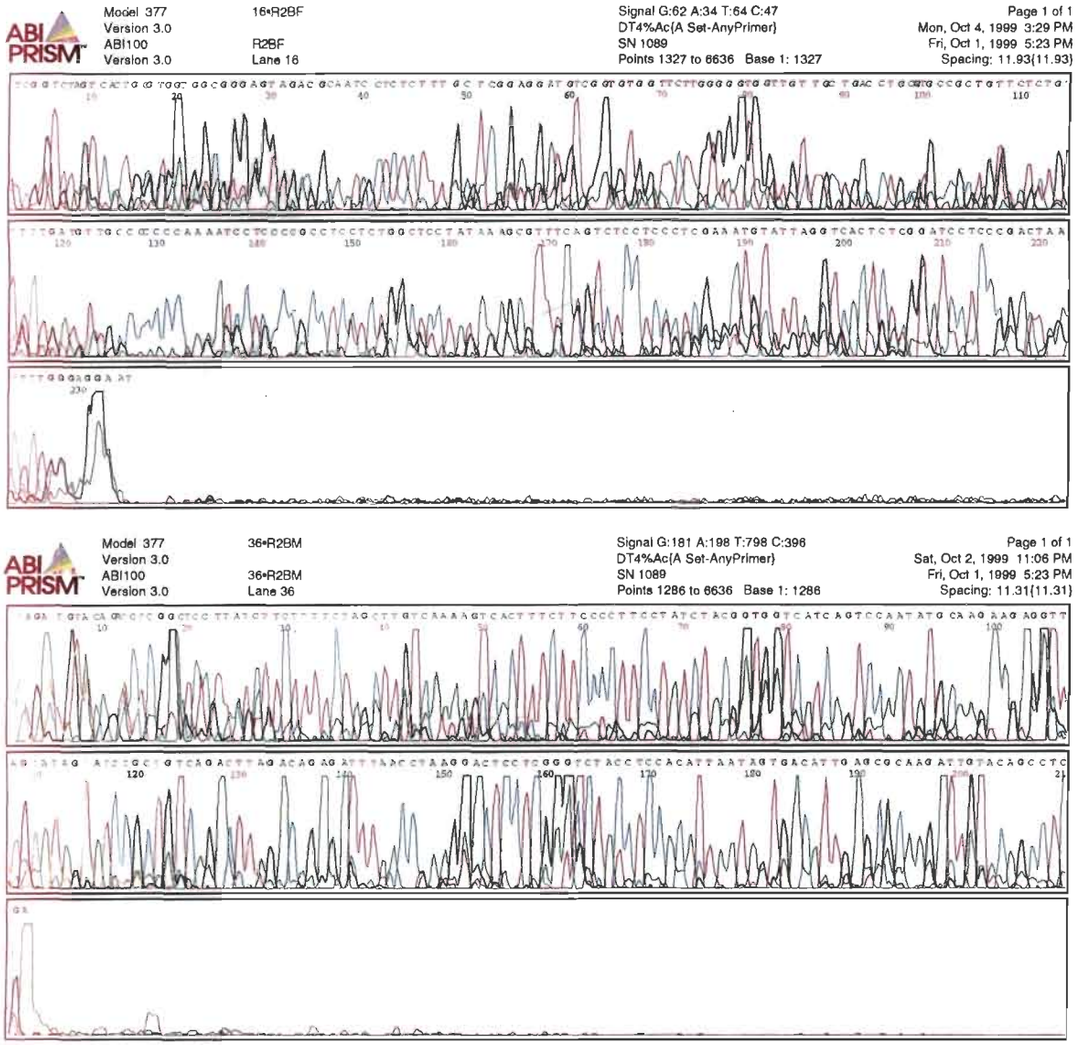


Fig. 7 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of MUKTESWAR/46

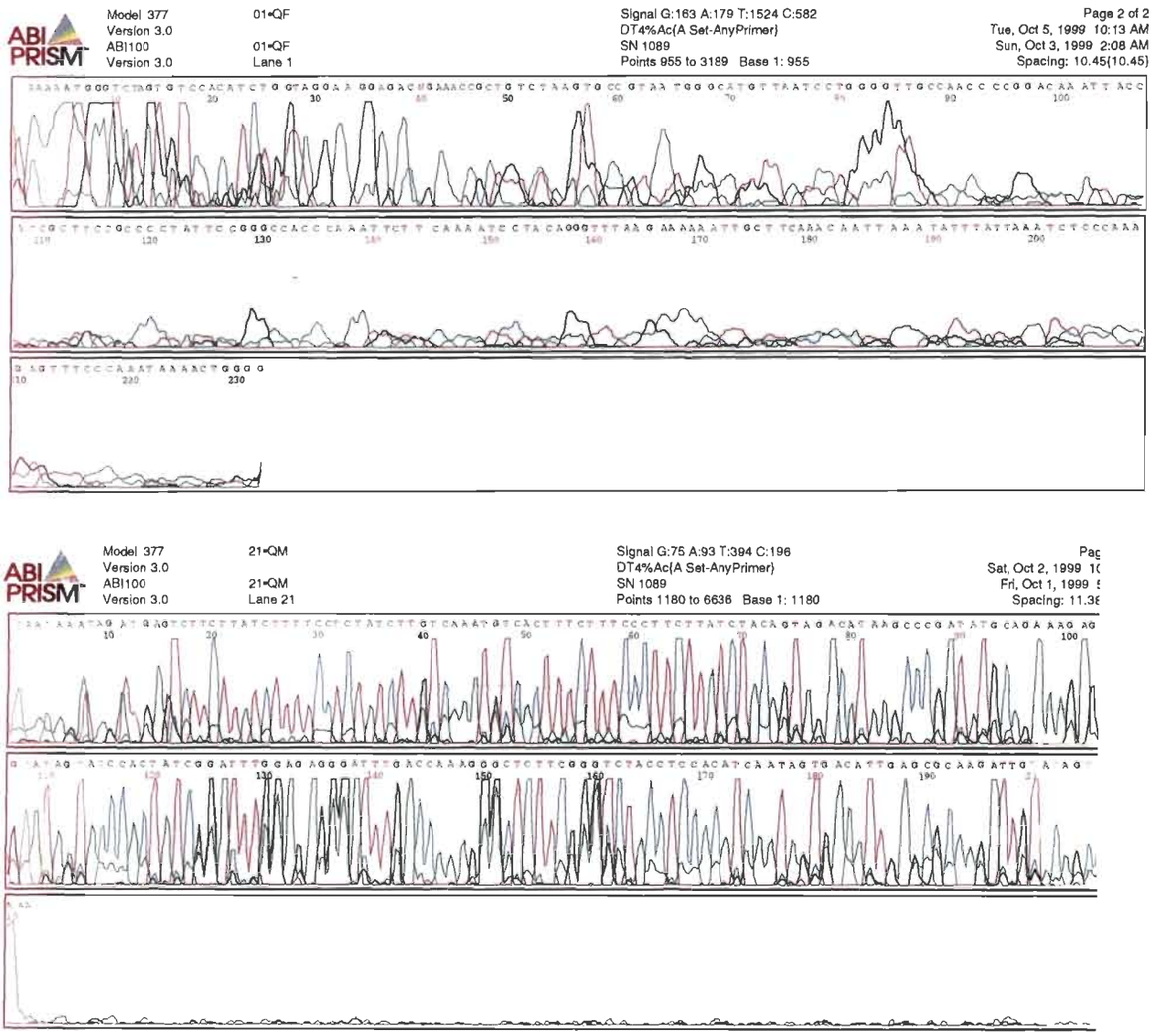
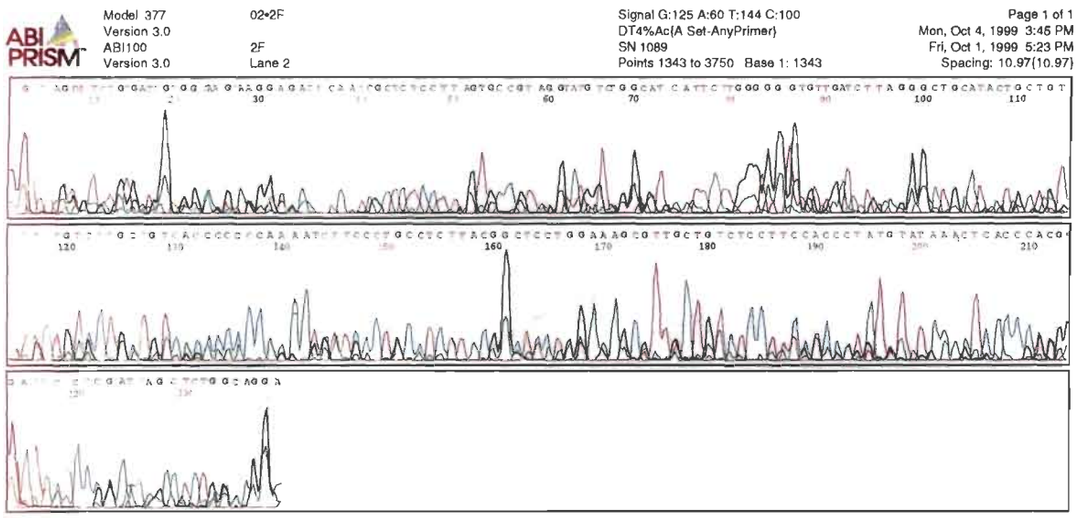


Fig. 8 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of JQC6/87



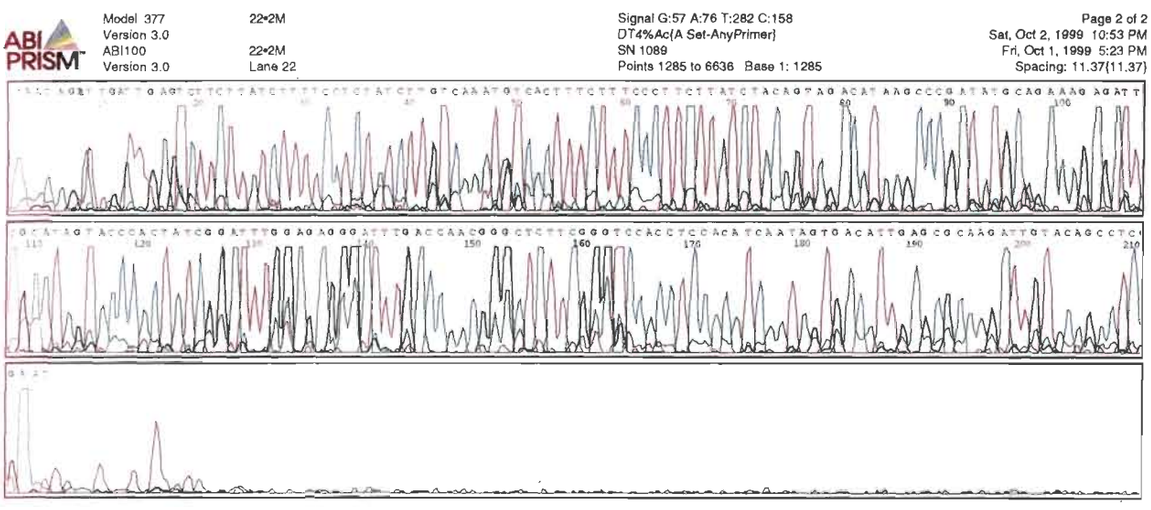


Fig. 9 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of LCN2/87

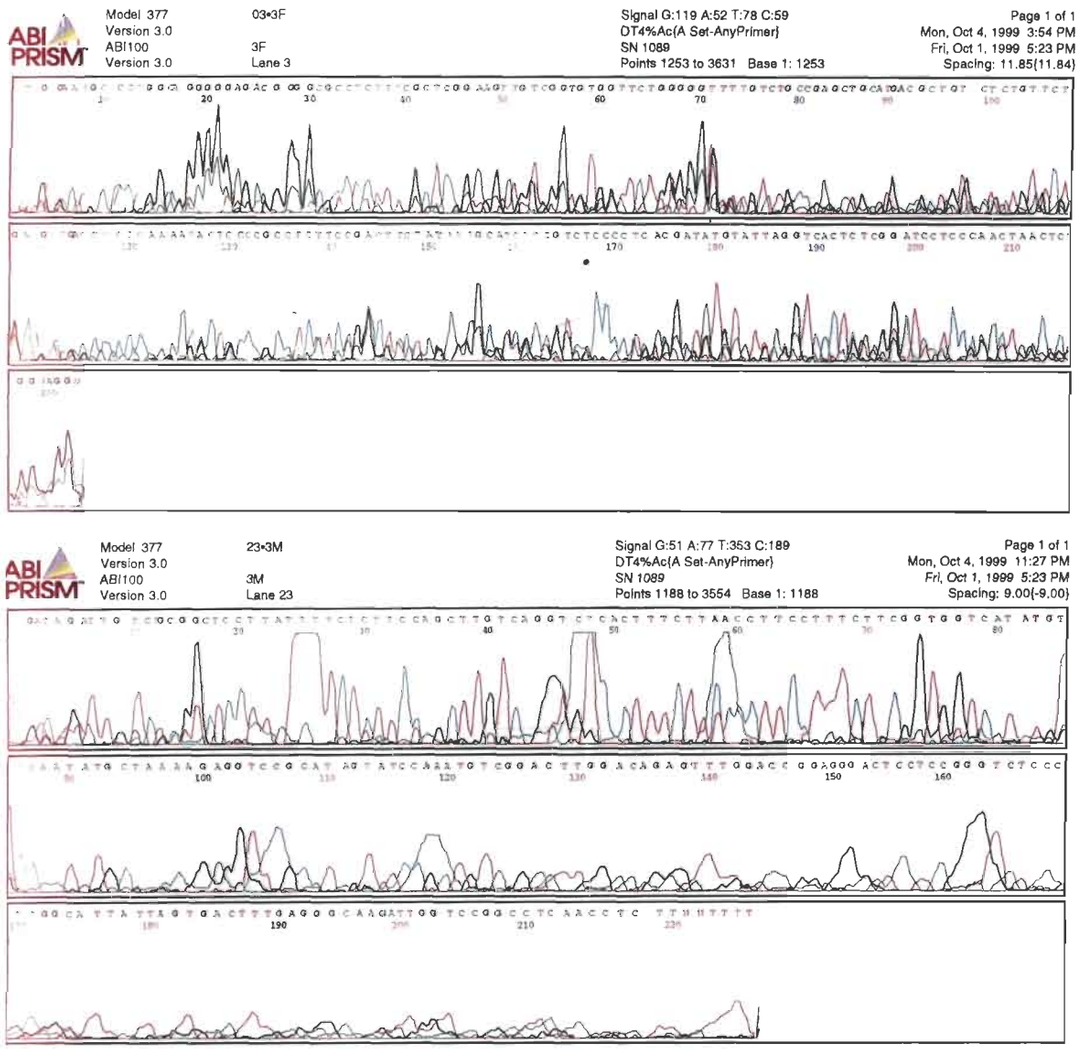
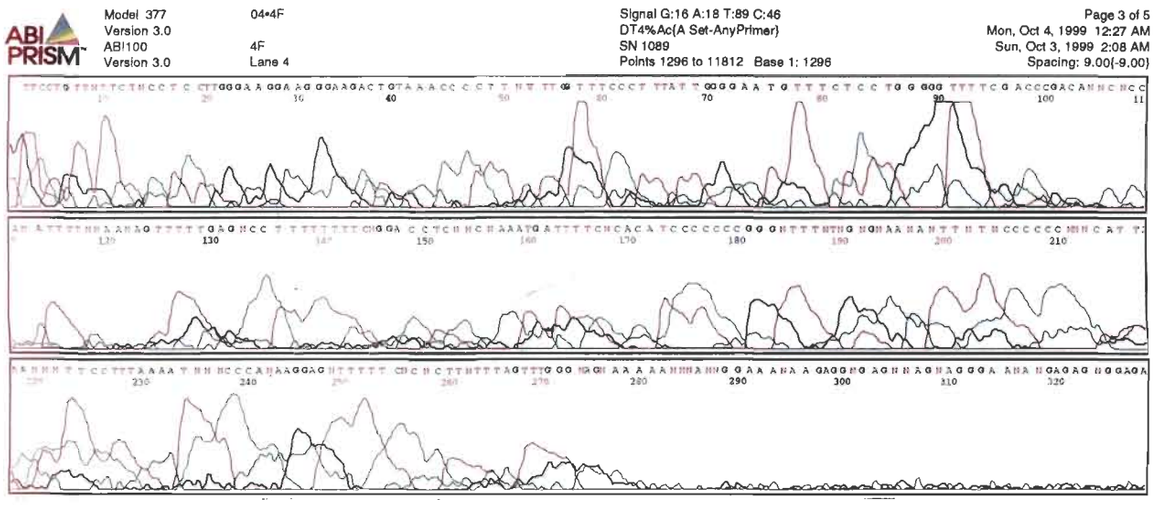


Fig. 10 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of LCN3/87



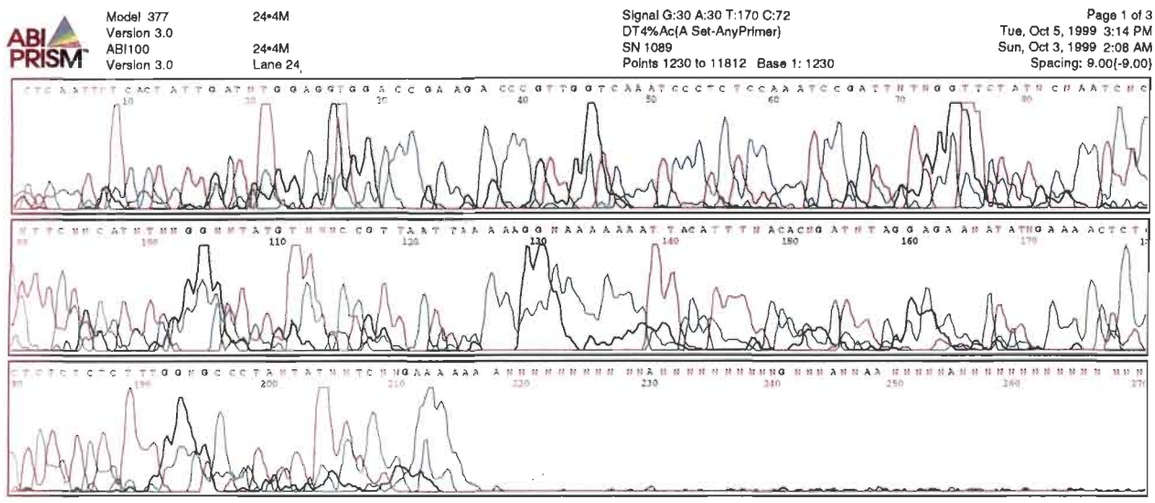
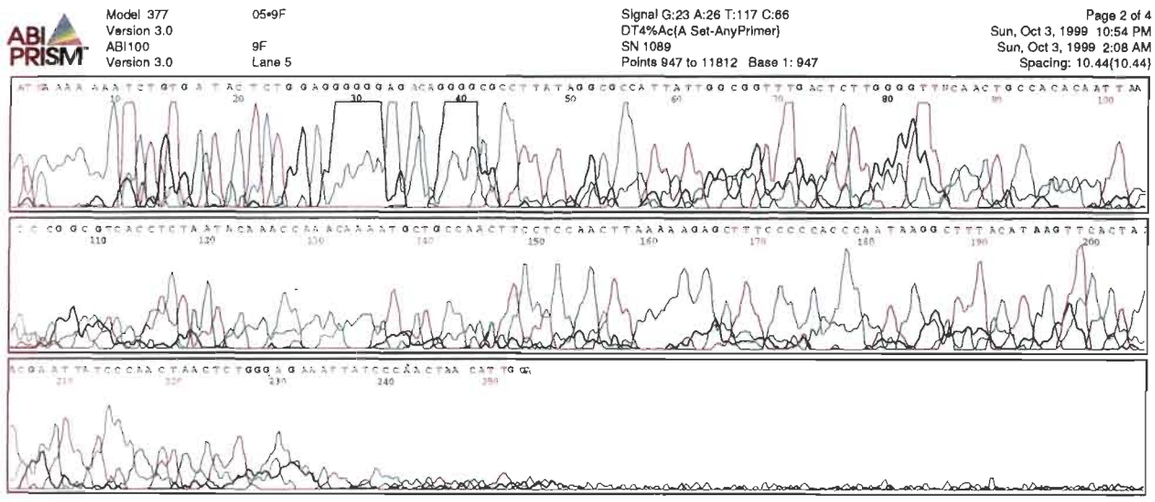


Fig. 11 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of BCC4/87



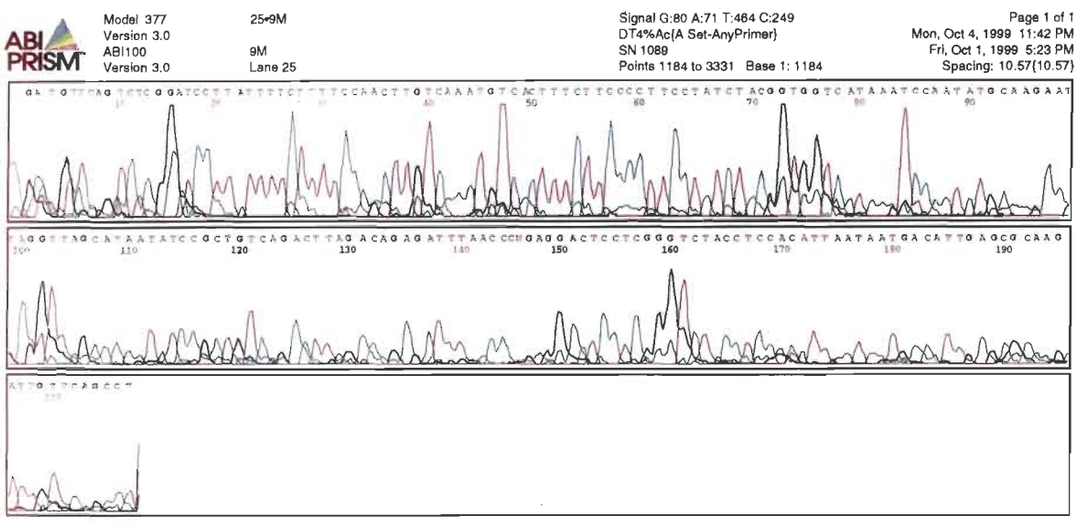


Fig. 12 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of LCC9/89

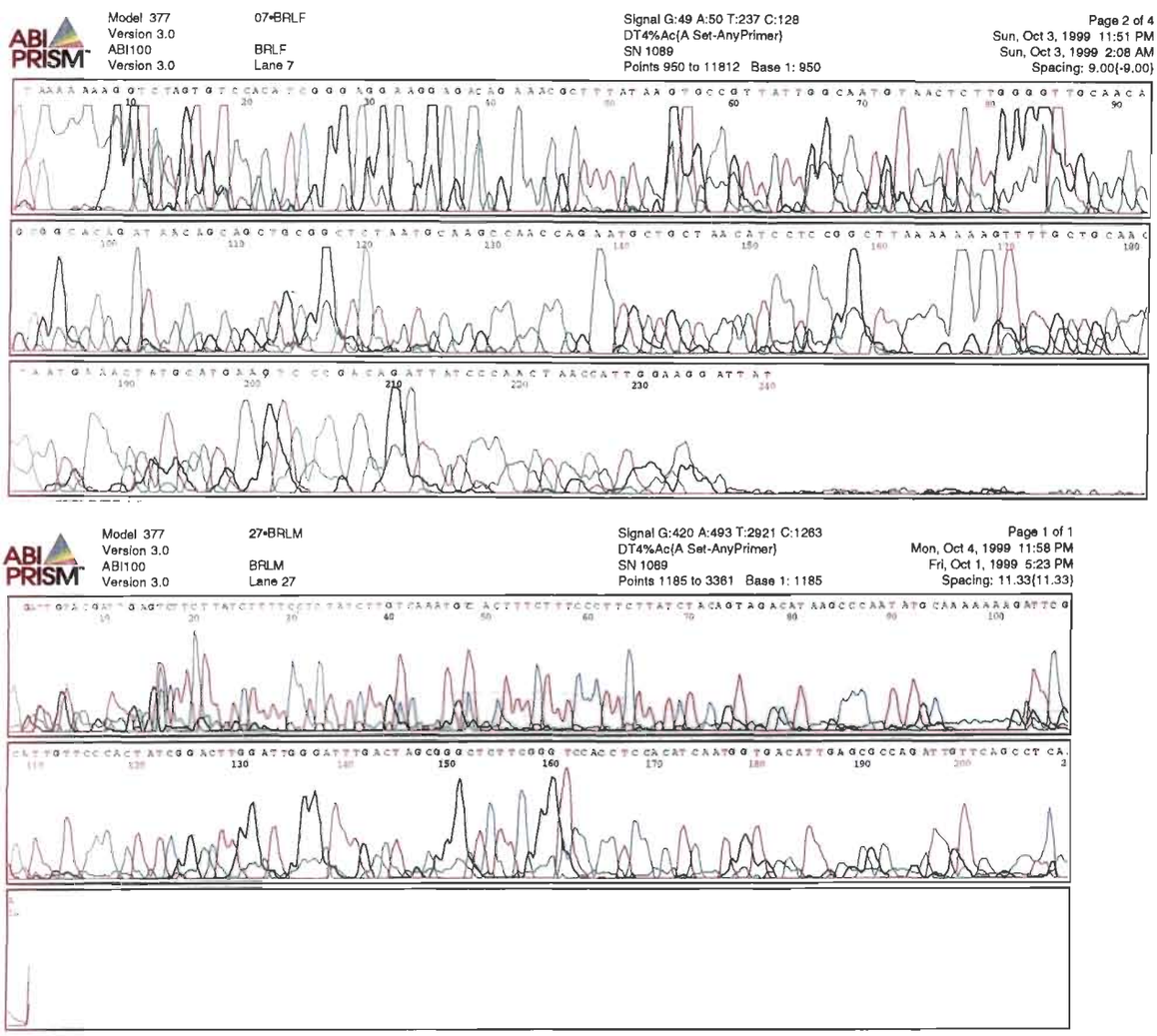


Fig. 13: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) *amplicons* of BRLY-58/93

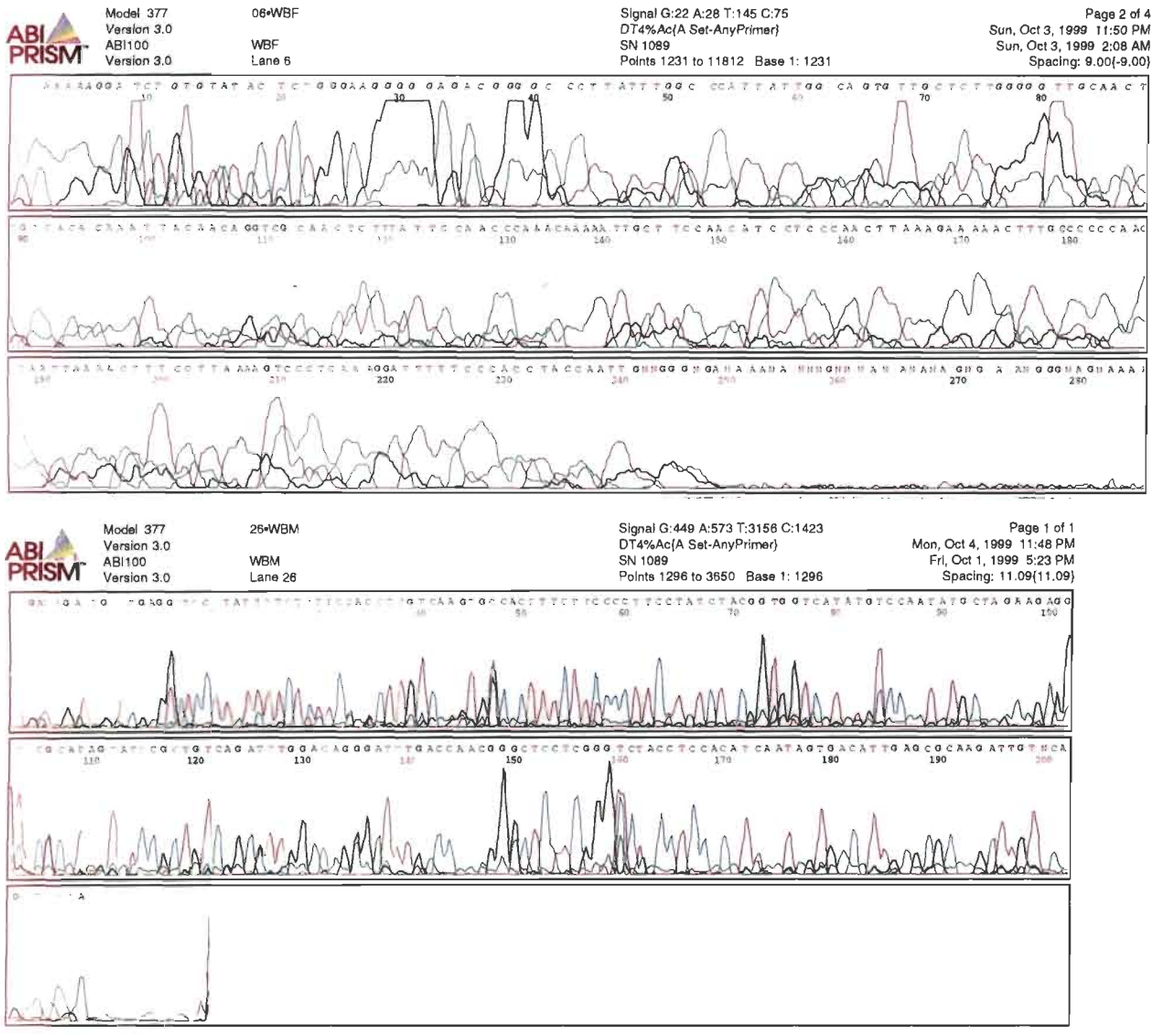
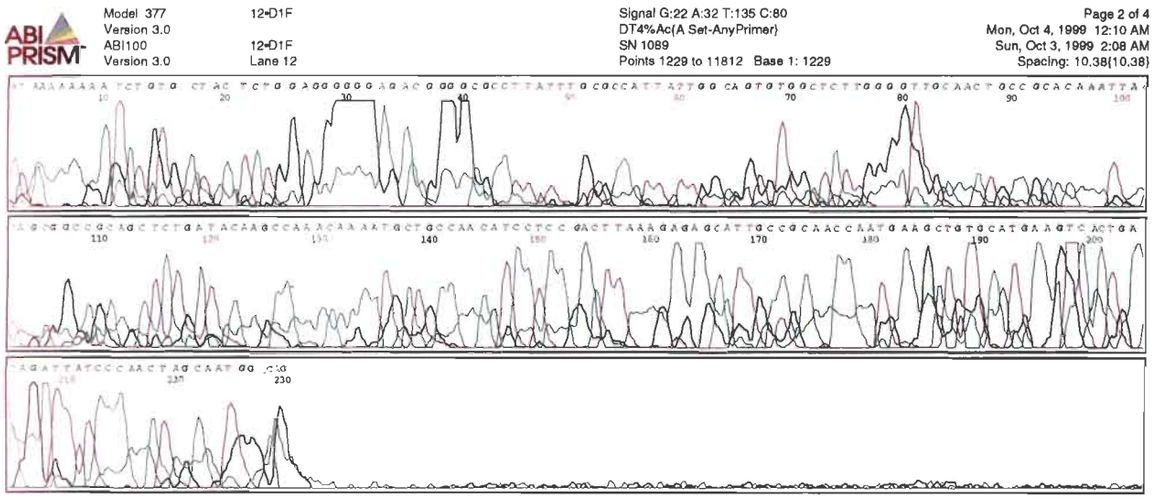


Fig. 14: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of WB-198/94



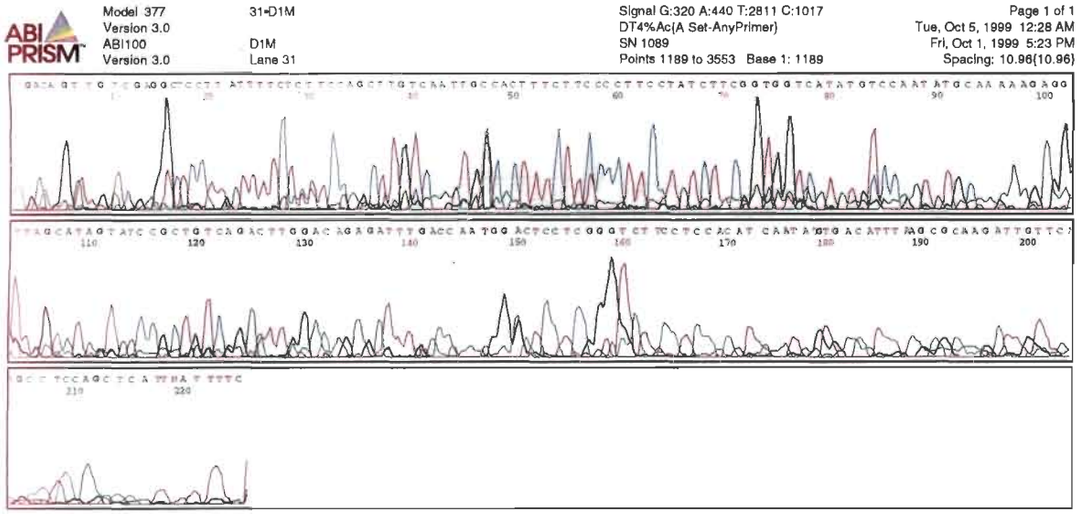
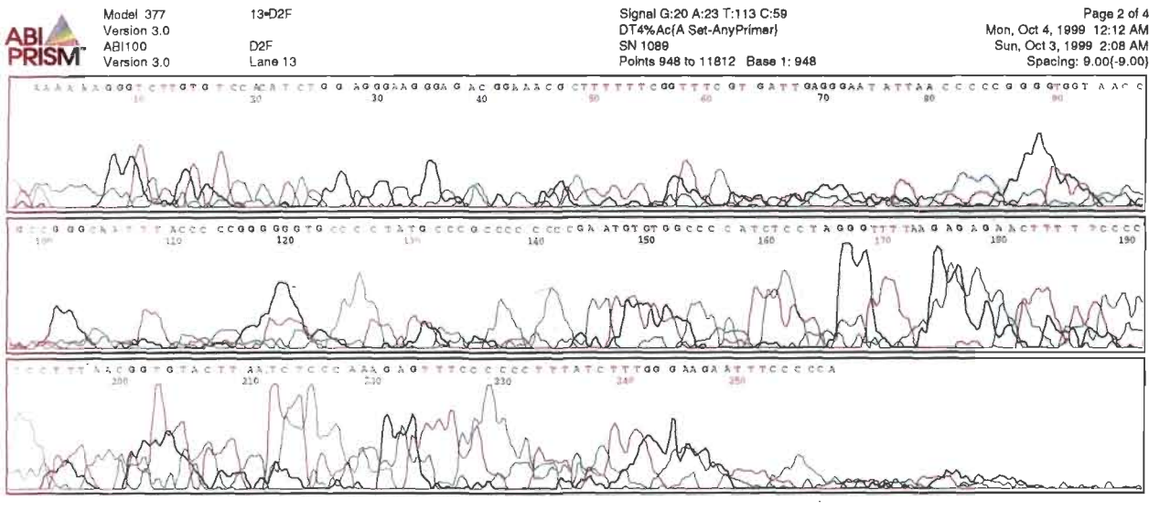


Fig. 15 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of D1/95



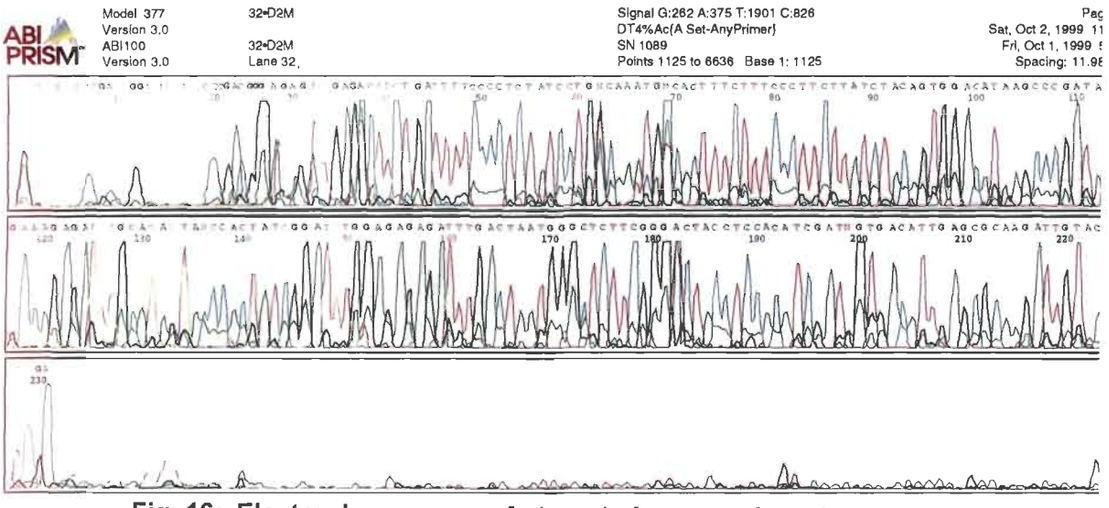
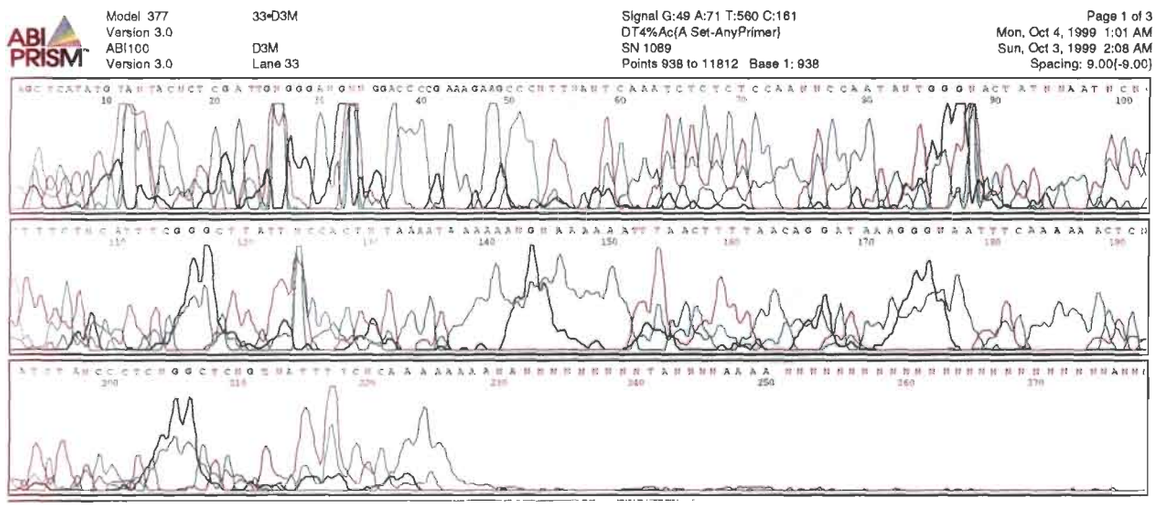


Fig. 16: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of D2/95



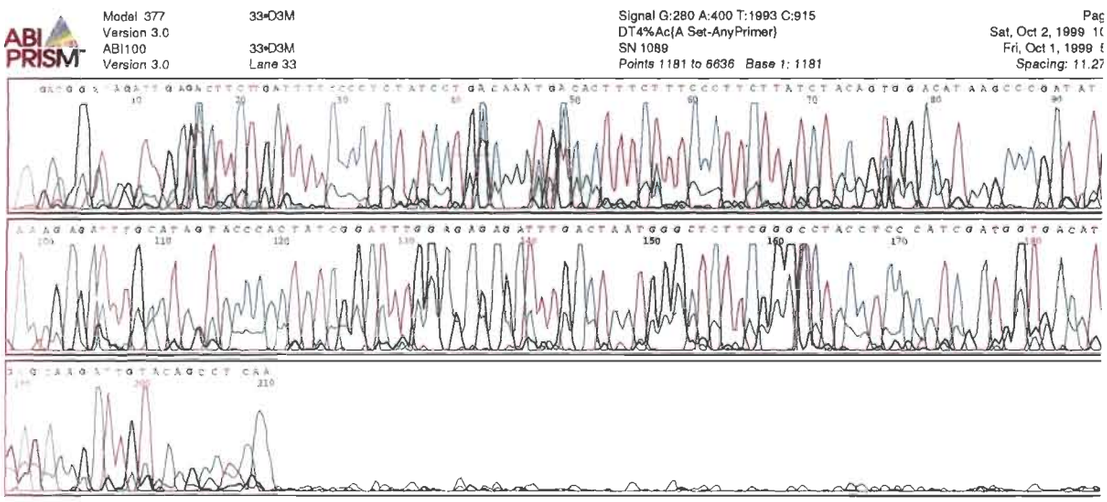
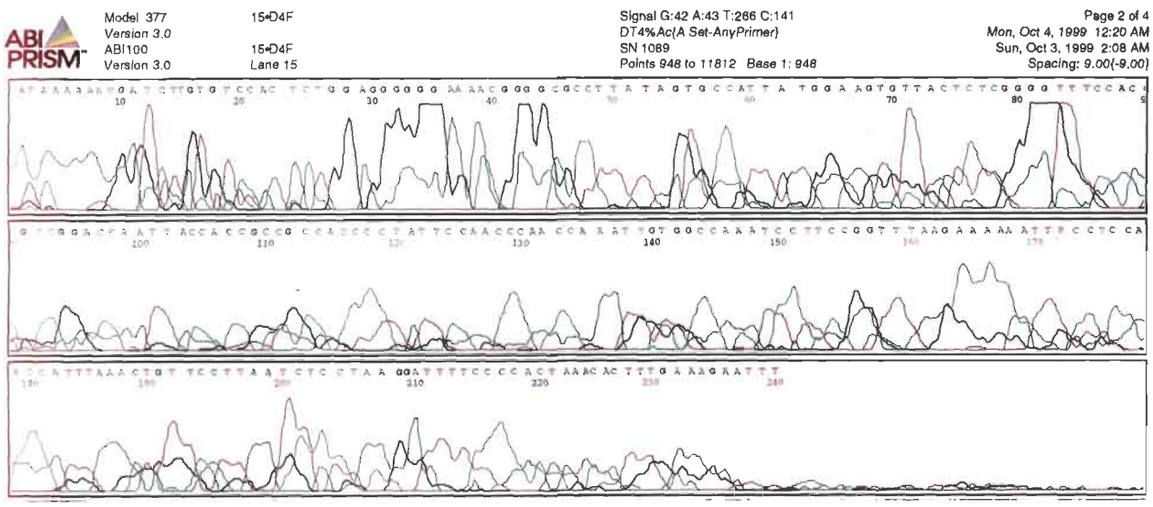


Fig. 17: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of D3/95



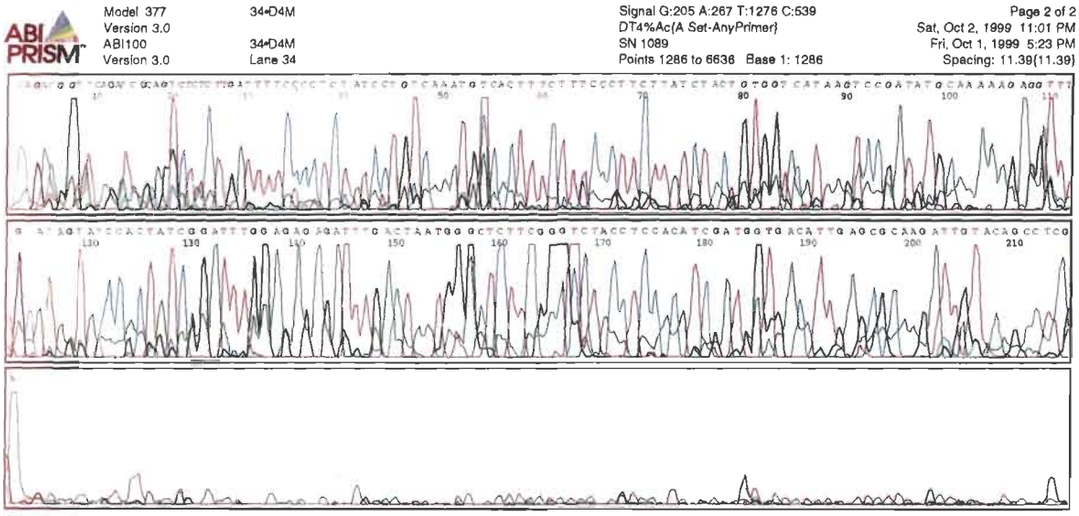
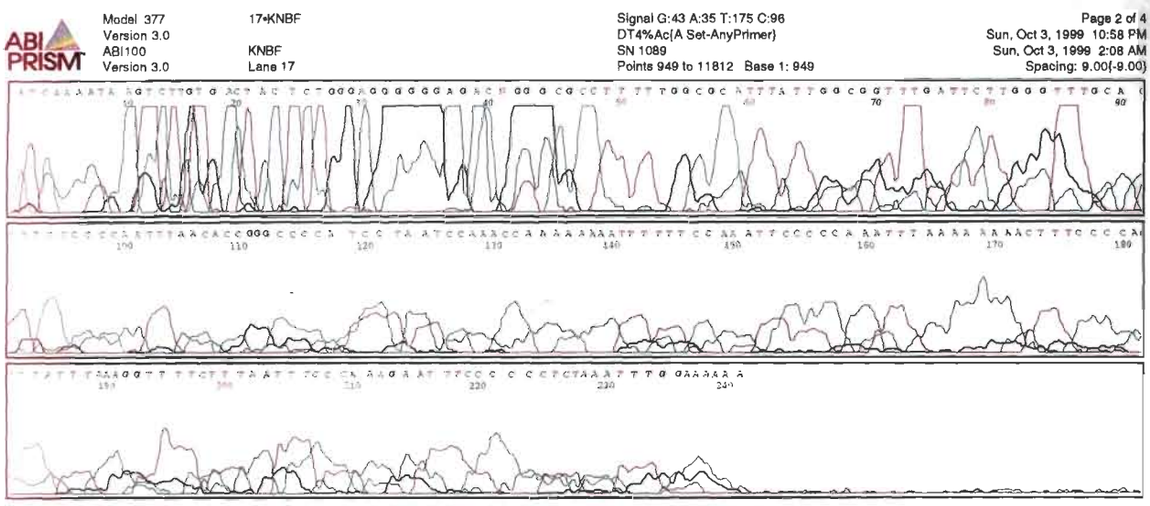


Fig. 18: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of D4/95



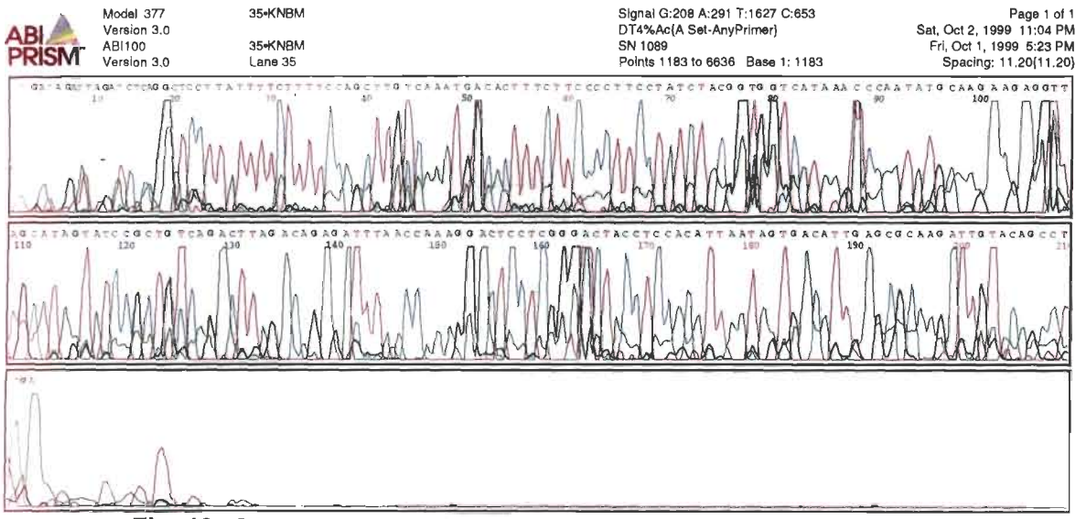
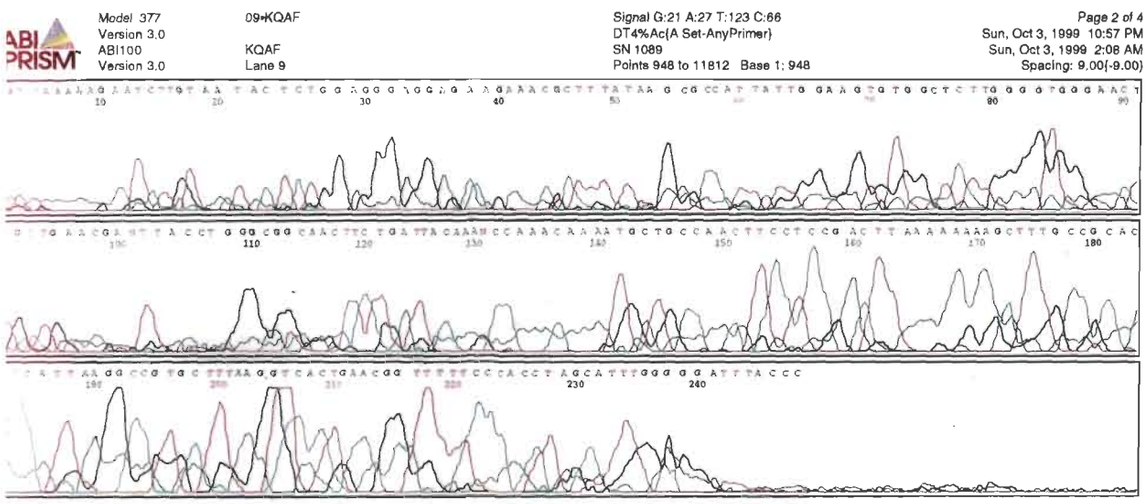


Fig. 19: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of KNBB/96



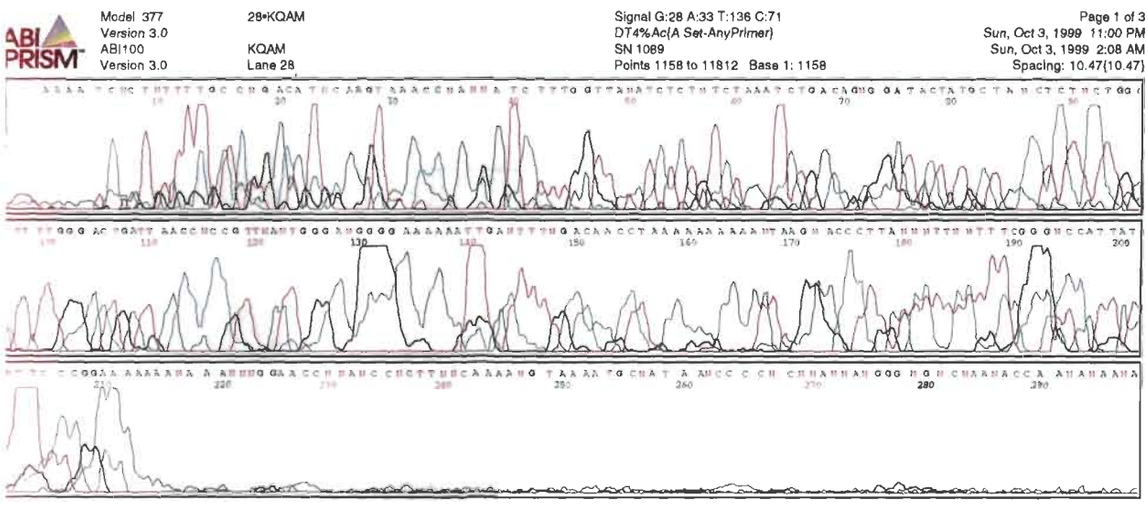
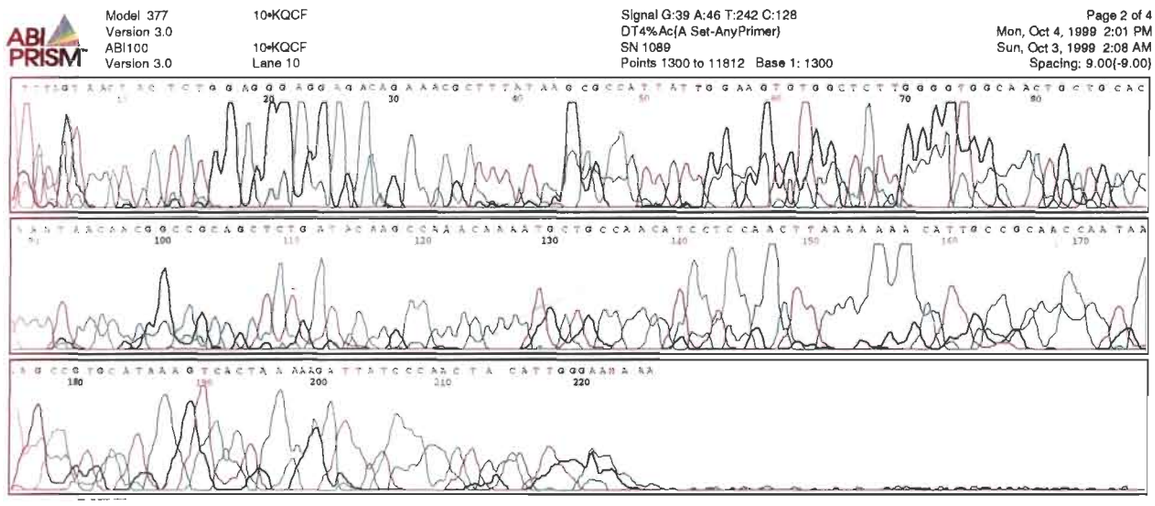


Fig. 20: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of KQA-ALW/98



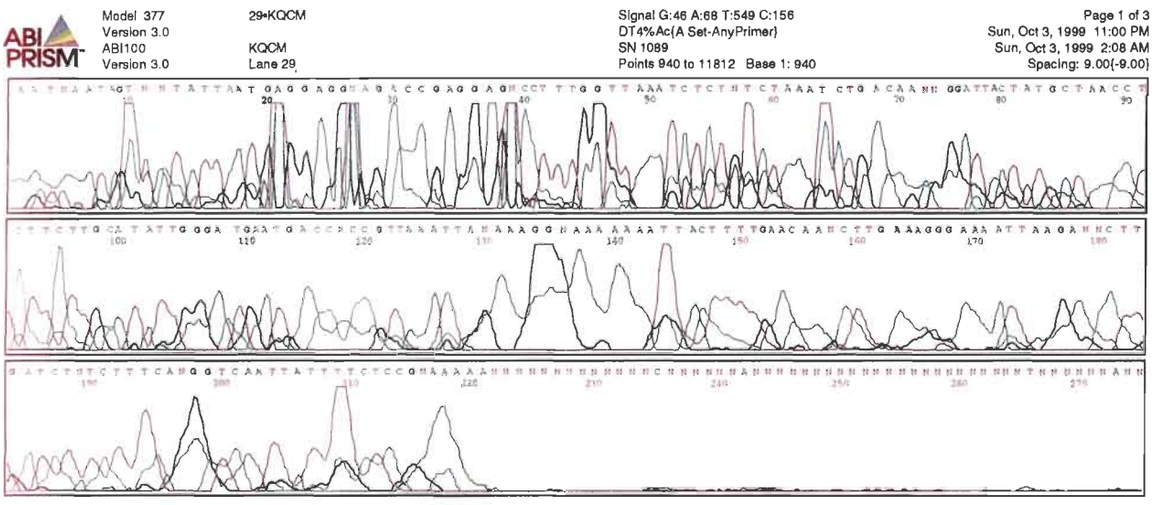


Fig. 21 : Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of KQC-CHE/98

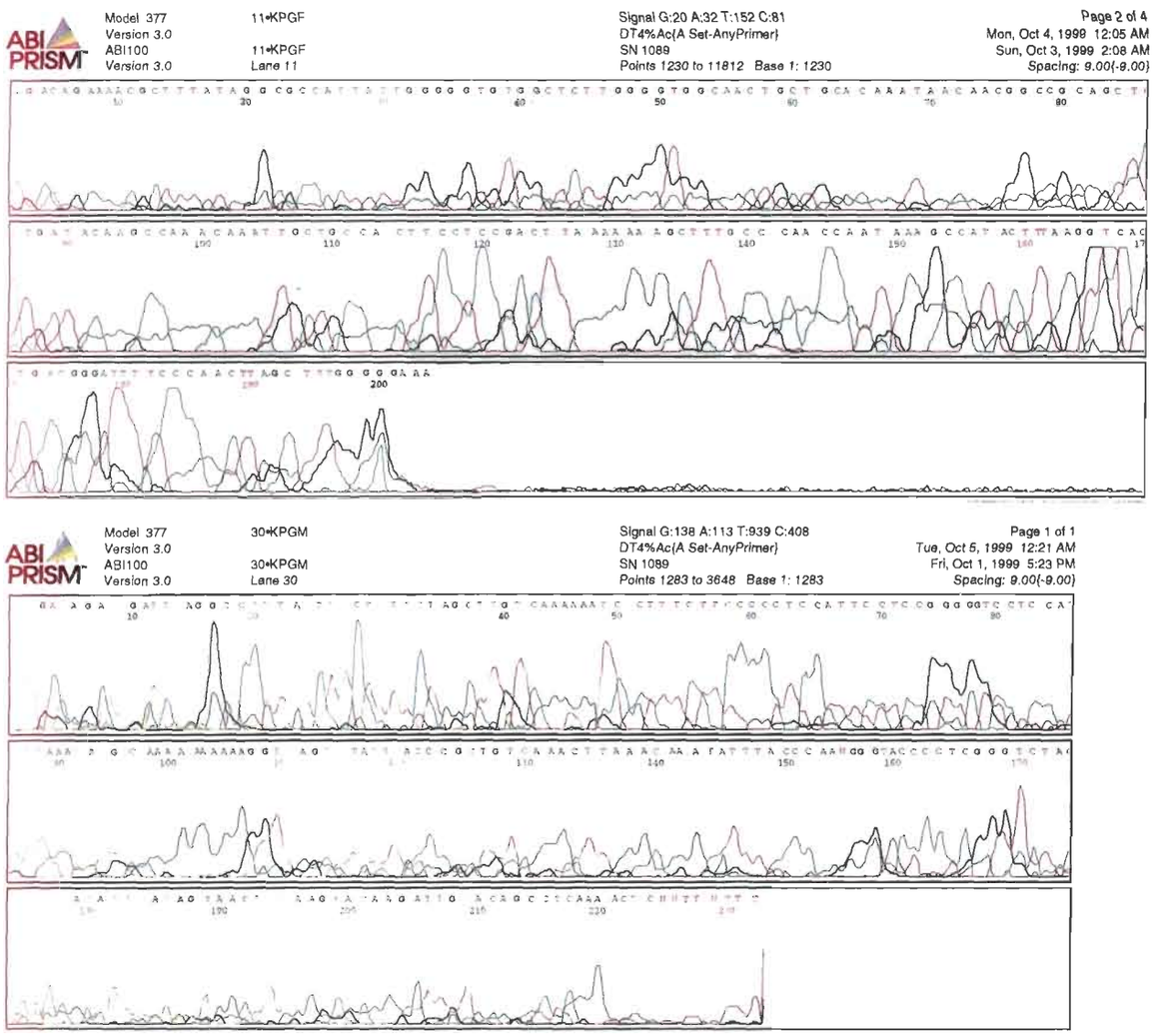


Fig. 22: Electrophoregrams : Automated sequencing of FPCS (A) and MPNLS (B) amplicons of KPG/98

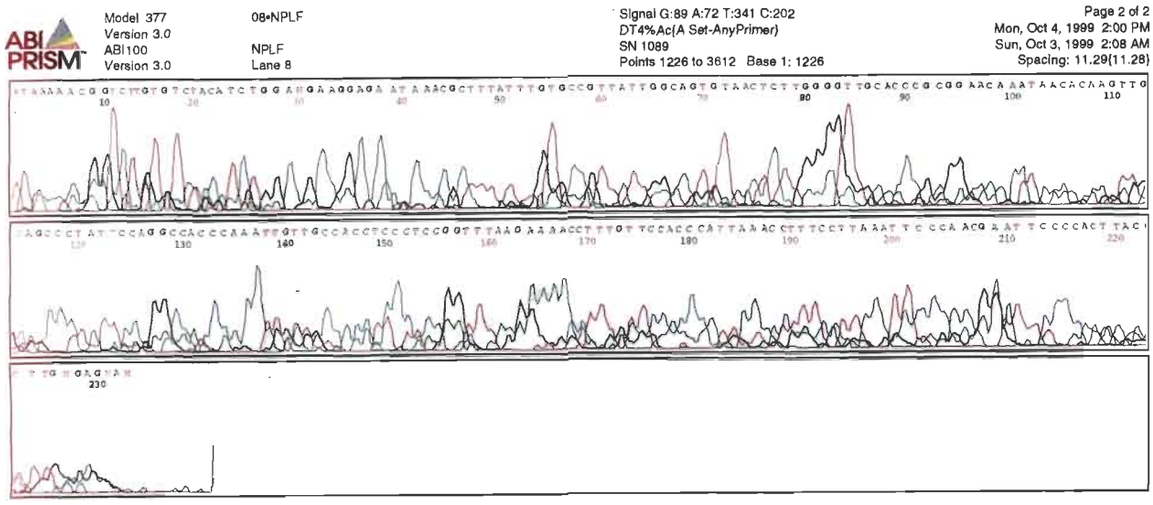


Fig. 23 : Electrophoregrams : Automated sequencing of FPCS amplicon of NPL-5/93

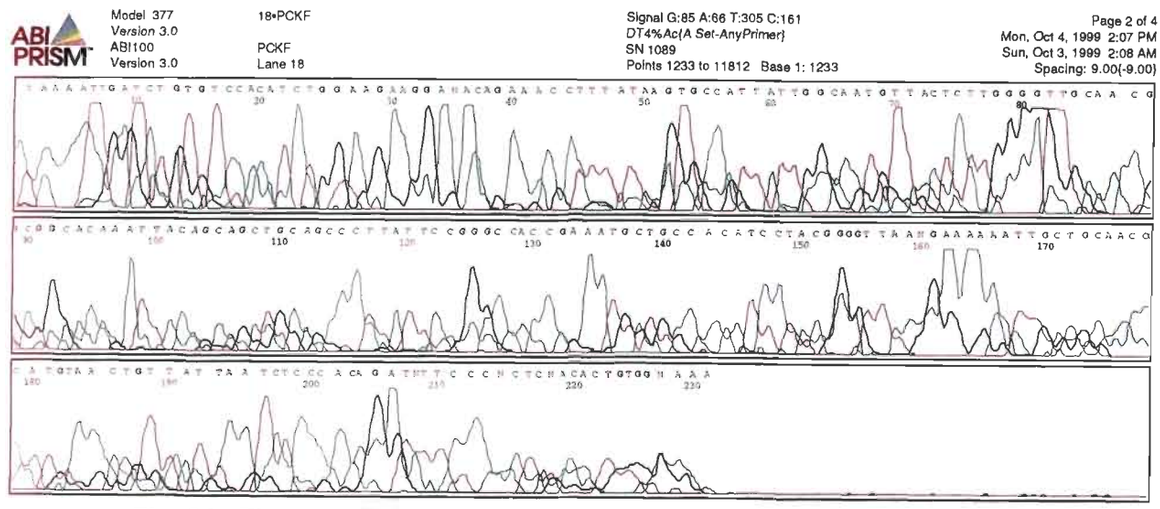


Fig. 24: Electrophoregrams : Automated sequencing of FPCS amplicon of PCKC/98

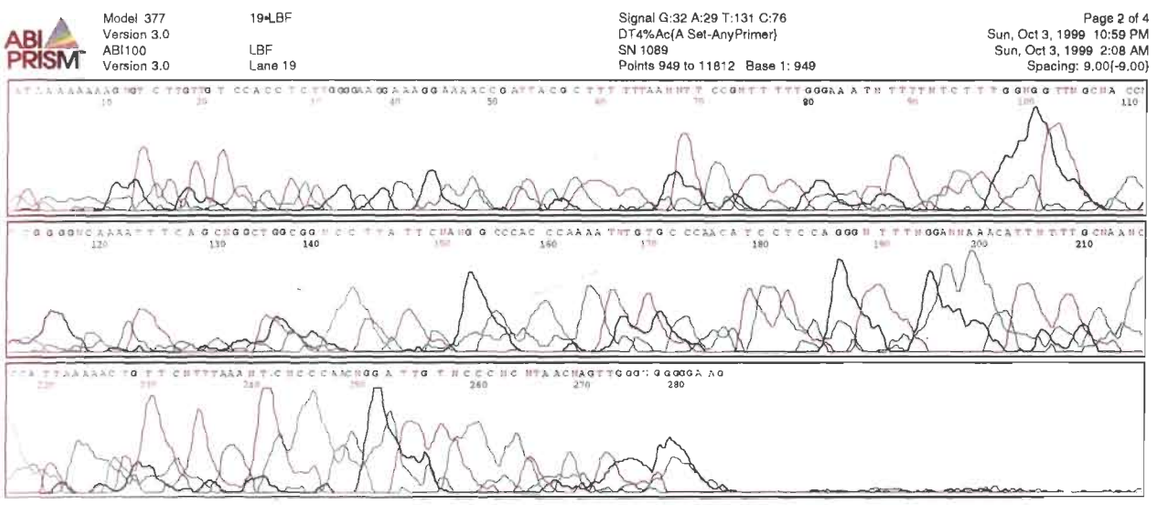


Fig. 25: Electrophoregrams : Automated sequencing of FPCS amplicon of LBC/99

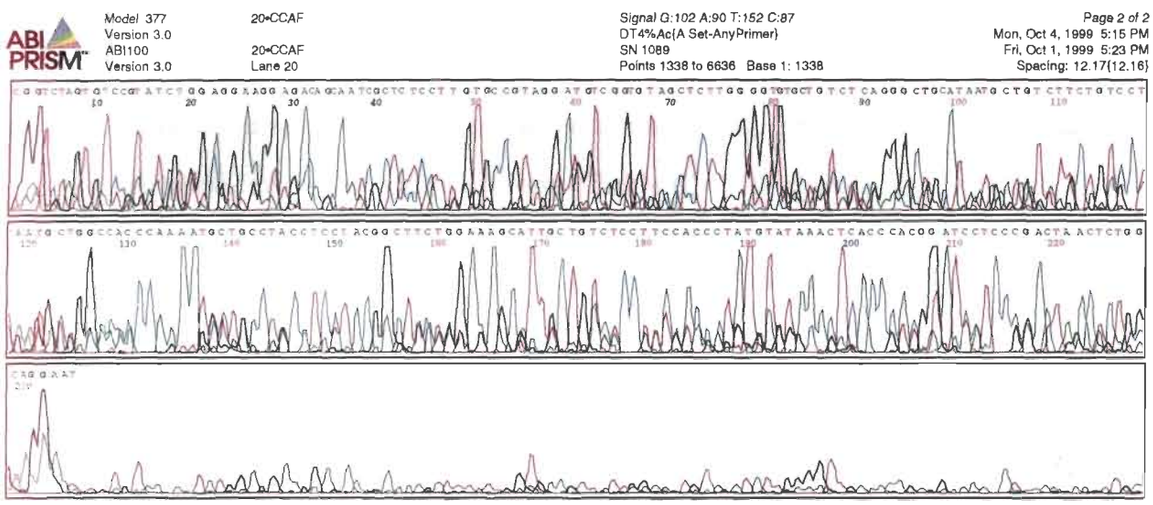


Fig. 26 : Electrophoregrams : Automated sequencing of FPCS amplicon of CCA/93

4.13.1 *Open reading frame (ORF) prediction*

The FPCS nucleotide sequence from all the analysed NDV isolates, on ORF prediction in all three reading frames, revealed that the second reading frame was without any termination codons (Fig.27).

The ORF prediction of all the MPNLS nucleotide sequences generated the graphic output in all three reading frames, with the first reading frame without any termination codons (Fig.27).

4.13.2 Conceptual translation of nucleotide sequence

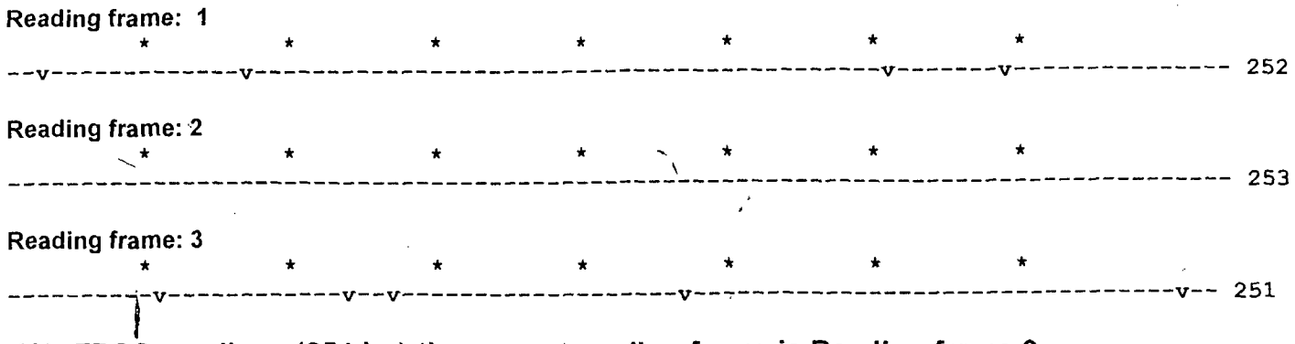
4.13.2.1 Fusion Protein Cleavage Site nucleotide sequence

The FPCS amplicon nucleotide sequence of NDV isolates were conceptually translated into aminoacid sequence starting from the second base of the sense strand oligonucleotide primer used for PCR. The FPCS aminoacid sequence of the NDV isolates were aligned (Fig. 28) and the FPCS sequence was used for pathotype prediction (Table 6).

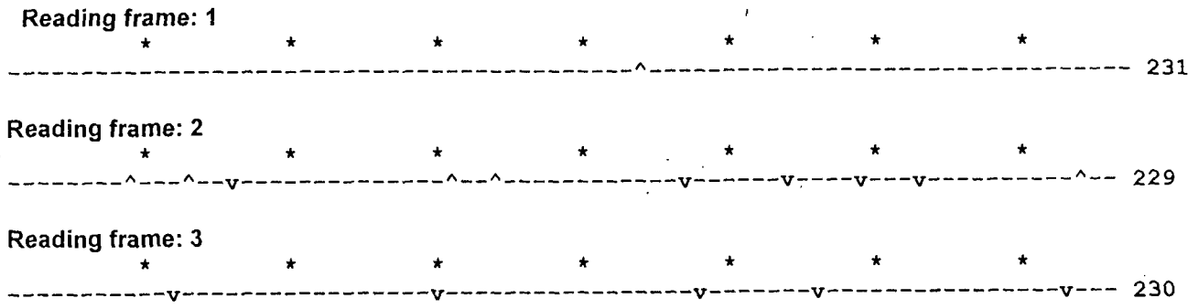
The predicted FPCS sequence of isolates LCC9/89, D1/95 and KNBB/96 were ^{109}SGGGKQ GRLIG^{119}, ^{109}SGGGRQ GRFIR^{119} and ^{109}SGGGRQ GRFLG^{119} respectively with GR sequence at positions 112 and 113 as well as 115 and 116 indicating lentogenic nature of the isolates. The WB-198/94 had a unique sequence ^{109}SGRGRQ GRLVG^{119} with R-for-G substitution at position 111, and GR sequence at 112, 113 and 115, 116 indicating lentogenic nature. The mesogen, Mukteswar/46 had the aminoacid sequence of ^{109}SGGRRQ KRFIG^{119} with K-for-R substitution at position 115, but shared 100% sequence similarity with Komorov strain at the FPCS. The R-for-G-substitution at the

Table-6 : Pathotype prediction of NDV isolates based on Fusion protein cleavage site amino acid sequence

ISOLATE/ STRAIN	Year	Host	Place	PATHOTYPE	FPCS-AMINOACID SEQUENCE
JQC6/87/V	1987	Japanese Quail	Chennai	>VELOGENIC	**SGRRRQ KRFVGA**
LCN2/87/V	1987	Layer	Namakkal	>VELOGENIC	**SRRRRQ RRFVGA**
LCN3/87/V	1987	Layer	Namakkal	<VELOGENIC	**SGRRRQ RRFVGA**
BCC4/87/V	1987	Broiler	Chen, VYD	>VELOGENIC	**SGRRRQ KRFVGA**
LCC9/89/V	1989	Layer	Chen, RH	LENTOGENIC	**SGGGKQ GRLIGA**
BRLY-58/93/V	1993	Chicken	Bareilly, UP	>VELOGENIC	**SGGRRQ KRFIGA**
WB-198/94/V	1994	Chicken	West Bengal	<VELOGENIC	**SGRGRQ GRLVGA**
D1/95/L	1995	Desi bird	Chennai	LENTOGENIC	**SGGGRQ GRFIRA**
D2/95/V	1995	Desi bird	Chennai	>VELOGENIC	**SGGRRQ KRFVRA**
D3/95/V	1995	Desi bird	Chennai	<VELOGENIC	**SGRRRQ KRFIGA**
D4/95/L	1995	Desi bird	Chennai	<VELOGENIC	**SGGRRQ GRLIGA**
KQA-ALW/98	1998	Japanese Quail	Kerala	>VELOGENIC	**SGGRRQ KRFIGA**
KQC-CHE/98	1998	Japanese Quail	Kerala	>VELOGENIC	**SGGRRQ KRFIGA**
KPG/98/V	1998	Pigeon	Kerala	>VELOGENIC	**SGGRRQ KRFIGA**
MUKTESWAR/ 46/M	1946	Chicken	Ranikhet, UP	>MESOGENIC	**SGGRRQ KRFIGA**
KNBB/96	1996	Chicken	Karnataka	LENTOGENIC	**SGGGRQ GRFLGA**
NPL-5/93/V	1993	Chicken	Nepal	>VELOGENIC	**SGGRRQ KRFIGA**
NDV-CCA	1993	Cell cultured	--	>VELOGENIC	**SGGRKQ KRFVGA**
NDV-PCK	1999	Peacock	Chennai	>VELOGENIC	**SGRRRQ KRFIGA**
NDV-LB	1998	Lovebird	Chennai	>VELOGENIC	**SGRRRQ KRFIGA**



(A): FPCS amplicon (254 bp)-the correct reading frame is Reading frame 2.



(B): MPNLS amplicon (232 bp)- the correct reading frame is Reading frame 1.

Fig. 27: SEQAIDII- Open Reading Frames in JQC6/87- FPCS(A) and MPNLS(B) Nucleotide sequence.

^ for start, v for terminator, - openframe

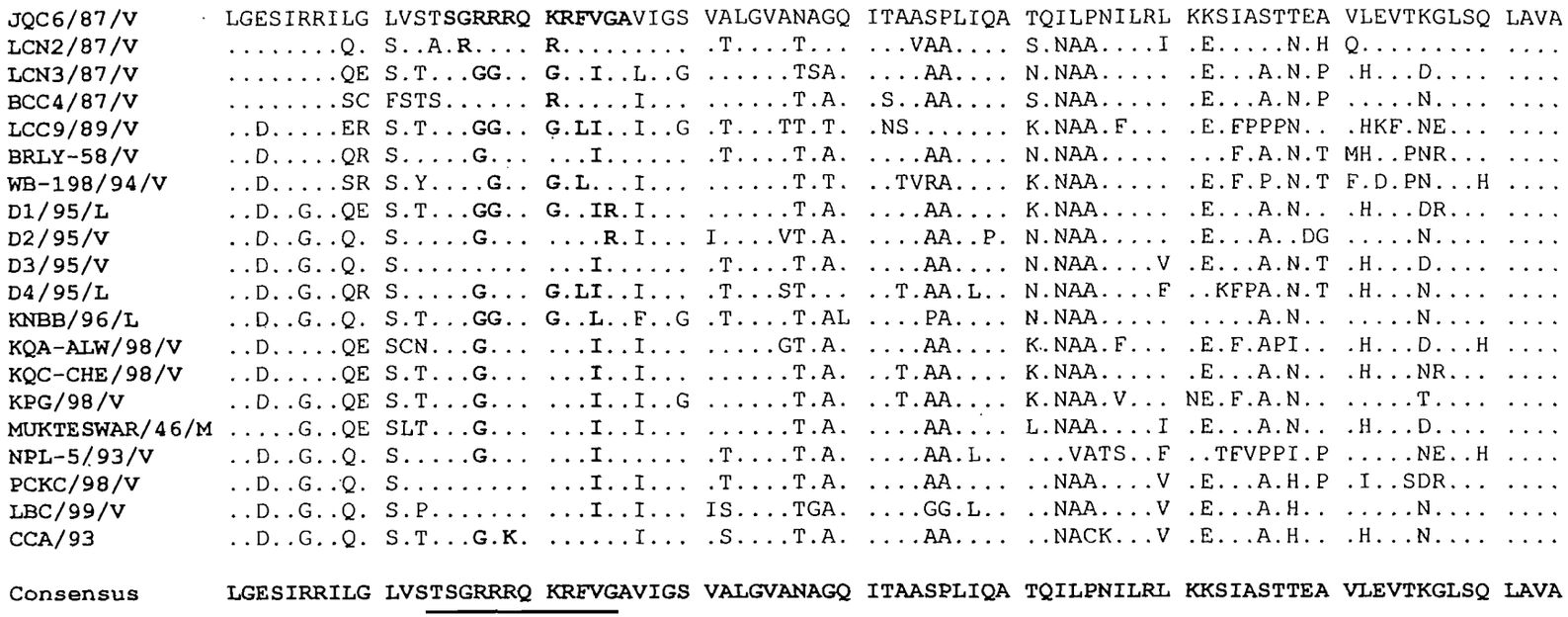


Fig. 28 : Predicted amino acid alignment of fusion protein cleavage site(FPCS) of NDV isolates from India and Nepal showing variations . The sequences were derived by conceptual translation of obtained primary nucleotide sequences. The fusion protein cleavage site sequence from positions 109 to 119 is underlined. The aminoacids are represented in standard IUPAC single-letter code.(FPCS aminoacids: S-Serine,G-Glycine,R-Arginine,Q-Glutamine,K-Lysine,F-Phenyl alanine, V-Valine, A-Alanine).

position 110 was found only in the case of isolate LCN2/87 along with V-for-I substitution at position 118. The V-for-I substitution at position 118 was also found in the isolates JQC6/87, LCN3/87, BCC4/87, WB-198/94, D2/95 and the cell culture passaged CCA/93 strain. The isolates LCN2/87 and LCN3/87 had the RR sequence at 112, 113 as well as 115, 116 position indicating more virulence, as reported by Seal *et al.* (1995). The K-for-R substitution at position 115 was found in the isolates JQC6/87, BCC4/87, KQA-ALW/98, KQC-CHE/98, KPG/98, D2/95, D3/95, BRLY-58/93, NPL-5/93, PCKC/98, LBC/99 and cell culture passaged CCA/93 strain.

The dibasic aminoacids RR at position 112 and 113 was found in isolates JQC6/87, LCN2/87, LCN3/87, BCC4/87, KQA-ALW/98, KQC-CHE/98, KPG/98, D2/95, D3/95, D4/95, BRLY-58/93, NPL-5/93, PCKC/98 and LBC/99 (Table 6) indicating velogenic nature, as described by Seal *et al.* (1995).

4.13.2.2 Matrix protein nuclear localization signal region amplicon nucleotide sequence

The conceptual translation of the MPNLS amplicon nucleotide sequence was from the first reading frame (base one) of the sense-strand oligonucleotide primer. The MPNLS amplicon aminoacid sequences were aligned (Fig.29) and the matrix protein nuclear localization signal was located, from position 247 to 263 in the aminoacid sequence. The D-for-N substitution at position 212 was found in isolates JQC6/87, LCN2/87, BCC4/87, BRLY-58/93, D2/95, D3/95 and KQA-ALW/98 shared by the chicken, quail and desi bird isolates. The E-for-K substitution at position 259 was identified in isolates JQC-6/87, LCN-2/87, LCN 3/87, BCC-4/87, LCC-9/89, BRLY-58/93 and KPG/98,

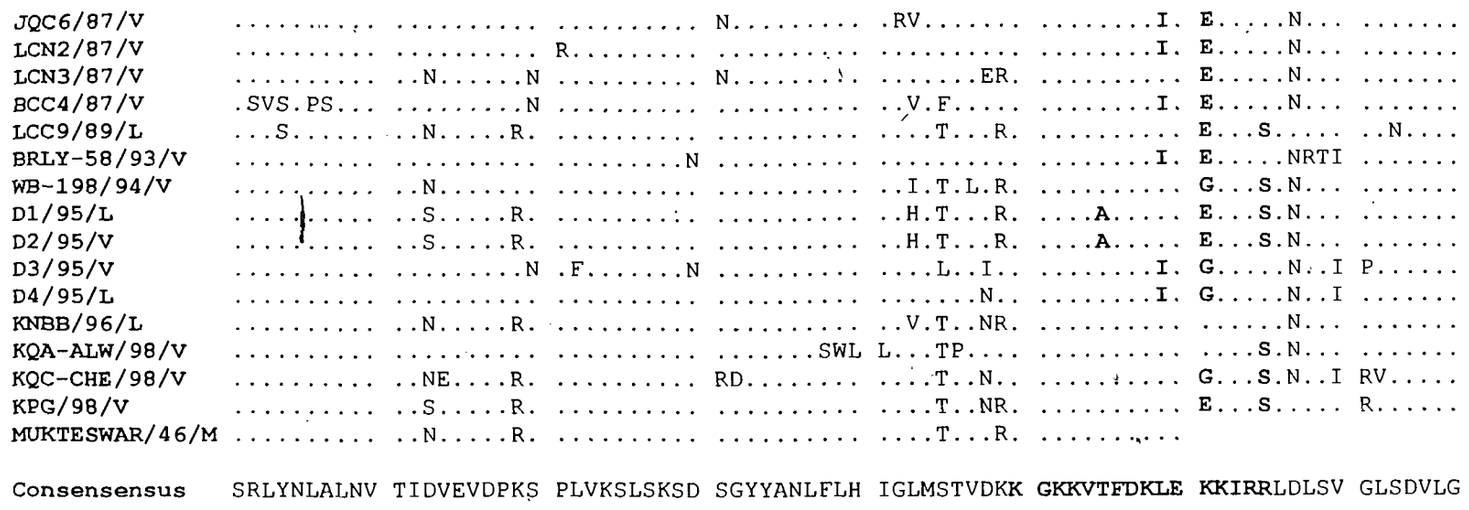


Fig. 29 : Predicted amino acid alignment of Matrix protein nuclear localization signal(MPNLS) region of NDV isolates from India showing variations . The sequences were derived by conceptual translation of obtained primary nucleotide sequences. The Nuclear localization signal from positions 247 to 263 is underlined. The aminoacids are represented in standard IUPAC single-letter code.(MPNLS amino acids: K-Lysine, G-Glycine, V-Valine, T- Threonine, F-Phenyl alanine, D-Aspartic acid,L-Leucine,E-Glutamic acid, I - Isoleucine).

while E-for-G substitution was found in isolates WB-198/94, D2/95, D3/95, D4/95 and KQC-CHE/98. The N-for-D substitution was found in case of isolates LCC9/89, KPG/98 and the vaccine strain Mukteswar/46. No particular sequence variation distinguishing lentogenic from velogenic or mesogenic viruses could be identified, other than sequence difference at position 259 the G-for-E substitution found in desi bird isolates and a quail isolate, which lacked in chicken isolates other than WB-198/94.

4.14 PHYLOGENETIC ANALYSIS OF OBTAINED NUCLEOTIDE SEQUENCES OF FPCS AND MPNLS AMPLICONS

Phylogenetic analysis of the obtained nucleotide sequences from the fusion protein and matrix protein genes were subjected to Neighbor-Joining tree building method with two different distance estimation methods. Jukes-Cantor and Kimura 2-Parameter distances.

The FPCS nucleotide sequences were aligned using CLUSTAL-W and a phylogenetic tree file was the output using Neighbor-Joining method of Saitou and Nei (1987). The phylogenetic tree file was plotted and output as graph by DRAWTREE and DRAWGRAM softwares (Fig.30).

The MPNLS nucleotide sequences were also subjected to similar analysis using CLUSTAL-W and the graphic output as tree was obtained (Fig. 31).

Contiguous nucleotide sequences of FPCS and MPNLS amplicons were subjected to phylogenetic analysis following multiple alignment using CLUSTAL-W and MEGA softwares. The phenogram and unrooted tree graphic

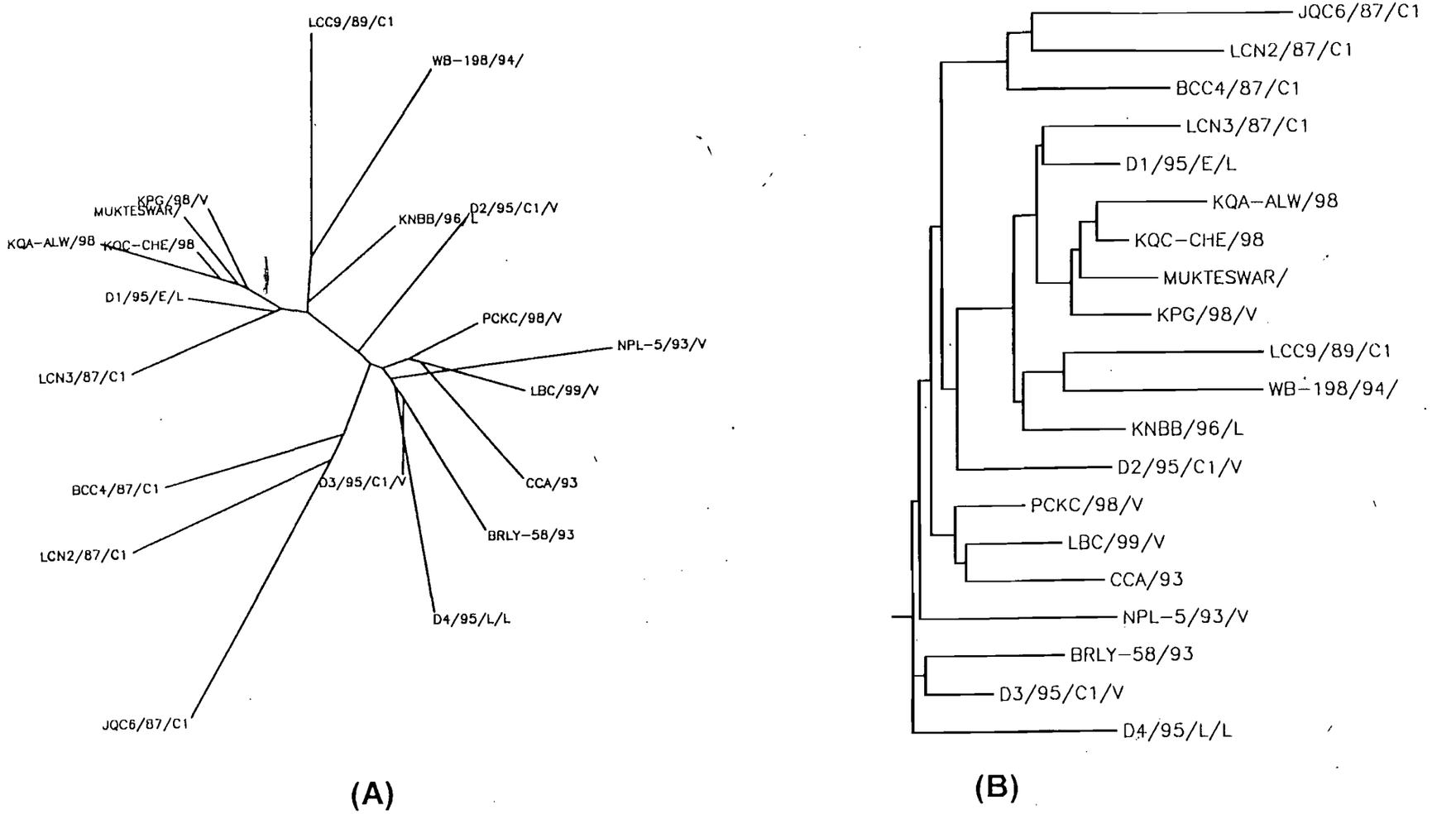


Fig. 30 : Phylogenetic relationship of NDV isolates of India and Nepal based on FPCS sequences : Gene tree generated by Neighbor-Joining method using CLUSTAL W, with DRAWTREE (A), and DRAWGRAM (B), Software's of PHYLIP Package

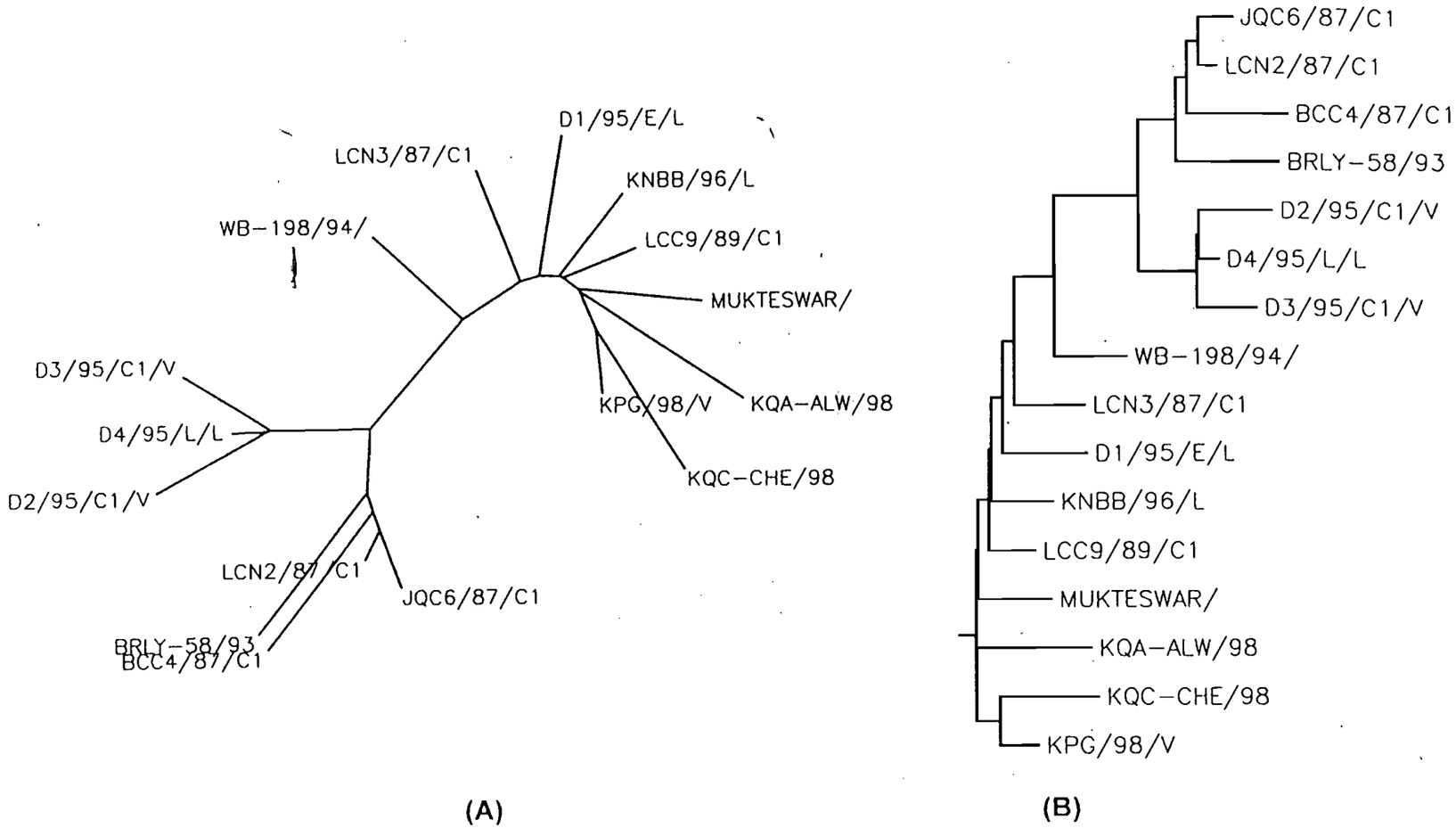
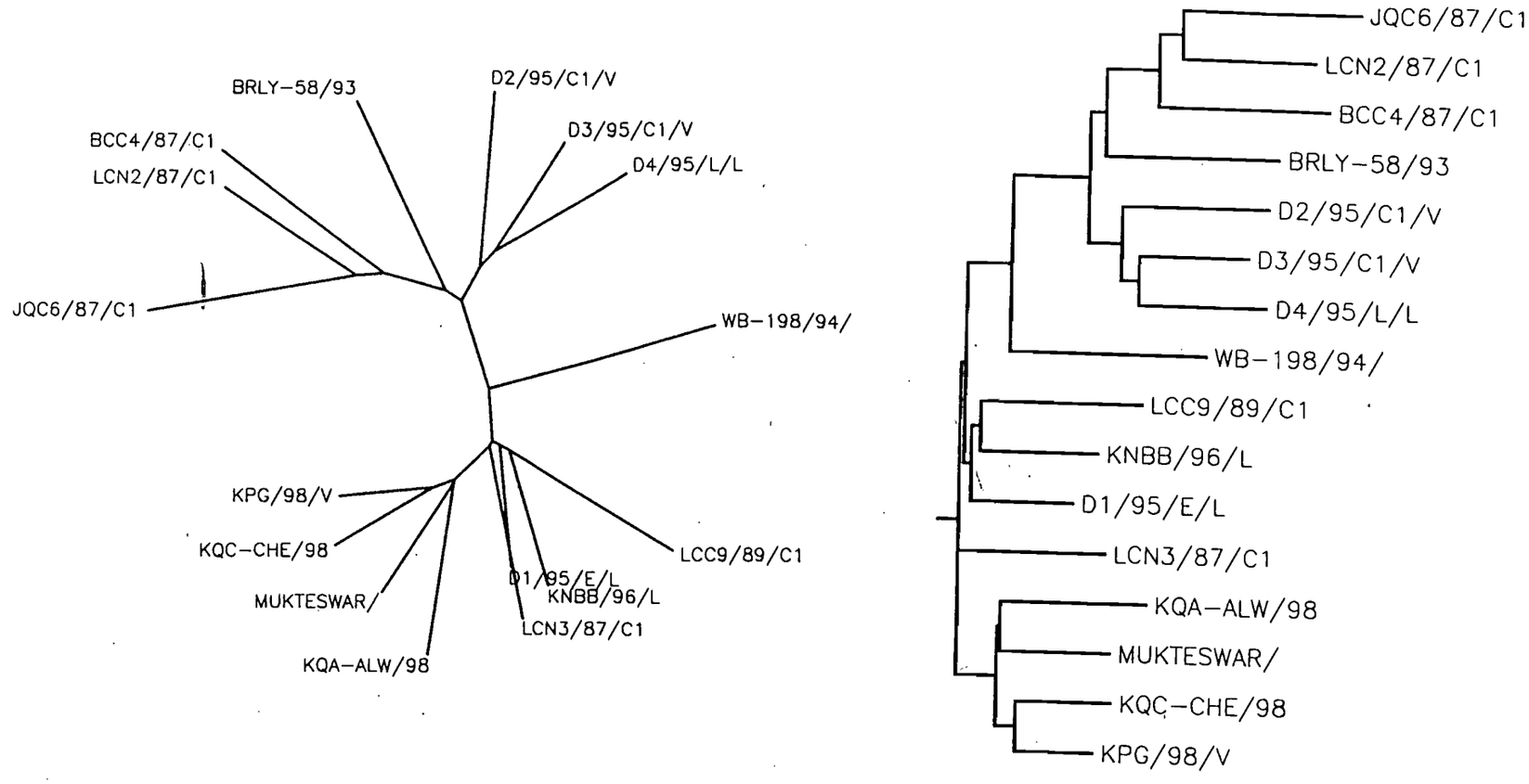


Fig. 31 : Phylogenetic relationship of NDV isolates of India based on MPNLS sequences : Gene tree generated by Neighbor-joining method using CLUSTAL W, with DRAWTREE (A), and DRAWGRAM (B), Software's of PHYLIP Package

output was obtained from CLUSTAL-W tree file output of contiguous FPCS and MPNLS nucleotide sequences (Fig.32). The graphic gene tree outputs from MEGA software were obtained using Neighbor-Joining tree building method along with Jukes-Cantor distance estimation method (Fig.33) as well as Kimura 2-Parameter distance estimation method (Fig.34). Both phylogenetic gene trees obtained by the Jukes-Cantor and Kimura 2-Parameter distance were tested by Bootstrap test with 2000 replicates as well as Standard Error test (Fig. 33 and 34).

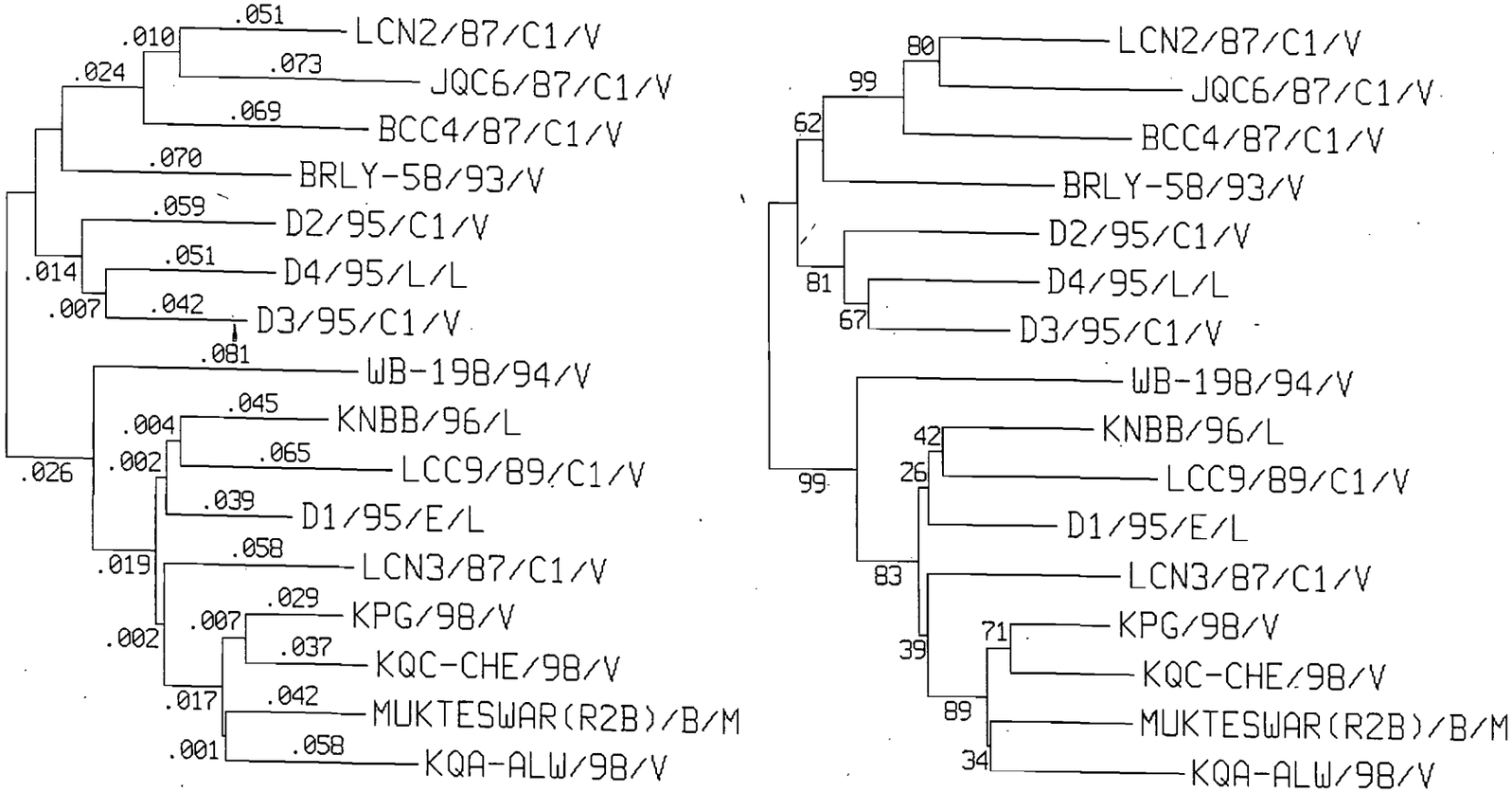
4.14.1 Phylogenetic relationships among Indian NDV isolates based on nucleotide sequence of FPCS amplicon

The FPCS nucleotide sequence of 18 field isolates, one vaccine strain (R_2B) Mukteswar/46 and one cell culture passaged CCA/93 strain on phylogenetic analysis yielded four major clusters (Fig.30). The early velogenic isolates viz. JQC6/87, LCN2/87 and BCC4/87 formed the first cluster. The early velogenic isolate LCN3/87 was closely linked to the lentogenic desi bird isolate D1/95. All the 3 Kerala isolates KQA-ALW/98, KQC-CHE/98 and KPG/98 were clustered closely along with Mukteswar vaccine strain. The LCC9/89 and the WB-198/94 isolates were grouped together, with the KNBB/96 lentogenic isolate joining the group later. The LCN3/87 group, Kerala isolates group and the LCC9/89 group formed the second cluster. The velogenic desi bird isolate D2/95 formed the major ancestor of the second cluster. The third cluster consisted of the PCKC/98, BCC-4/99 and the CCA/93 strains. The NPL-5/93 isolate formed the major ancestor for the first three clusters. The fourth cluster was made together by the BRL-58/93, the desi bird isolates D3/95 and D4/95..



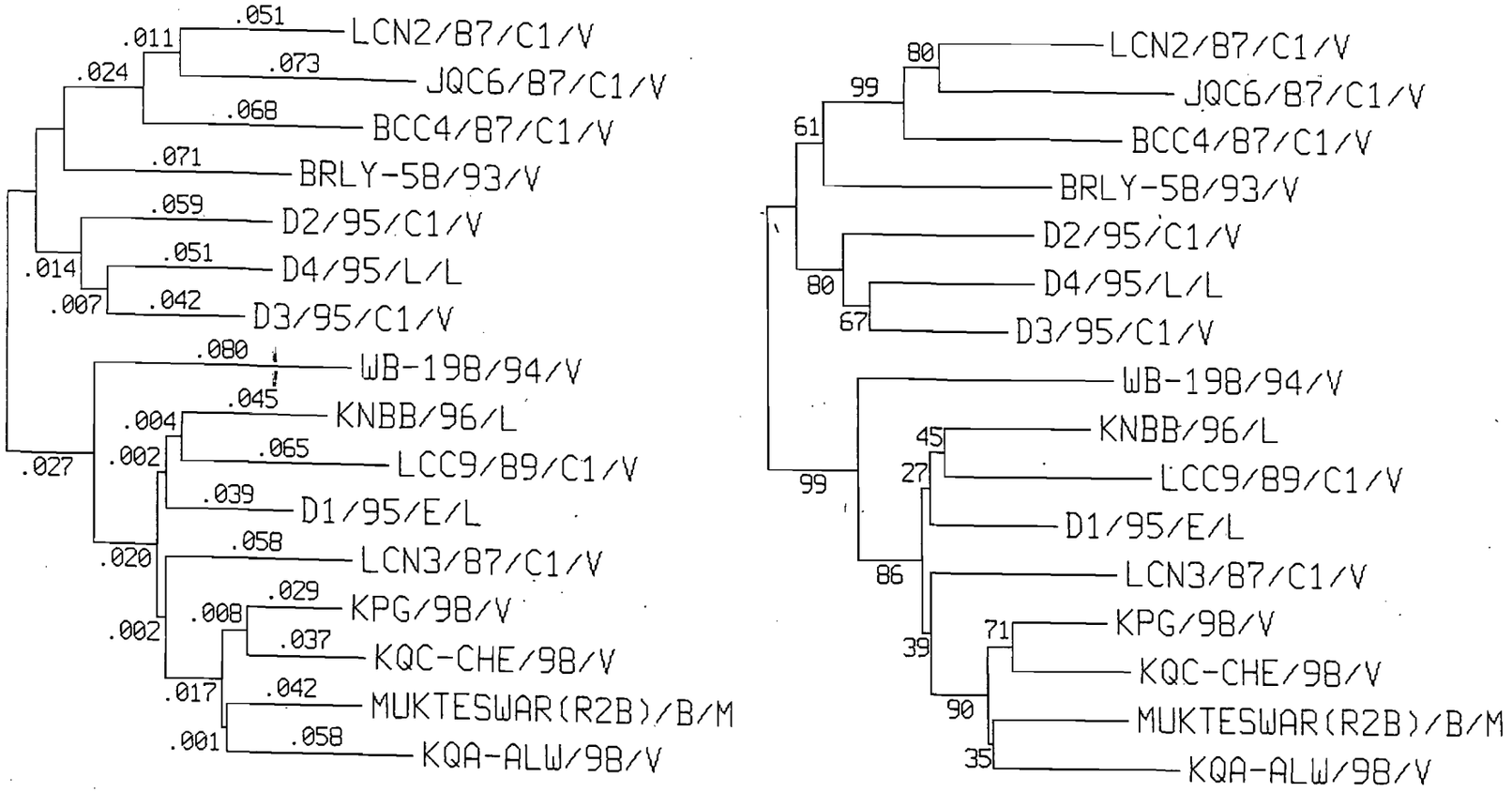
(A) (B)

Fig. 32 : Phylogenetic relationship of NDV isolates of India based on Contiguous FPCS+MPNLS sequences: Gene tree generated by Neighbor-Joining method using CLUSTAL W, with DRAWTREE (A), and DRAWGRAM (B), Software's of PHYLIP Package



(A) (B)

Fig. 33 : Phylogenetic relationship of NDV isolates of India based on Contiguous FPCS+MPNLS sequences: Gene tree generated by Neighbor-Joining tree building method with Jukes-Cantor Distance estimation method (A), and Bootstrap analysis with 2000 replicates (B), using MEGA Software. Distance measures are the percent chance of a nucleotide substitution at one site (A). Bootstrap confidence levels (BCL) are listed for branch points as a percentage (B).



(A) (B)

Fig. 34 : Phylogenetic relationship of NDV isolates of India based on Contiguous FPCS+MPNLS sequences: Gene tree generated by Neighbor Joining tree building method with Kimura 2-parameter Distance estimation method (A), and Bootstrap analysis with 2000 replicates (B), using MEGA Software. Distance measures listed are the percent chance of a nucleotide substitution at one site (A). Bootstrap confidence levels (BCL) are listed for branch points as a percentage (B).

The phenogram was compared to a unrooted tree with spatial *configuration of branches for relationships of the NDV isolates (Fig.30).* The isolates PCKC/98, NPL-5/93, LBC/99, BRLY-58/93, D4/95, D3/95 along with CCA/93 strain were closely related. The early velogenic isolates JQC6/87, LCN2/87 and BCC4/87 formed the next closely related cluster branching from the exotic and desi bird isolates cluster. The Kerala isolates cluster along with one lentogenic desi bird isolate D1/95, LCN3/87 velogenic isolate and the vaccine strain Mukteswar/46 formed a distant cluster from the exotic - desi bird and early velogenic isolates cluster. The LCC9/89, WB-198/94 isolates along with KNBB/96 formed the next closely branching cluster along with the Kerala isolates cluster (Fig.30).

4.14.2 Phylogenetic relationship among Indian NDV isolates based on nucleotide sequence of MPNLS amplicon

The MPNLS amplicon nucleotide sequence of 15 field isolates and one vaccine strain Mukteswar on phylogenetic analysis generated three major clusters (Fig.31). The early velogenic isolates JQC6/87, LCN2/87 and BCC4/87 were grouped in a single cluster along with BRLY-58/93. The three desi bird isolates D2/95, D3/95 and D4/95 were grouped together in the second cluster. The third cluster was made up of Kerala isolates KQA-ALW/98, KQC-CHE/98 and KPG/98 along with vaccine strain, Mukteswar/46. The isolates LCC9/89, KNBB/96, D1/95, LCN3/87 and WB-198/94 were diverging from the connecting branch between the three major clusters (Fig.31). The desi bird isolates and the early velogenic isolates were observed diverging equality from the branching point and were closely related with one another (Fig.31).

4.14.3 Phylogenetic relationship among Indian NDV isolates based on contiguous nucleotide sequences of FPCS and MPNLS amplicons

Contiguous FPCS and MPNLS nucleotide sequences subjected to phylogenetic analysis using CLUSTAL-W and MEGA softwares.

4.14.3.1 Phylogenetic Analysis using software CLUSTAL-W

The CLUSTAL-W phylogenetic analysis generated four different clusters, with the first two clusters viz. early velogenic isolates and desi bird isolates closely related to each other. The third cluster comprised of lentogenic isolates D1/95, KNBB/96 and LCC9/89, while the fourth cluster encompassed three Kerala isolates along with vaccine strain Mukteswar. The WB-198/94 formed the major ancestor of the early velogenic isolates and the desi bird isolates clusters. The LCN3/87 isolate was closely related to the lentogenic isolates group. The recent BRLY-58/93 isolate was found in the first cluster along with the early velogenic isolates JQC6/87, LCN2/87 and BCC4/87 (Fig.32).

4.14.3.2 Phylogenetic analysis using software MEGA

Using Neighbor-Joining tree building method with Jukes-Cantor distance, two major lineages was found with two different clusters in each lineage (Fig.33). The first lineage comprised of the early velogenic isolates cluster and the desi bird isolates cluster. The recent isolate BRLY-58/93 went

into the early velogenic isolate cluster. The JQC6/87 isolate was more closely related to LCN2/87 isolate and the BCC4/87 isolate was the next closely related in that cluster. The desi bird isolates D3/95 and D4/95 were more closely related and the D2/95 isolate joining the cluster after D3/95. The lentogenic field isolates (based on nucleotide sequence) LCC9/89, KNBB/96 and D1/95 formed the first cluster of the second lineage. The other cluster in the second lineage consisted of three Kerala isolates KQA-ALW/98, KQC-CHE/98 and KPG/98 along with vaccine strain Mukteswar/46. The Bootstrap test carried out 2000 replicates of the NJ and Jukes-Cantor distance method tree confirmed the two major lineages with conservation among the clusters observed in each lineage (Fig.33).

On using the Kimura 2-Parameter distance with the Neighbor-Joining method, the phylogenetic tree obtained (Fig.34) was similar topographically compared to that obtained with Jukes-Cantor distance estimation method (Fig.33). The Kimura 2-Parameter-NJ tree on Bootstrap analysis using 2000 replicates yielded the tree with essentially same configuration (Fig.34) The standard error '*t*' test carried out for both the trees obtained with Jukes-Cantor and Kimura 2-Parameter distances using NJ method. The trees obtained expressed the confidence levels from '*t*' test (CP) as confidence proportion (Fig.35). The Bootstrap tree (Fig.33,34) was similar to the Standard Error '*t*' test tree (Fig. 35).

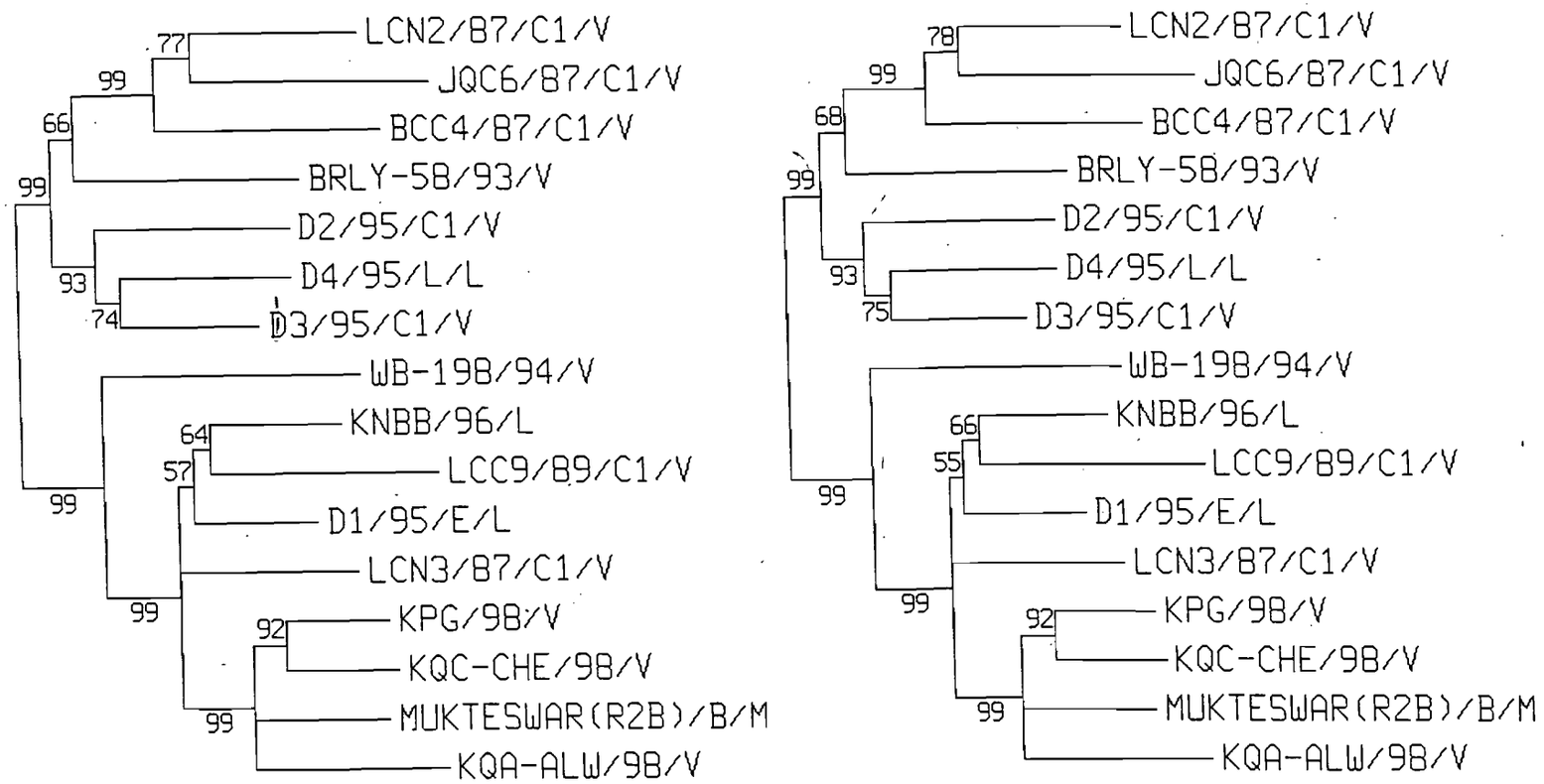


Fig. 35 : Phylogenetic relationship of NDV isolates of India based on Contiguous FPCS+MPNLS sequences: Standard Error tests for Gene tree generated by Neighbor Joining tree building method with for Jukes Cantor Distance estimation method (A), and Kimura 2-parameter Distance estimation method (B), using MEGA Software. Confidence levels from standard Error, t-test are listed for branch points as Confidence Probability CP).

discussion

CHAPTER - 5

DISCUSSION

Newcastle disease (ND) is a highly contagious viral disease of poultry of great economic importance, decisively influenced by virulence of the organism. Latently infected hens, which are not sufficiently immunized or which harbor the virulent field virus in the respiratory tract inspite of humoral antibodies, are the agent reservoir in domestic poultry. In India ND is endemic, owing to the dense hen populations, open hen houses and small distance between farms, making the eradication of the epizootic probably not possible (OIE Manual - NDV, 1992). Control measures in this kind of enzootically infected areas aim at reducing or preventing economic losses. Understanding the epizootiology of NDV in context with the close nexus between free-living virulent virus reservoirs like desi birds, pigeons, psittacine species and wild birds with the domestic poultry is of great importance, to effectively control the disease. Rapid NDV strain identification and differentiation is essential for deploying effective control measures during an outbreak and also to establish vaccine identity, due to the indiscriminate use of several live vaccines in India. Differentiation of NDV isolates during outbreaks as vaccinal or enzootic virus is important for enforcing strict zoo-sanitary and isolation measures, and to prevent the importation of epizootic virus into vaccinated populations.

Conventionally, NDV isolates are characterized by virulence characters like MDT, ICPI and IVPI and strain differentiating characters like stability of haemagglutinins at 56° C, agglutination of mammalian erythrocytes, rate of elusion of chicken erythrocytes and variations of structural polypeptides (Hanson and Brandly, 1955 ; Allan *et al.*, 1978; Alexander, 1989; Kumanan *et al.*, 1992; Vijayarani *et al.*, 1992; Raghavan *et al.*, 1998). Although some of these techniques are effective in assessing the virulence of the isolates, they are time consuming and require the use of more than one technique. Sometimes, the results of many of the above techniques need to be corroborated for effective pathotyping.

Monoclonal antibodies have also been used for specific identification of vaccine strains and epizootic viruses and to place the epizootic virus isolates into meaningful epizootiological groups (Alexander, 1990). Other molecular biological techniques for epizootiological classification viz., genotyping by restriction site analysis of specific gene fragment (Ballagi-Pordany *et al.*, 1996; Lomniczi *et al.*, 1998), phylogenetic analysis of nucleotide sequence of different gene regions (Heckert *et al.*, 1996; Seal *et al.*, 1996; Yang *et al.*, 1997) and pathotyping based on FPCS (Seal *et al.*, 1995; 1998) have been carried out to provide microepidemiological classification of NDV field isolates.

In the context of above developments, NDV isolates from different regions of India, isolated at various time periods have been subjected to epizootiological classification based on PCR-RFLP of the Fusion gene and phylogenetic analysis of contiguous FPCS and MPNLS amplicon nucleotide sequences obtained by RT-PCR from the analysed NDV isolates. These analyses would lead to effective pathotype prediction, differentiation of vaccine virus from epizootic virus and control of ND. These studies also help in considerable reduction in live bird testing.

5.1 NEWCASTLE DISEASE VIRUS ISOLATES

Newcastle disease virus isolates subjected to this study were isolated during a period of past 13 years from 1987 to 1999. Since the isolates were of only 13 years period the Neighbor-Joining method using the principle of minimum evolution was applied for the phylogenetic study (Saitou and Imanishi, 1989). NDV isolates were also obtained from diverse regions of India namely Tamil Nadu, Kerala, Karnataka, West Bengal, Uttarpradesh and one originating from the adjacent country Nepal.

The phylogenetic analysis of these representative isolates from different regions was carried out with nucleotide sequence estimation of the selected NDV isolates. The recently obtained exotic bird isolates such as PCKC/98 (Peacock) and LBC/99 (Love-bird) were directly included in the sequence estimation and analysis to increase the diversity of the NDV isolates origin included in this study. Further, the isolates from commercial poultry were obtained from different type of birds viz., broilers, layers, as well as Japanese quails. The desi bird isolates from backyard poultry have also been included in this study to evaluate the phylogenetic relationship between them and the other isolates from commercial poultry.

5.2 CONVENTIONAL CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES

The conventional methods of assessing virulence and strain differentiation are based on estimating the Mean death time (MDT), Intracerebral pathogenicity index (ICPI) and Intra-venous pathogenicity index

(IVPI). NDV strains and isolates have been grouped on the basis of their MDT as velogenic, if MDT is less than 60 hours, 60-90 hrs as mesogenic and greater than 90 hrs as lentogenic (Hanson and Brandly, 1955). The MDT of all tested isolates were found to be in range from 42.0 to 56.5, with one isolate NPL-5/93 having a MDT of 66.0 hrs. The major pathotype observed based on MDT test was velogenic, with one mesogenic isolate. The desi bird isolates, D1/95 and D4/95 which were lentogenic based on Mab typing results of the earlier studies (Raghavan *et al.*,1998), still gave high MDT values.

The ICPI test is a sensitive measure of virulence with velogenic strains indices ranging from 1.75 to 2.0, while mesogenic and lentogenic isolates indices range from 0.8 to 1.46 and 0.0 to 0.25 respectively (Hanson and Brandly, 1955).

The Nepal isolate, NPL-5/93, typed as mesogenic by MDT and ICPI tests was having the FPCS amino acid sequence 'GR' at positions 115 and 116 indicating moderate virulence. The LCC9/89 isolate which was velogenic on MDT and ICPI test was found to have lentogenic FPCS aminoacid sequence. Similar differences between biological assay and molecular biological determination has been reported by Oberdorfer and Werner (1998) with the pigeon variant virus, PPMV-1. Also, marked increase in ICPI and IVPI values were observed in case of pigeon variant NDV, PPMV-1 from natural infections of chickens (Alexander,D.J., and G. Parsons, 1986).

The lentogenic desi bird isolate D4/95 expressing high ICPI was found to be a mesogenic pathotype on FPCS amino acid sequence evaluation, with two pairs of dibasic aminoacids found at 112, 113 and 115, 116 positions. Lentogenic field isolates showing considerable variation in virulence with high

ICPI was inhibited by Mab against La Sota like viruses (Werner *et al.*,1999). The D4/95 isolate typed as lentogenic based on Mab typing, was different from the MDT and ICPI observations, which suggested it to be of mesogenic pathotype. The other lentogenic desi bird isolate D1/95 was typed similarly on FPCS amino acid sequence evaluation. The isolates WB-198/94, BRLY-58/93, KQA-ALW/98, KQC-CHE/98 and KPG/98 were typed as velogenic on MDT and ICPI tests which correlated with FPCS amino acid sequence based typing. The mesogenic vaccine strain Mukteswar (R_2B) could be differentiated from Komarov strain, based on the high MDT value in Mukteswar strain compared to Komarov strain, although both had similar FPCS amino acid sequence.

The lentogenicity of KNBB/96 isolate was found to correlate both in MDT and ICPI tests and also in FPCS amino acid sequence.

5.3 POLYMERASE CHAIN REACTION FOR THE FPCS AND MPNLS REGIONS

The PCR for FPCS using the degenerate oligonucleotide primers amplified the FPCS region from genomes of divergent NDV isolates. While the FPCS region primers amplified the expected product of 254 bp from all the NDV isolates analysed, the MPNLS primers could not amplify the expected product of 232 bp from isolates NPL-5/93, PCKC/98 and LBC/99, probably owing to sequence variation at the primer binding site in these isolates. Seal *et al.* (1995) have reported that MPNLS region amplicon could not be obtained using the degenerate primers from RNAs of pigeon NDV as well APMV-2 and 3 with the protocol followed. The isolates PCKC/98 and LBC/99 were from peacock and love-bird (Passeriformes) which were exotic birds compared to the natural host chicken. The NPL-5/93 isolate was from chicken and probably still

had substantial differences of nucleotides at the primer binding site (or) by possible transfer of pigeon NDV to the chicken, which led to no amplification of MPNLS amplicon. The degenerate primers may be altered to pick up the pigeon NDV sequence, for further efficient amplication of MPNLS region from all pathotypes and pigeon NDV isolates.

5.4 RESTRICTION ENDONUCLEASE ANALYSIS OF FUSION GENE (1349bp) AMPLICON

Restriction site analysis of fusion gene fragment (1349bp) of diverse NDV strains was carried out by Ballagi-Pordany *et al.* (1996) and Lomniczi *et al.* (1998). They have described eight different groups/genotypes of NDV isolates viz. I-VII and RI-3, (Fig.36) based on Hinf I, BST OI and Rsa I Fusion gene fingerprint patterns (Table 1).

Restriction endonuclease analysis of Indian NDV isolates fusion gene amplicon using Hinf I, Bst OI and Rsa I resulted in three identical groups viz., II, IV and VI, as described by Ballagi-Pordany *et al.* (1996) and Lomniczi *et al.* (1998). Apart from the distinct Group VI describing few isolates, grouping possibilities of VI with either V or RI-3 genotypes were found in few isolates. Group II was the major genotype and consisted of isolates LCC5/87, LCC9/89, LCC15/89, KQA-ALW/98, KQC-CHE/98, KPG/98, D1/95, D5/95 along with vaccine strain Mukteswar/46. LCN3/87 was the only isolate in the genotype Group IV, while Group VI had JQC6/87 and BRLY-58/93 isolates. The combination of Group VI with Group V consisted of isolates D2/95, D3/95 and D4/95, while the Group VI with RI-3 comprised of LCN2/87 and LCC5/87.

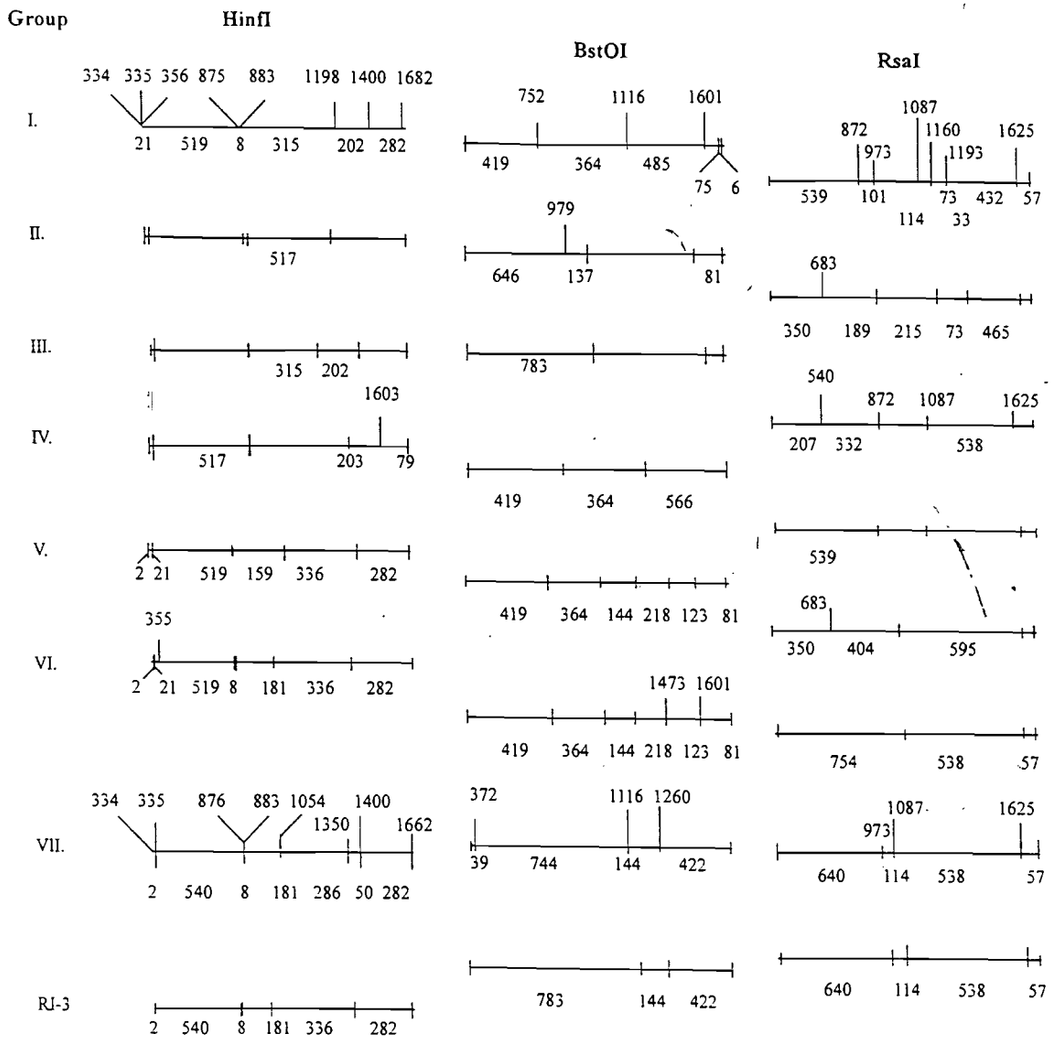


Fig. 36 : . Physical maps of restriction fragments and cleavage sites of the PCR products of the F genes of representative NDV strains.(Ballagi-Pordány *et al.*,1996 and Lomniczi *et al.*,1998)

Group II was described as 'old' (prior to 1960s) North American isolates by Ballagi-Pordany *et al.*, (1996). Moreover Group II consisted of isolates irrespective of virulence with lentogenic, natural mesogenic and velogenic isolates. Similarly in this study, the velogenic isolates LCC5/87, KQA-ALW/98, KQC-CHE/98, KPG/98, the mesogenic strain Mukteswar/46 and lentogenic isolates like LCC9/89, D1/95 and D5/95, were placed under Group II on the basis of fusion gene restriction site analysis as described by Ballagi-Pordany *et. al.*, (1996).

Group IV described by Ballagi-Pordany *et al.*, (1996) consisted of early European strains viz., Herts 33 and Italien, of the first panzootic (starting in the late 1920's) and their descendants with some modifications. In this study, the LCN3/87 chicken isolate was the only one classified in this Group IV, having similarity with European strains of the first panzootic.

The Group VI encompassed strains from the Middle East in the late 1960s and later isolates from Asia and Europe. Pigeon Paramyxovirus formed a distinct subgroup in Group VI which were responsible for the third panzootic (Ballagi-Pordany *et al.*, 1996). In this study, the Japanese quail isolate JQC6/87 and the chicken isolate from Bareilly BRLY-58/93 were classified into this Group VI, responsible for current panzootic. The quail isolate had a Rsa I restriction site in 754 bp fragment in addition to that described for Group VI, leading to two bands of 539 and 215 bps in size.

The desi bird isolates D2/95, D3/95 and D5/95 were classified as Group VI with both Hinf I and Rsa I pattern correlating with patterns described by Ballagi-Pordany *et al.*,(1996) but with few mutations of restriction sites leading to the grouping as VI. A Hinf I restriction site in the 519 bp band led to two fragments of 380 and 139 bps, while presence of three Bst OI sites at 1260, 1478 and 1601 nts lead to generation of 218, 144, 123 and 81 bp fragments instead of 422 and 144 bps described for Group VI by Ballagi-Pordany *et al.*,(1996).

The presence of Rsa I site at 1625 nt led to generation of 538 and 57bp fragments instead of the standard 595bp fragment of the Group VI. With additional mutations, the isolates D2/95, D3/95 and D5/95 correlate with Group V, which describes the strains originating in imported psittacines and in epizootics of chicken at the early 1970's (Ballagi-Pordany *et al.*, 1996). The additional mutations suspected were, in the presence of HinfI site at 883 nt leading to 181 and 8 bp fragments instead of 189 bp standard fragment. The absence of 683 nt Rsa I site led to a single fragment of 754 bp in these described isolates instead of the standard 404 and 350 bps described for Group V (Ballagi-Pordany *et al.*, 1996).

The isolate LCN2/87 and BCC4/87 classified in the Group VI with presence of Hinf I restriction site in the 519 bp standard leading to 380 and 139 bps in the LCN2/87 isolate. The Bst OI site at 1478 nt and 1601 nt were absent leading to generation of 422 bp fragment in the LCN2/87 isolate instead of 218, 123 and 81 bps, as in the standard Group VI. The presence of Rsa I site at 683 nt led to two fragments of 539 and 538 instead of the standard 754bp fragment.

The isolates LCN2/87 and BCC4/87 were also correlated with group RI-3 described by Lomniczi *et al.*,(1998) which consists of NDV strains from Indonesia and Taiwan. The presence of Hinf I sites at 355 and 335 nts led to the formation of 3 bands of 380, 139 and 21 bps instead of the standard 540 bp fragment.

A Bst OI site at 752 nt was present in the isolates LCN2/87 and BCC4/87 leading to generation of 419 and 364 bp fragments, instead of the standard 783 bp fragment of Group RI-3. The absence of Rsa I site at 1087 nt and presence of 872 nt Rsa I site led to the formation of 539 and 215 bp fragments in the isolates LCN2/87 and BCC4/87, instead of the standard 640 and 114 bp fragment of Group RI-3 (Lomniczi *et al.*, 1998).

5.5 PHYLOGENETIC ANALYSIS OF NDV ISOLATES BASED ON FUSION GENE (1349bp) FINGERPRINT ANALYSIS

In this study, the Hinf I : UPGMA phylogenetic grouping differentiated the vaccine strains 'B1', La Sota, 'F' and 'K' from the NDV field isolates analysed. The mesogenic vaccine strain Mukteswar/46 was correlated along with the Kerala isolates viz., KQA-ALW/98, KQC-CHE/98 and KPG/98, as observed in restriction site grouping (Group II) described by Ballagi-Pordany *et al.* (1996) and Lomniczi *et al.* (1998). The early velogenic isolates JQC/87, LCN2/87, BCC4/87 were grouped along with lentogenic and velogenic desi bird isolates namely, D2/95, D3/95 and D4/95 suggesting close relationship between these chicken and desi bird isolates. The isolates LCN3/87, LCC5/87, LCC9/89 and LCC 15/89 were grouped along with lentogenic desi bird isolates D1/95 and D5/95 (Fig.3).

The Bst OI : UPGMA Phylogenetic grouping had the early velogenic isolates JQC6/87, LCN2/87, BCC4/87 and a recent BRLY-58/93 velogenic isolate grouping together showing close relationship and possible re-emergence of these early isolates. The lentogenic vaccine strains went along with three Kerala field isolates and formed the major ancestor branch to all field isolates. Present isolation of these Kerala isolates (1998) indicates the possible re-emergence of early strains like LCN3/87, BCC4/87, LCC9/89, LCC15/89 and WB-198/94 owing to the close relationship between them (Fig.4).

The Rsa I : UPGMA phylogenetic grouping revealed close relationship of recent desi bird isolates with the early velogenic chicken isolates JQC/87, LCN2/87 and LCN3/87, suggesting the maintenance of velogenic chicken isolates in the desi birds over a time period of five years. The Rsa I phylogenetic grouping could not effectively differentiate the vaccine strains from field isolates (Fig.5).

5.6 RESTRICTION ENDONUCLEASE ANALYSIS OF FPCS AND MPNL AMPLICONS

The analysis of short stretch of FPCS (254 bp) and MPNLS (232bp) amplicon regions, compared to the larger fusion gene (1349bp) amplicon by restriction site analysis, suggested that short regions were not effective in classifying the isolates based on the presence or absence of restriction sites. The use of unique and rare restriction enzymes in the short stretch of the amplified genome may obviate the classification and typing based on restriction sites analysis of FPCS and MPNLS regions (Plate 12, 13, 14 and 15).

5.7 PATHOTYPE PREDICTION BASED ON FUSION PROTEIN CLEAVAGE SITE AMINO ACID SEQUENCE

The FPCS aminoacid sequence estimated from the NDV field isolates was efficiently used for pathotype prediction (Nagai *et al.*, 1976a).The isolates *D1/95 and KNBB/96 were classified as avirulent isolates based on FPCS amino* acid sequence and well correlated with the conventional tests like MDT, ICPI and IVPI (Table 7). The LCC9/89 isolate typed as lentogenic based on FPCS amino acid sequence, was earlier classified as velogenic by conventional tests and was 'C1' Mab group describing virulent isolates probably owing to the isolation of pigeon variant virus PPMV-1 from chicken.

The KPG/95 isolate had a FPCS amino acid sequence motif of ^{111}RRQ KRF^{116}, similar to that described for PPMV-1 pigeon variant isolated from naturally infected chickens (Werner *et al.*, 1999). The two other Kerala isolates KQA-ALW/98 and KQC-CHE/98 both from quails were also found to have the similar FPCS amino acid sequence motif as that of PPMV-1 pigeon variant, suggesting isolation of PPMV-1 variants from Japanese Quails. The PCKC/98 and LBC/99 FPCS aminoacid sequence were found to be of velogenic nature owing to the presence of dibasic aminoacids RR at positions 112 and 113. The velogenic nature of these isolates from exotic birds indicates the potential source of virulent virus to domestic poultry.

Table-7 : Grouping of of NDV isolates and commonly used vaccine strains by the predicted FPCS Cleavage site amino acid sequence

GROUP	ISOLATE/ STRAIN	FPCS-AMINOACID SEQUENCE
1	NDV – Q	SGRRRQ KRFVGA
	NDV – 2	SRRRRQ RRFVGA
	NDV – 4	SGRRRQ KRFVGA
2	NDV – 3	SGRRRQ RRFVGA
	NDV – 9	SGGGKQ GRLIGA
3	NDV -WB	SGRGRQ GRLVGA
4	NDV-R2B	SGGRRQ KRFIGA
	NDV-KQA	SGGRRQ KRFIGA
	NDV-KQC	SGGRRQ KRFIGA
	NDV-KPG	SGGRRQ KRFIGA
	NDV-BRL	SGGRRQ KRFIGA
	NDV-NPL	SGGRRQ KRFIGA
5	NDV-R2B	SGGRRQ KRFIGA
	Komarov	SGGRRQ KRFIGA
6	B1	SGGGRQ GRLIGA
	F	SGGGRQ GRLIGA
	LaSota	SGGGRQ GRLIGA
	NDV-D4	SGGRRQ GRLIGA
	NDV-KNB	SGGGRQ GRFLGA
	NDV-D1	SGGGRQ GRFIRA
7	NDV-D2	SGGRRQ KRFVRA
	NDV-D3	SGRRRQ KRFIGA
8	NDV-CCA	SGGRKQ KRFVGA
	NDV-PCK	SGRRRQ KRFIGA
	NDV-LB	SGRRRQ KRFIGA
9	NDV – Q	SGRRRQ KRFVGA
	NDV-CCA	SGGRKQ KRFVGA
	P3G	SGGGRQ GRLIGA
10	NDV-PCK	SGRRRQ KRFIGA
	NDV-LB	SGRRRQ KRFIGA
	NDV-D3	SGRRRQ KRFIGA

5.8 PHYLOGENETIC RELATIONSHIPS OF NDV FIELD ISOLATES BASED ON OBTAINED NUCLEOTIDE SEQUENCE DATA

5.8.1 Phylogenetic relationship among NDV isolates based on FPCS amplicon nucleotide sequences

The early velogenic isolates JQC6/87, LCN 2/87 and BCC4/87 belonging to the same period, though from different host origins like Japanese quail, layer and broiler chicken were grouped together in a single cluster (Fig.30). Isolates JQC6/87 and BCC4/87 were from Chennai, while isolate BCC4/87 was from Namakkal (Salem). The presence of all these isolates in Tamil Nadu indicates the existence of the same genotypes in different regions simultaneously. The close relationship between the early velogenic chicken isolate LCN3/87 from Namakkal, with the recent desi bird isolate D1/95 from Chennai, and the velogenic desi bird isolate D2/95 from Chennai suggests the maintenance of the early velogenic isolates in desi birds.

The three Kerala isolates of Japanese quail and pigeon origin were grouped together indicating similar genotype in existence in that region. The two quail isolates KQA-ALW/98 and KQC-CHE/98 were grouped together, though from different places viz., Alwaye and Chenganur in Kerala. The mesogenic vaccine strain Mukteswar forming the ancestor of quail isolates group indicates possible use of this vaccine, which would have led to the generation of these quail isolates. The KPG/98 pigeon isolate from Kerala was the recent major ancestor for both the quail isolates, from the same region. The KPG/98 was having similar FPCS amino acid sequence compared to that of PPMV-1 pigeon variant viruses, described by Collins *et al.* (1993; 1994). This clearly suggests that the PPMV-1 type might have viruses spread from pigeons to Japanese quails population.

The LCC9/89 chicken isolate from Chennai is grouped along with WB-198/94, West Bengal isolate showing the existence of similar genotypes of viruses in different regions and time period. The Karnataka lentogenic field isolate KNBB/96 has close relationship with the West Bengal and Chennai isolates suggesting the possible grouping based on chicken origin of isolates.

The exotic bird isolates PCKC/98 from peacock and LBC/99 from Love-bird were grouped together indicating sequence similarity of these velogenic isolates. The CCA/93 cell culture passaged virus of the JQC6/89 Japanese quail isolate was linked to the exotic bird isolates probably owing to sequence similarity acquired by the CCA/93 strain during cell culture passages. The exotic bird isolates formed the major ancestor branching for the early velogenic isolates and Kerala isolates of different host origin including desi bird isolates indicating a possible theory of dissemination of velogenic virus to these commercial poultry from the exotic birds.

The two velogenic desi bird isolates D3/95 and D4/95 were closely linked to the Bareilly isolate BRLY-58/93, and formed of the progenitor type viruses branching, similar observations have been made by Seal *et al.*,(1998) suggesting chicken / Australia / AV / 32 has possible progenitor for exotic bird isolates. The Nepal isolate NPL-5/93 was branched near the Bareilly isolate. BRLY-93, during the same period, which indicates the existence of these similar genotypes in these adjoining regions.

5.8.2 Phylogenetic relationships among NDV isolates based on MPNLS amplicon nucleotide sequences

The phylogenetic analysis pattern of MPNLS amplicon nucleotide sequence revealed subtle changes, as to that obtained with FPCS amplicon nucleotide changes owing to the slow evolution and high conservation of MPNLS sequences. Compared to other viral genes the matrix protein gene is not greatly affected during attenuation in Paramyxoviruses.

The Bareilly isolate BRLY-58/93 grouped together with early velogenic isolates, JQC6/87, LCN2/87 and BCC4/87, suggests the close relationship of these isolates with conservation of MPNLS region sequences.

The desi bird isolates D2/95, D3/95 and D4/95 were grouped together indicating conservation observed among these isolates of same host origin. The three Kerala isolates were again grouped together indicating good homology and conservation of these isolates MPNLS region nucleotide sequences

The results obtained in this study have also indicated that the lentogenic isolates LCC9/89, KNBB/96, D1/95 were closely related, suggesting the conservation of MPNLS region sequences in these lentogenic field isolates. The major observation of this MPNLS nucleotide sequence analysis being the, equally balanced grouping of the early velogenic isolates and the recent desi bird isolates (Fig.29), describing effectively, the maintenance of the velogenic viruses in the desi birds. The theory was mainly supported by conservation of MPNLS sequence among these isolates grouping them together.

5.8.3 Phylogenetic relationships among Indian NDV isolates based on contiguous FPCS and MPNLS amplicon nucleotide sequences

The use of contiguous FPCS and MPNLS region amplicon sequences for phylogenetic analysis (Seal *et al.*, 1995 ; 1998) provided good, balanced evaluation of the NDV isolates based on two regions of their genome.

5.8.3.1 Phylogenetic Analysis using software CLUSTAL-W

The early velogenic isolates JQC6/87, LCN2/87 and BCC4/87 grouping, conserved in both the stand alone analysis of FPCS and MPNLS sequences, was proved to be the same in the contiguous FPCS:MPNLS sequence analysis. The Bareilly isolate, BRLY-58/93, supported by MPNLS analysis into the early velogenic group, was found to be conserved on contiguous FPCS : MPNLS sequence analysis. The desi bird isolates grouping was similarly grouped in MPNLS and the contiguous FPCS:MPNLS sequence analysis (Fig.32). The Kerala isolates group along with the vaccine strain Mukteswar was found conserved in all the FPCS, MPNLS and contiguous FPCS:MPNLS sequence analysis.

The grouping of the early velogenic isolate LCN2/87 of chicken origin with the recent desi-birds isolate D1/95 suggests the close relationships of these isolates from different hosts.

The LCC9/89, KNBB/96 and D1/95, all lentogenic field isolates from different regions as well as from different time periods were closely linked

indicating the conservation of FPCS and MPNLS sequences in these isolates. The branching pattern initiated from in between the Kerala isolates group and the lentogenic field isolates group to their immediate descendants.

5.8.3.2. Phylogenetic Analysis using software MEGA

The use of the software MEGA gave flexibility to use two widely used distance estimation methods viz., Jukes-Cantor and Kimura 2-Parameter distances, used for phylogenetic analysis with Neighbour-Joining method for tree construction of NDV nucleotide sequences (Collins *et al.*, 1996 ; Seal, 1996).

Two major lineages observed for the NDV isolates from India (Fig.33 & 34) was similar to that expressed by Seal *et al.*, (1995) analysing the North American isolates (Fig.1). The first lineage with early velogenic chicken isolates and recent desi bird isolates clusters grouped together, strongly recommends the theory of maintenance of the velogenic viruses in the desi birds. The second lineage with the lentogenic field isolates and the Kerala isolates cluster indicates close relationships of these isolates from three different states of South India, isolated during different time periods. The Kerala isolates grouped together with mesogenic vaccine strain and the pigeon variant virus PPMV-1 correlation with these field isolates has to be taken into consideration, in suggesting the spread of the current pigeon panzootic in India. The layer chicken velogenic isolate, LCN3/87 isolated during 1987 in Tamil Nadu formed the recent major ancestor to the Kerala isolates with a proposed theory of spread of this velogenic virus from Namakkal in Tamil

Nadu to Kerala over a period during the past 12 years. Collection of more isolates from the states from different regions and time of isolation along with basic epizootiological data of outbreaks may concrete this proposed theory of spread of velogenic viruses.

The West Bengal velogenic chicken isolate WB-198/94, is the major ancestor of the second lineage groups/clusters. The distant nucleotide sequence of WB - 198/94, compared to that of South Indian isolates sequence, correlates well with distant geographical location of WB-198/94 isolate, from the isolates of South India.

The phylogenetic relationships estimated by both the Jukes-Cantor distance and Kimura 2-Parameter distances with Neighbor-Joining method of tree construction was conserved, proving the truthful phylogenetic grouping of analysed NDV isolates (Fig.33 & 34). The Bootstrap replicates and Standard Error too confirmed a similar phylogenetic classification of the analysed NDV isolates (Fig.35).

5.9 PHYLOGENETIC RELATIONSHIPS OF INDIAN NDV ISOLATES WITH NDV STRAINS OF THE WORLD

Contiguous FPCS and MPNLS amplicon nucleotide sequences from Indian NDV isolates were compared with 45 NDV sequences already available in the Gen Bank database. The Neighbor joining tree building with Jukes cantor distance yielded phylogenetic tree with two major lineages (Fig.37). In the first lineage the Indian velogenic NDV isolates LCN2/87, JQC6/87 and BCC4/87 were closely related to velogenic isolates Herts/33, Itally/Milano, Timneh/Zimbabwe/90 (Seal *et al.*, 1996 ; 1998). The desi bird isolates D2/95,

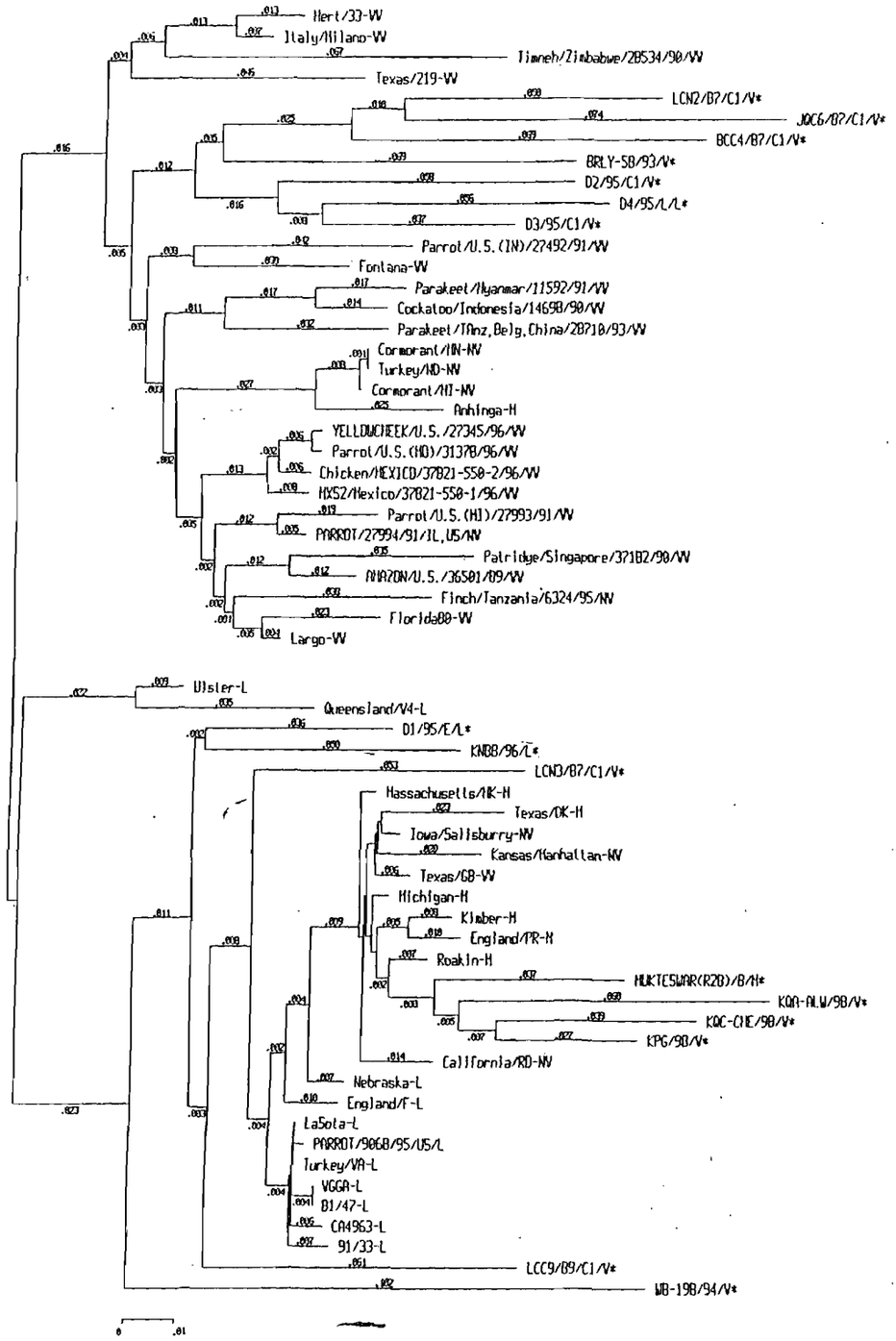


Fig. 37 : Phylogenetic relationship of NDV isolates of India with world strains of NDV based on Contiguous FPCS+MPNLS sequences: Gene tree generated by Neighbor Joining tree building method with Jukes Cantor Distance estimation method using MEGA Software. Distance measures listed are the percent chance of a nucleotide substitution at one site.

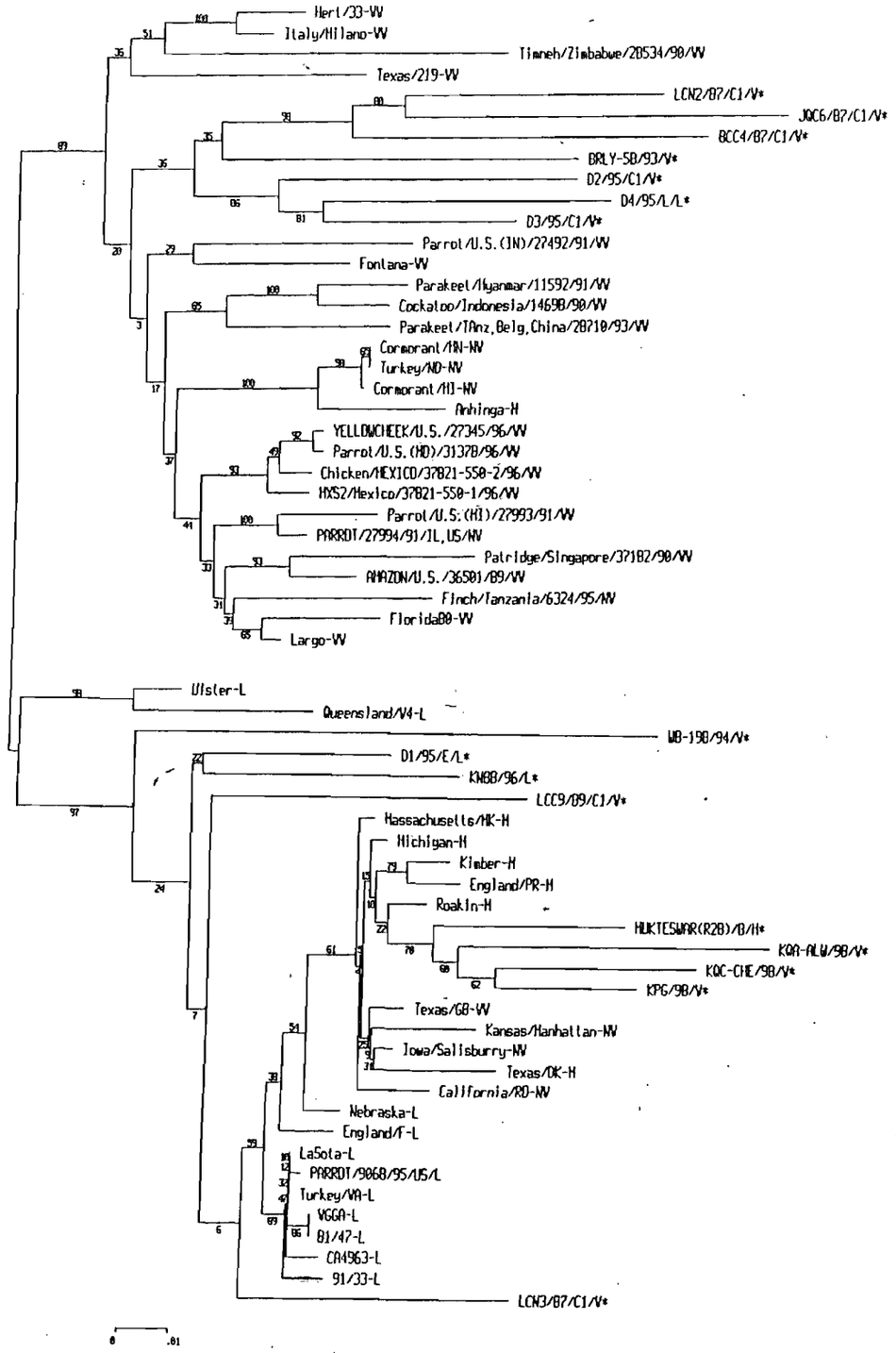


Fig. 38 : Phylogenetic relationship of NDV isolates of India with world strains of NDV based on Contiguous FPCS+MPNLS sequences: Bootstrap test for Gene tree generated by Neighbor Joining tree building method with Jukes Cantor Distance estimation method using MEGA Software. Bootstrap confidence levels (BCL) are listed for branch points as a percentage.

D4/95 and D3/95 were branched close to the velogenic parrot and Fontana isolates from U.S.A. The second lineage encompassed the Kerala isolate cluster along with vaccine strain Mukteswar grouping together with other mesogenic isolates viz. Kimber, England/PR, Michigan and Roakin (Seal *et al.*, 1996 ; 1998). The isolate LCN3/87 formed the recent ancestor of the second lineage followed by LCC9/89. The D1/95 and KNBB/96 lentogenic isolates were grouped together and were found to be closely related to other lentogenic strains Queensland/V4. The WB/98/94 formed the ancestor branch after LCN3/87 isolate. The NJ-Jukes-Cantor distance tree on Bootstrap analysis using 2000 replicates yielded similar clustering and lineage (Fig.38).

It is concluded that, in the present study, NDV isolates from different regions and from different time points were subjected to strain differentiation using both conventional and molecular methods. It is clearly evident from the results, that the molecular methods have a clear edge over the conventional methods in predicting the nature of the isolate. However, this study is just a beginning of a detailed study involving several NDV isolates from various parts of the country which in turn will give us some clues in programming future control measures for this dreaded poultry disease.

summary

CHAPTER - 6

SUMMARY

Phylogenetic analysis of diverse Newcastle Disease Virus (NDV) isolates of India was carried out using Restriction site analysis and Nucleotide sequencing of Fusion and Matrix protein gene regions, to classify them into different clusters and to correlate them epizootiologically.

Monoclonal antibody (Mab) typed NDV isolates from Tamil Nadu along with NDV isolates from different states of India, from different time periods and a mesogenic vaccine strain-Mukteswar, were subjected to RT-PCR and Nucleotide sequencing of Fusion protein cleavage site (FPCS) region and Matrix protein Nuclear localization signal (MPNLS) region. Preliminary analysis of representative NDV strains, by RT-PCR-RFLP of a 1349 nucleotide long region of Fusion protein gene using restriction enzymes Hinf I, Hae III, Bst OI, Rsa I, Taq I, and Mbo I was carried out, to differentiate the isolates. The fusion gene fingerprint distance measures obtained using Jaccard's distance coefficient, were analyzed with the software MEGA using UPGMA method. The Phylogenetic trees obtained using Hinf I distance measures differentiated the Mab typed homogenous velogenic isolates group, as well as vaccine strains from field isolates. NDV isolates from the period 1987-89 were found to form the ancestral group for the recent isolates indicating the re-emergence of similar strains after a period of three years. Possible re-emergence of virulent NDV clones after a period of three years suggested.

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Phylogenetic analysis of 20 FPCS & 16 MPNLS amplicon sequences on stand-alone basis using CLUSTAL-W, revealed four & two major clusters respectively, while phylogenetic analysis of contiguous FPCS & MPNL sequences using Jukes-cantor (JCD) and Kimura 2-parameter distance (K2-PD) and Neighbor joining (NJ) tree construction, revealed two major lineages. The first lineage encompassed three desi bird isolates (D2, D3 and D4) are grouped in a single cluster along with early (80's) velogenic isolates-Q, 2,4 and one recent ('93) isolate-BRL. The WB isolate ('90's) formed the ancestor of the second major lineage with one Karnataka & desi lentogenic field isolate (D1) are grouped along with a early (80's) velogenic isolate - ND-9, all lentogenic in nature. Three Kerala isolates grouped together, along with R2B Vaccine strain, while the '87 velogenic chicken isolate-ND-3, formed the recent ancestor of the recent Kerala isolates ('98) and R2B. Both Distance methods-JCD & K2-PD on NJ tree building generated identical branching patterns of NDV isolates analyzed.

The Indian isolates shared phylogenetic relationship with already existing two major global phylogeny lineages in a well-balanced pattern with both velo, meso & lentogenic isolates going into both the lineages. The desi bird velogenic isolates were grouped along with the other exotic bird velogenic isolates viz., parrot, cockatoo and parakeet. The Indian early velogenic isolates grouped along with other velogenic isolates from Texas, Italy and Herts. All the 3 Kerala isolates grouped together, along with R2B Vaccine strain went into the mesogenic cluster of field isolates and vaccine strains

Phylogenetic analysis of FPCS and MPNLS nucleotide sequence has been found effective in discriminating the homogenous monoclonal antibody typed isolates group into various clusters and also between vaccine strains and field isolates. Existence and circulation of at least two major lineages and different clones/ genotypes of virulent NDV in–India is suggested. Effective pathotype prediction has been carried out using predicted amino acid sequence analysis of FPCS. The LCC9/89 isolate was found velogenic on MDT and ICPI was found to have lentogenic FPCS amino acid sequence, suggesting the isolation of pigeon PMV-1 variant from chicken. Other analysed isolates for FPCS amino acid sequence correlated with the conventional Mab pathotyping tests. Also the D4/95 isolate typed as lentogenic by conventional tests was found to be mesogenic type on FPCS amino acid sequence correlating with high ICPI recorded. The Indian NDV isolates displayed unique diversity in the global phylogeny map showing conservation among them, compared to other isolates of world.

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Phylogenetic Analysis of Indian Field Isolates and Vaccine Strains of Newcastle Disease Virus

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Abstract

PURPOSE: In India, first reported outbreak of highly virulent Newcastle disease occurred as early as 1927 and spread all over the subcontinent rapidly. Extensive vaccination has greatly reduced the incidence of the disease, but the existence of free-living virulent virus reservoirs like desi birds and pigeons poses constant threat to domestic poultry population. Very little information is available on epizootiological classification of Indian Newcastle Disease Virus (NDV) isolates. Velogenic NDV isolates collected over a period of 10 years on Monoclonal antibody (mAb) typing revealed a major homogenous cluster of 'C1' group. Others were 'E'&'L' groups consisting of virulent NDV isolates from healthy free-living desi birds. Monoclonal antibody typing was not efficient in grouping isolates into different clusters to correlate them epizootiologically. Hence this study was undertaken to (i) further differentiate the homogeneously typed mAb group of virulent isolates, (ii) obtain a database of Fusion protein gene fingerprints of NDV isolates from different parts of the country and (iii) to identify and shed light on the phylogenetic relationship of different clones of NDV in circulation/existence in India.

METHODS: RT-PCR for part of the Fusion protein gene encompassing the clevage site was carried out for 16 field isolates obtained over a period of 11 years, from different parts of the country and common vaccine strains used in India. Restriction Fragment length polymorphism (RFLP) was carried out with restriction enzymes Hinf I, Hae III, Bst OI, Rsa I, Taq I, and Mbo I. The Molecular weight of the obtained bands were estimated using Kodak 1D image analysis software. Using Jaccard's distance coefficient the distance measures were calculated and analyzed with the software MEGA, to obtain Phylogenetic trees using UPGMA and Neighbour joining methods.

RESULTS: Homogenous 'C1' mAb grouping of 7 virulent NDV isolates were differentiated into two different clusters comprising of 6 different groups. The restriction enzymes Hinf I, Hae III, Bst OI, Rsa I, Taq I and Mbo I differentiated the NDV isolates and vaccine strains into 6, 5, 4, 3, 3 & 3 groups respectively. Vaccine strains studied grouped together in a single cluster, with LaSota and B1 of mAb group 'E' grouping similarly. NDV isolates from the period 1988-90 were found to form the ancestral group and re-emergence of similar strains

after a period of three years is observed.

CONCLUSIONS: PCR- RFLP analysis of the Fusion gene was found effective in differentiating even the homogenous mAb typed 'C1' group and also differentiating the vaccine strains from that of virulent field isolates. Existence and circulation of atleast 6 different clones of NDV in India is suggested. Re-emergence of virulent NDV clones after a period of three years suggests the maintenance of the same in free-living birds and re-infection of the domestic population. Identification of new Fusion protein genotypes with unique pattern different from that already published, puts Indian isolates for the first time in the Global Phylogeny map of NDV.

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