Isolation, characterization of Ranikhet Disease (RD) virus along with seroepidemiological study in West Bengal

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In
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By

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WEST BENGAL UNIVERSITY OF ANIMAL AND FISHERY SCIENCES

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CERTIFICATE

This is to certify that the thesis entitled ***"Isolation, Characterization of Ranikhet Disease (RD) virus along with seroepidemiological study in West Bengal"*** *submitted by* ***Dr. Ujjwal Biswas****, in partial fulfillments of the requirements for the* ***'Degree of Doctor of Philosophy in Veterinary Epidemiology and Preventive Medicine'*** *of the West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my personal supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma.*

The assistance and help received during the course of investigation have been duly acknowledged.

Dated, Kolkata.

The 29th Day of Dec: 2006

Chanchal Guha

(Prof. Chanchal Guha)
Chairman,
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Dedicated to
My Family

APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

We, the undersigned having been satisfied with the performance of Ujjwal Biswas in the viva-voce Examination, conducted today, the 8th March.....2007, recommend that the thesis be accepted for the award of the Degree of Doctor of Philosophy in Veterinary Epidemiology and Preventive Medicine.

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(Dr. Ujjwal Biswas)

ABBREVIATIONS

EID_{50}	:50% embryo infected dose
FAO	:Food and Agriculture Organization
gm	:Gram
HA	:Haemagglutination
HI	:Haemagglutination Inhibition
hr.	:Hour
ICPI	:Intra Cerebral Pathogenicity Index
IVPI	:Intra Venous Pathogenicity Index
Min	:Minute
ml	:Milliliter
MDT	:Mean Death Time
ND	:Newcastle Disease
NDV	:Newcastle Disease Virus
NS	:Normal Saline
OIE	:Office of International des Epizooties
PBS	:Phosphate Buffer Saline
PPLO	:Pleuropneumonia like Organism
RD	:Ranikhet Disease
SPF	:Specific Pathogen Free
VVNDV	:Viscerotropic Velogenic Newcastle Disease Virus
µl	:Microliter
µm	:Micrometer
%	:Percentage
^0c	:Degree Centigrade

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Chapter – 1

Introduction

INTRODUCTION

The poultry industry has formed a major component of livestock industry in West Bengal. There are three types of poultry farms existing viz. commercial, small holder and backyard types. The bulk of production (80%) of poultry meat and egg comes from small subsistence farms (small holder and backyard types) rather than large commercial operation (Roy, 2005). This backyard system of poultry farming has been practiced for centuries among the rural families (Leong and Jalaludin, 1982) and it is essentially a no cash operation. The birds are generally owned and managed by women and children and are often essential elements of female headed and poor household (Alders and Spradbrow, 2001). This traditional system of poultry production will continue to form a substantial portion of poultry farming in south-east Asia.

In West Bengal, poultry farming is very successful, because there are about 20 thousand small poultry farms which are directly employment provider to 20 thousand families in rural sector. Out of which 20% is being in organized sector, providing input like day-old chicks, poultry feed, medicines, vaccines and are also involved in marketing of chicken which are essential and nearly one lakh economically weaker section people in the state of West Bengal, are directly employed (Roy, 2005).

In this context, it is worth mentioning that the total poultry population in West Bengal is 385.5 lakhs as per the census 2005 in comparison to India's poultry population of 12,000 lakhs. Growth of

poultry industry is 8% in West Bengal in comparison to 9.09% in the Country and this supports the states economic turn over of Rs.800 crores in 2005 (Roy, 2005).

The chicken are slow growing therefore, health protection of the chicken is very important. However, due to unconfined type of management, disease control is difficult. The main losses are due to New- castle disease which can result in 90-100% mortality in individual affected flocks.

Newcastle disease is included in list A diseases of OIE (Alexander, 2000). This highly fatal devastating viral disease is endemic in tropical and subtropical countries and affecting developing poultry production (Spradbrow, 1990). As per the report of FAO, about 50% countries of Asia, there is prevalence of velogenic strains of ND virus. On world scale, about 20% of countries have velogenic strains of ND virus. ND, specially the velogenic form is an extremely important problem of village chicken in the tropical countries (Spradbrow, 1999; Aini, 1990), where the New- castle disease is enzootic, outbreak of the disease regularly result in mortality of 50-100% and it causes highest economic losses by death and loss of production. In India, the ND is known as Ranikhet disease. This threatening ND problem in poultry rearing in India and in parts of South-East Asian countries can be controlled by:

(a) Either prevent by the susceptible birds from becoming infected and

(b) To reduce the number of susceptible birds.

These objectives can be achieved by strict bio-security measures and vaccination.

Vaccination is a most effective means of controlling ND and has been used throughout the world since 1940 (Beard and Hanson, 1984). In 1955, the National Veterinary Research Institute (INVRI) in Maputo, Mozambique commenced production of live, attenuated ND

vaccine and has produced vaccines based on strain F, B1, LaSota and Koumarov.

Now a days, various pharmaceutical private agencies and State Biologicals are producing ND vaccines in India, consisting of live attenuated LaSota strain, B_1 strain, F-strain (originated from Weybridge) and inactivated R_2B strain. These vaccines are used mainly in the commercial poultry sectors (Intensive poultry farms) and have limited applications in rural area (Extensive production system) due to problems of heat lability of vaccine strain of viruses large dose presentation, affordability, cold chain for effective administration of the vaccine and ignorance of the farmers. Moreover, in areas where ND is endemic, disease control through vaccination is greatly cost effective intervention and has been given a high priority by farmers. Yet such measures appear not to be effective in many cases as frequent report of ND outbreak in vaccinated flocks are all over the state. Village chicken farmers are disheartened by the loss of large number of their birds due to ND outbreak that often occur on an annual basis. If the losses caused by ND, can be controlled by any means, farmers will be most benefitted and concerned to improve poultry husbandry further.

Therefore, to prevent such type of economic loss by sudden outbreak of ND, it is indeed to develop a simple but absolutely effective vaccine against ND.

In view of the above facts, the present programme was considered on the basis of the following objectives:

1. Sero-epidemiological studies of ND outbreaks in West Bengal.
2. Isolation and identification of ND virus from different outbreaks in West Bengal.
3. Serotyping of the isolate as per the standard methods.
4. Attempt will be made to develop a potent improved attenuated vaccine.

Chapter – 2

Review of Literature

REVIEW OF LITERATURE

2.1. History and distribution of Newcastle Disease (ND)

In 1926, Newcastle disease (ND) was recognized in two widely separated locations: Java, Indonesia and Newcastle upon Tyne, England (Alexander, 1997). In 1927, ND was reported in Ranikhet in India. Today ND is prevalent all over India.

There are three theories on the origin of ND virus (Alexander 1988). The first possibility is that a major mutation of a precursor virus of low virulence took place which resulted the appearance of virulent ND virus. The second is that the disease was present in poultry in South East Asia for a long time , but because it affected only poultry raised at a village level, it had little or no attention and it was only with the development of large scale poultry operations the disease with enormous economic losses was noticed. The third possibility could be that ND virus was present as enzootic in species other than poultry and it was only when these species and poultry birds were brought together by chance, the disease emerged.

2.2. Prevalence of ND strain and outbreak in village chicken.

Nguyen (1982) reported that viscerotropic form of ND outbreaks were most frequent during the winter season in Vietnam and the most common source of infection was through diseased birds sold at village markets.

Chowdhury *et al.* (1982) collected 662 samples from suspected bird for Newcastle disease from 19 districts in Bangladesh. Out of

these 67.4% yielded NDV. In total 150 isolates were taken for characterization and 120(80%) found to be velogenic by MDT.

Ezeokoli *et al.* (1984) reported that in backyard management systems unvaccinated birds around 16-24 weeks of age (i.e. grower) had the highest risk of NDV infection than unvaccinated adults and chicks.

Adu *et al.* (1985) reported that in Nigeria, velogenic strains of NDV were prevalent in traditionally managed poultry.

Bell and moulodi (1988) conducted a study in Morocco with 100 suspected village chickens. NDV could be isolated from 41 samples, 12 isolates were characterized and all were found velogenic.

Mishra (1992) reported that Seasonal influence on the incidence rate and severity of Newcastle disease outbreaks in Nepal to be higher in summer compared to other seasons and it was observed that ND causes 90% mortality with great economic losses estimated at 74.77 million NRs (Nepal Rupees) in 1990 and all Pathotype of NDV were present.

Musiime (1992) also reported that ND infections in Kenya were more common during cold and dry periods.

Martin, (1992) reported that velogenic NDV was responsible for the majority of ND outbreaks in village poultry.

Asadullah (1992) reported that ND outbreaks in Bangladesh occurred throughout the year with a peak during the winter (Nov-Feb).

Barman (2002) reported that number of ND outbreaks were higher in Bangladesh during winter (Nov-Jan) compared to the dry season (Aug- Oct). The low temperature during the winter could influence the disease occurrence i.e. the survivability of ND virus in cold and humid condition was more compared to the dry season.

Barman (2002) examined 24 ND suspected samples in Bangladesh and out of these 8 samples (33.33%) were found positive.

Table 1. Showing epidemiological observation on Ranikhet disease in West Bengal, India

YEAR	No of outbreaks	Attack	Death	CFR (%)	Morbidity (%)	Mortality (%)
2001-02	205	24668	12913	52.35	16.40	8.59
2002-03	218	27407	13089	47.76	16.75	8.00
2003-04	306	33057	13986	42.31	13.99	5.92
2004-05	272	30016	15073	50.22	14.25	7.16
2005-06	400	27416	9987	36.43	10.50	3.82

Annual report, Epidemiological Unit, Directorate of Animal Resource and Animal Health, Govt. of West Bengal, IAH & VB, Kolkata-37.

Table 2. Showing antibody titre of poultry serum collected from different farms of West Bengal estimated by HI test (two folds dilution).

Year	Number of farm	Total number of samples tested	Total /Percentage of serum sample showing HI test		
			≥ 1:64 (2^6) good protective titre	1:32 (2^5) satisfactory titre	≤1:16 (2^4) Poor protective titre
2002-03	Non-vaccinated field sample	208	28 (13.5%)	-	180 (86.5%)
	Vaccinated field sample	458	129 (28.1%)	271 (59.2%)	58 (12.6%)
2003-04	21 (vaccinated)	1504	1044 (69.61%)	254 (16.89%)	206 (13.7%)
2004-05	31 (vaccinated)	1978	1437 (72.65%)	313 (15.82%)	228 (11.53%)
2005-06	30 (vaccinated)	1914	1516 (79.21%)	143 (7.47%)	255 (13.32%)

- 9-12% of birds per flock were tested.
- The reason of the titre may be due to intercurrent disease, immunosuppressive factors such as IBD infection and other concomitant infection, Mycoplasma infection as well as due to presence of mycotoxin in the feed after from this, some error in management practice may be one of the contributory factors for low titre.

Annual Activity Report,
R.D. immune Status Studies Laboratory, IAH & VB (R&T),
Directorate of Animal Resource and Animal Health,
Govt. of West Bengal.

Biswas *et al.* (2006) conducted a cross - sectional survey to asses immune status of 471 Sonali- chicken (age above 2 months) in Bangladesh and reported that 7.86% chicks had shown protective level of titre (2^5), 44.16% had good protective titre ($\geq 2^6$) and 47.98% had below protective titre ($\leq 2^4$).

2.3. Isolation and Pathotyping of Newcastle disease virus

Viet, M. H. (1978) isolated 36 ND viruses from 134 samples from sick chicken in Vietnam and all were viscerotropic velogenic. Among those one (VN 91) had the MDT value 37 hrs, ICPI value 1.77 and IVPI value 2.67.

Danials *et al.* (1987) isolated 23 Newcastle disease virus from different outbreak in Indonesia out of which19 were velogenic, 2 mesogenic and 3 lentogenic pathotypes determined by pathogenecity tests i.e. ICPI, MDT and IVPI.

Alexander (1989) described the correlation between the pathotype and pathogenicity indices of ND virus in the following manner:

Table 3.Showing correlation between the pathotype and pathogenicity indices of ND virus

Pathotype	Range of Indices			Example of virus
	MDT(hrs)	ICPI	IVPI	
Viscerotropic velogenic	<60	1.5-2.0	2.0-3.0	Hests33 NY, Parrof 70181
Neurotropic velogenic	<60	1.5-2.0	2.0-3.0	Texas GB
Mesogenic	60-90	1.0-1.5	0.0-0.5	Roakin, Mukteshwar
Lentogenic	>90	0.2-0.5	0.0	B_1, LaSota, F strain
Asymptomatic	>90	0.0-0.2	0.0	Ulster2c, V_4

Kumanan *et al.* (1992) isolated 9 ND viruses from ND outbreaks of different farms in Tamilnadu, India and characterized their pathogenecities and found to be highly virulent for chickens

Raghavan *et al* (1998) isolated 11 Newcastle disease viruses from apparently ailing desi chicken and characterized 10 isolates as velogenic and one was mesogenic

OIE (2000) also recommended that the ND virus having an ICPI value in day old chicks 0.7 or greater is to be considered as virulent.

Roy *et al.* (2000) isolated 5 Newcastle disease viruses from different farms of Tamilnadu, India and all isolates were characterized as velogenic on the basic of MDT, ICPI and IVPI. All the viruses were stable at 56^0c, haemagglutination of equine erythrocyte and its reaction with a panel of monoclonal antibodies.

Howard (2000) carried out a survey in Newzeland from 1976 to 1995 on poultry bird and isolated 4 positive cases of Paramyxovirus-1 having the ICPI value 0.0.

Mishra *et al.* (2000) isolated viscerotropic velogenic strain based on pathogenecity index from an outbreak in Rajasthan, India.

Cattoli *et al.* (2001) isolated 32 Newcastle disease virus from the 2000 Italian epidemic and characterized by monoclonal antibody binding pattern and nucleotide sequencing. The pathogenecity of six of these isolates was assessed by means of the ICPI, exhibited ranging from 1.6 to 2.0.

Clavijo *et al.* (2001) reported that one mesogenic strain Newcastle disease virus was isolated in 1999 from an outbreak in Canada showing the symptom of leg and wing paralysis and more than 100 birds died..

Mishra *et al.* (2001) isolated Newcastle disease virus from chicken and guinea fowls and characterized them as viscerotropic velogenic on the basis of the MDT, ICPI, IVPI, cloacal and conjunctival mean death time.

Yu *et al.* (2001) isolated Newcastle disease virus from 6 outbreaks in chicken and characterized genotypically and found that 4 isolates were viscerotropic velogenic, one neurotropic velogenic and one mesogenic.

Liu *et al.* (2003) isolated 29 New castle disease viruses from outbreaks in chicken and goose flocks in several region of China during 1998-2001 and viruses were characterized pathotypically and genotypic-ally. All except one of those strains were velogenic.

Li and Zhang (2004) reported isolation of 15 ND virus in China and characterized them. Out of which, 5 were vaccine strain (lentogenic Pathotype), 3 were mesogenic and 7 were velogenic Pathotype.

2.4. Newcastle Disease vaccine

Commission of the European Communities (1993) clearly and strictly had given the recommendation that for preparation of live attenuated vaccine master seed must have an ICPI value of < 0.4 if EID50 no fewer than 10^7 or ICPI value < 0.5 of EID50 no fewer than 10^8 .

Aini, *et al.* (1986) selected V_4 strain (having the property of non-pathogenecity, thermostable, immunogenecity and naturally transmissible among surrounding chicken) for preparation of vaccine (V_4 UMP). Twenty vials containing the V_4 New Castle Disease virus was thawed into ice bath and kept into water bath at $56^0 \pm 0.5^0$c. At various intervals two vials were removed from the water bath, the contains pooled and the infectivity determined by inoculating 0.1ml of virus (allantoic fluid) into 5, 10 days old SPF chicken eggs. Four days after inoculation the eggs were chilled at 4^0c for four hours, the allantoic fluid was harvested and the viral haemagglutinin was detected by the Haemagglutination (HA) test. The HA positive allantoic fluid containing virus that survived the longest period at 56 ± 0.5^0c was collected, pooled and centrifuged for 30 minutes at 2000 rpm. The supernatant was collected, filtered using at 0.45 micrometer Millipore filter, dispensed in 1 ml glass vial and kept at -20^0c. The process was repeated several times until the virus that survived at 50^0c for 9 hours that was obtained as stock virus, had a titre of 10^6 EID_{50}/0.1 ml and was designed as V_4-UPM.

Seang (1987) clearly describe that prior to 1982, the 'F' strain uncloned vaccine seed was passaged from time to time in non-specific pathogen free eggs without a proper seed lot system for vaccine production. In 1982 a new seed was cloned from the existing seed by limiting dilution and further plaque purified. A master seed lot and a working seed lot were produced in SPF eggs. Both master seed lot and working seed lot were characterized and tested in-vitro and in-vivo and also subjected to different purity test before being freeze dried for storage and subsequent production.

Seang (1987) described that during production of vaccine 10^4 EID_{50}/0.1 ml of virus was inoculated into 9-10 days embryonated eggs via intra allantoic route and incubated and harvested after 96 hours post inoculation and one night after chilling at 4^0c. The vaccine should be sealed in ampoules frozen at -20^0c for storing or freeze dried

in vials with 5%lactose and 0.1% polyvinylpyrilidon (PVP) m.w $7x10^5$ as stabilizer in equal volume.

Spradbrow (1992) recommended V_4 strain (a lentogenic strain of Newcastle disease having the property of immunogenecity, thermostability, avirulent and transmissibility) for preparation of a live vaccine coated on food, fed to scavenging chicken but local factor extremely important in affecting their success.

The Commission of the European Communities (1993) has decided (93/152/EE) that the master seed for live vaccine would be tested and should have to show ICPI of <0.4 if no fewer than 10^7 mean Egg Infective dose (EID_{50}) are administered to each bird, or less than 0.5 if no fewer than 10^8 EID_{50} are administered to each bird and for inactivated vaccine ICPI <0.7 if no fewer than 10^8 EID_{50} are administered to each bird.

The Council of Europe (1997) and OIE (2004) recommended the safety test for production of live vaccine as follows: The use of day old chicks for the testing of vaccine involves the inoculation of 10 or more originated from SPF flock. Ten doses of live vaccine should be administered supra-conjunctivally to each chick and then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine.

The Council of Europe (1997) and OIE (2004) recommended on the potency test of Newcastle disease vaccine. The methods involved the vaccination of 20 day old SPF chicken using the minimum recommended dose (10^3 to 10^4 EID_{50}/0.1ml of virus per bird). After 14 -21 days each vaccinated bird and 10 control birds should be challenged with 10^5 LD_{50} of ND challenge virus. The vaccine is passed if at the end of 10 days, 90% of the vaccinated chicken survived with no signs of disease but all the controls should die within 6 days.

Seang (1987) recommended a field dose of vaccine via normal route and observed for any noticeable reaction within 2 weeks and then challenged with $10^{6.5}$ EID_{50} of virulent virus. The vaccine would be passed if 90% challenged birds survived.

Barman (2002) mentioned the Newcastle disease virus along with ICPI and IVPI values used as live vaccine.

Table 4. Showing Newcastle disease virus along with ICPI and IVPI values used as live vaccine.

Virus	Pathotype	ICPI	IVPI
LaSota	Lentogenic	0.4	0.0
F strain (Asplin's)	Lentogenic	0.25	0.0
Hitchner B1	Lentogenic	0.20	0.0
V_4	Lentogenic	0.0	0.0

The OIE Standards Commission (2000) has similarly recommended for live vaccine that while in principle vaccine should have an ICPI <0.7 in order to account for interassay and inter laboratory variability a safety margin should be allowed so that vaccine master seed virus strains should not have an ICPI exceeding 0.4.

King (2001) concluded that field isolates with a more thermostable haemagglutinin could be derived by selection from the heterogeneous Newcastle disease virus population in vaccine strain and that minor antigenic changes may be a result of that selection.

Alders and Spradbrow (2001) recommended thermostable 1-2 strain vaccine for vaccinating village chickens to be given eye drop.

OIE (2004) also recommended 10^3 to 10^4 EID_{50}/0.1ml of virus working seed should be inoculated in to the allantoic cavity of 9-10 days old embryonated eggs and then incubated at 37°c. Eggs containing embryos that die within 24 hours should be discarded. The incubation time should depend on the virus strain being used and would be predetermined to ensure maximum yield with the minimum number of embryo deaths. The infected eggs should be chilled at 4°c for atleast 4 hours or over night before being harvested. The top of the eggs should be removed and the allantoic fluid aspirated after pushing

of the embryo down below. Fluid should be stored immediately at 4°c and tested for bacterial contamination before lyophilisaion of live vaccine.

OIE (2004) recommended that live virus of low virulence (lentogenic) or of moderate virulence (mesogenic) may be used for the vaccination of poultry depending on the disease situation. Within lentogenic group there is considerable range of virulence. Immunogenecity increase when the pathogenecity of live virus increase. Newcastle disease Asplin's "F" strain has originated from Wey Bridge, UK, 1953 and ND, Mukteswar strain was obtained from India in 1949 for vaccine production (Seang, 1987)

2.5. ND Vaccination dose, route and HI titre response

Schmidt and Schmidt (1955) studied the relationship between HI titre and protection capacity for a 10 month period after vaccination. They found that a variable percentage (8.5-95%) of birds having HI titre up to 16 failed to resist the challenge infection against virulent NDV and those having titres 32 and above resisted the challenge infection

Rao and Agarwal (1962) observed that day old chick immunized with F strain vaccine through intranasal route, induced protection against NDV infection 48 hours after vaccination and 100% of the birds survived challenge exposure of virulent NDV, 2, 3 and 4 month after primary vaccination. They suggested that a second vaccination performed I/M at 6 weeks after primary vaccination with Mukteswar strain would provide prolonged protection.

Shakour *et al.* (1971) evaluated routes of administration and virus concentration of B_1 vaccine. The B_1 vaccine in a dose $4x10^4$ EID_{50} was given to day-old chick in drinking water, induced 90-100% protection when birds were challenged 3 weeks later. A similar dose afforded 80% protection up to 7 weeks by intraocular administration. Intranasal administration of 10^3 EID_{50} protected 70% of the chicks for 4 weeks and 50% for 8 weeks. They also reported that HI antibodies

were detected in serum (max Titre 1:64) from the 9th day after vaccination. A gradual fall in the titers was noticed from 4th week and no antibodies were demonstrated at 11th week.

Vindevogel *et al.* (1975) made a comparative study on the immunity conferred in 6 day old chicks, carrying maternal antibodies, by using beak dipping and through drinking water with B_1 strain or lasota strain (Live lentogenic strain).HI titre were determined at weekly intervals in chick for 8 weeks. Twenty birds were challenged by intranasal inoculation with virulent virus. Mortality on the 7th day was higher among chicks vaccinated through drinking water than by beak dipping. Vaccination by beak dipping protected from the 7th day to the 8th week whereas vaccination through drinking water protected from the 14th day. The total mortality of all chicks challenged was higher among those vaccinated with B_1 strain compared to lasota.

Eidson *et al.* (1976) studied B_1 and Lasota vaccine strain applied intratracheally or by spray in different age group of chicks possessing varying levels of maternal antibodies. A satisfactory antibody response was induced in a group of day old chicks and in most cases they were resisted to challenge with Texas GB strain. All groups of spray vaccinated chicks showed a marked increase in antibody titre following vaccination at one day of age. The highest serum antibody titre was achieved most rapidly by spray method and by drinking water method gave the lowest titre after the great delay.

Semov *et al.* (1976) observed that administration of Lasota vaccine to broiler chicks by the spray method on the 4th day and twice in the drinking water on the 21st day of life, produced better immunity against ND than single administration in the drinking water; a period during which the decline of maternal antibodies in unvaccinated chicks made these susceptible to ND.

Satyanarayan *et al.* (1977) reported that maternal antibodies were insufficient to give protection against ND beyond 48 hrs of hatching. Immunization of chicks at the age of 3rd day, 5th day and 7th day by nasal route with F strain vaccine gave protection even at the

end of the 1st week without showing significant rise in HI titre, probably due to the interference phenomenon by F strain virus rather than specific antibodies. Chicks of all three groups survived till the end of 8th week, despite of low titre.

Chulan *et al.* (1982) reported that 2000 chickens vaccinated with lyophilized ND F vaccine at 1,21 and 42 days showed sufficient protection when challenged at day 14,35, 56 and 70 days with virulent strain of Newcastle Disease virus administered intramuscularly, intranasally or by contact A high percentage of vaccinated birds were protected even though antibody levels were persistently low.

Ronohardjo (1984) estimated maternal immunity (antibody) in 300 day old chicken from 15 commercial hatcheries at the age of 2 and 6 days and at 2, 4, 5, and 6 weeks where the parent flocks had received intensive vaccination. The antibodies were high (58%) in initial stage (2 days of age) and 42% had no detectable antibodies and undetectable at 4 week of age in all chicken.

Saifuddin *et al.* (1986) reported that the intra ocular vaccination in day old chickens with B_1 vaccine followed by re-vaccination with Komarov strain by I/M route at 6th to 7th week of age provided a safe and efficient programme of vaccination and the administration of vaccine should be repeated with a minimum interval of 7to 8 month.

Aini *et al.* (1987) vaccinated 100 chicken at the age of 3 weeks and 6 weeks with food pellet vaccine orally (V_4 – UPM vaccine) at the rate of 10gm of feed per bird (10 gm feed containing 10^6 EID_{50} of the virus). Birds were challenged with viscerotropic velogenic New Castle Disease virus designated AF2240-220 (Chulan *et al,* 1982) with 10^6 EID_{50} of the virus intramuscularly. About 90% of the chicken vaccinated with the pellet ND vaccine were protected against challenge with the virulent NDV.

Ibrahim *et al.* (1987) calculated a dose for chicken was 10 gm of the pelletted vaccine containing an equivalent of the 10^6 EID_{50} of the vaccine virus and repeated the same dose at 3 weeks interval. The

efficiency of the vaccine evaluated either by monitoring the incidence of ND in the vaccinated flocks or by challenging the vaccinated chickens with the virulent NDV 3 weeks after the second vaccination. They also reported that 60% of the vaccinated chickens were protected when challenged at the laboratory with the virulent NDV and there was no incidence of ND in the flocks where the trial was conducted for a period of atleast one year.

Satter *et al.* (1988) reported that vaccination through subcutaneous route of day old chick with Lasota, Komarov and Mukteswar strain gave 100% protection against challenge at day 23rd while these vaccine strains in drinking water gave 94.3%, 99.6% and 94.3% protection respectively.

Biswas *et al.* (1996) made a comperative study on the protection of indigenous chickens against Newcastle Disease induced by Australian NDV_4-HR and local convenient vaccine (F- strain and Mukteswar strain). Blood samples were collected at interval from all the chicken and tested by HI test. The conventional vaccine apparently conferred higher protection in the birds (93-94%) than NDV_4-HR vaccine (provided 67-88%). NDV_4-HR administered in drinking water and ocular method provided better protection (86-88%) than the other roles.

Ullah (2001) reported that oral vaccination of day old chicks with B_1 and F strain and revaccination with Komarov and Mukteswar strain at 8 weeks of age provided a safe and efficient vaccination.

Rahman (2002) reported an experiment with Newcastle vaccine in two different sub-sites with two different treatments. Treatment 1 was vaccinated at day 10 and day 24 with live F strain vaccine, at day 31 (half dose) and at day 120 (full dose with inactivated vaccine). Treatment 2 was vaccinated at day 10, 24, 60, with live F strain vaccine and day 120 with inactivated vaccine. Blood samples were collected at day 10, 24, 31,60, 120 and 150 days for HI antibody titre and found that treatment-1 provided better humoral response than treatment-2.

2.6. CHALLENGE

Aini *et al.* (1987) randomly selected 200 chicks in two groups i.e. 100 chicks in each group. Group-I was vaccinated with food pellet vaccine at the age of 3 weeks and 6 weeks and group-II was unvaccinated control. Both groups were challenged at the age of 8 weeks with VVNDV (designated 1 pol AF 2240-220). Ten chickens from each group were infected intramuscularly by inoculating each bird with 10^6 EID_{50} of the virus. Contact challenge was accomplished by allowing 10 chickens from each group to mingle in the same room with unvaccinated chickens which had been challenged either intramuscularly or by contact were kept in two separate isolation unit. All chickens were observed for 14 days post challenge.

Spradbrow (1987) randomly selected 35 days old chicken in 2 groups. Group-I having (vaccinated group) control 20 chickens and group-II having 15 chickens (10 controls and 5 donors) i.e. non-vaccinated. At the day 21, 20 chickens of Group-I vaccinated with 10^6 EID_{50} dose of virus and revaccinated on 35 days. At 49days, 5 donor chickens were inoculated with lethal dose of local NDV and combined group-I & II vaccinated control and donor in one pen. Donor group acted as a source of challenge and observed up to 63 days for ND.

Barman (2002) randomly selected 92 birds into three groups i.e. Group-I (30 birds vaccinated with local vaccine), Group-II (30 birds vaccinated with imported vaccine) and Group-III (32 birds unvaccinated and challenged all birds with 1 ml /bird of field isolated NDV velogenic strains containing dose 10^5 EID_{50}/ml ten dose through intramuscular route. All the chickens were observed for 14 days for any death.

Chapter – 3

Materials and Methods

MATERIALS AND METHODS

3.1. Seroprevalence of Newcastle disease in affected and non-affected flock:

Seroprevalence study was undertaken to assess the presence of antibodies against Newcastle disease virus in different districts of West Bengal during the period from April, 2004 to March, 2006. In the present study, antibodies against Newcastle disease was determined by employing Haemagglutination Inhibition (HI) test (OIE, 2004).

3.1.1. Collection of serum sample

One thousand eight hundred fifty five serum samples were collected from 259 broiler flocks / farms which were suspected for Newcastle disease. All birds of the affected flocks were vaccinated at the age of 5-7 days with either F-strain or LaSota strain vaccine intranasally. Out of 259 broiler farms, 147 were single flock rearing farm (all-in and all-out rearing system) and 112 were multi-flock rearing farms. Five serum samples from each affected flocks (Total 1295 samples) and 5 samples from other non-affected flocks (Total 560 samples) were collected for sero-surveillance study.

3.2. Prevalence of Newcastle Disease in West Bengal

Studies on prevalence of Newcastle disease outbreak was undertaken in broiler farms and free range rearing flocks (backyard) which were clinically infected with the specific causative agent i.e. Paramyxovirus type-1 along with morbidity and mortality pattern during the period throughout the state of West Bengal. Tissue samples

were collected from dead / live infected birds and stored at -20°C before being used.

3.3. Isolation of Newcastle Disease virus

3.3.1. Collection of sample

Three hundred and eighty five samples were collected from 259 broiler flocks and 126 from free range rearing on the basis of clinical sign and macroscopic lesions, in West Bengal, during the period from April 2004 to March 2006. Samples from recently dead birds or moribund birds containing lung, kidney, intestine, spleen, brain, liver, trachea and air sac as a pooled samples were collected aseptically during necropsy examination. Oro-nasal swabs from live or sick birds showing respiratory signs and nervous signs were also collected aseptically as per the recommendation of OIE, 2004.

3.3.2. Preparation of Inoculums

Tissue samples were homogenized using tissue grinder with the help of sterile glass wool. A 10% (w/v) suspension of tissue samples was prepared with sterile PBS (pH 7.2). Oro-nasal swabs were also transferred to sterile PBS. The suspension was clarified by centrifugation at 1000 g for 10 minutes. The supernatant fluid was collected and 50 microgram gentamycin per ml was added and kept for 30 minutes. Then the antibiotic treated suspension was passed through 0.45 micro meter membrane filter to make it free from bacteria and mycoplasma (OIE, 2004).

3.3.3. Virus culture in SPF embryonated eggs.

As per the recommendation of OIE 2004, 0.2 ml of filtrated (after centrifugation and filtration of tissue suspension) of each sample was inoculated into allantoic cavity of five 9th to 11th day old embryonated specific pathogen free chicken egg (SPF chicken eggs were procured from SPF Egg division of Venkey's India Ltd, Pune,

India) and incubated at 37°C without turning (Fig.1,). Those inoculated eggs were candled twice daily for embryopathy. Any embryo death within 24 hours of inoculation was assumed to be non-specific and discarded.

After 24 hrs incubation, dead embryonated SPF eggs were chilled at 4°C atleast for 4 hrs and allantoic fluids were collected aseptically for observing haemagglutination activity. Allantoic fluid which had given a negative reaction were further processed for another passage in SPF eggs and subsequently discarded.

3.4. Identification of Virus

As recommended by OIE, 2004, positive haemagglutination inhibition activity of infective allantoic fluid with known positive antisera of Newcastle disease (ND positive antiserum was procured from Division of standardization, IVRI, Izzatnagar,UP,India) confirm the diagnosis of Newcastle disease virus. As such haemagglutination test was performed as per the standard methods (OIE, 2004). For the titration, allantoic fluid was collected from each and every inoculated dead embryonated egg. Spot haemagglutination test was performed for positivity of all individual isolates of virus as per the standard method as stated above.

3.5. Pathotyping of the isolates of Newcastle disease virus

For confirmation of Newcastle disease outbreak, clinical signs, post mortem examination followed by isolation and serotyping was done. The virulence of the isolates were determined by following in-vivo tests i.e. Mean death time (MDT) (Fig. 2), Intracerebral pathogenecity index (ICPI) (Fig. 3), and Intravenous pathogenecity index (IVPI) as per the standard methods (OIE, 2004).

Twelve viral isolates were sent to OIE, FAO and National Reference Laboratory for Newcastle disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'

Fig. 1 Showing sample inoculation in embryonated SPF eggs for isolation of virus.

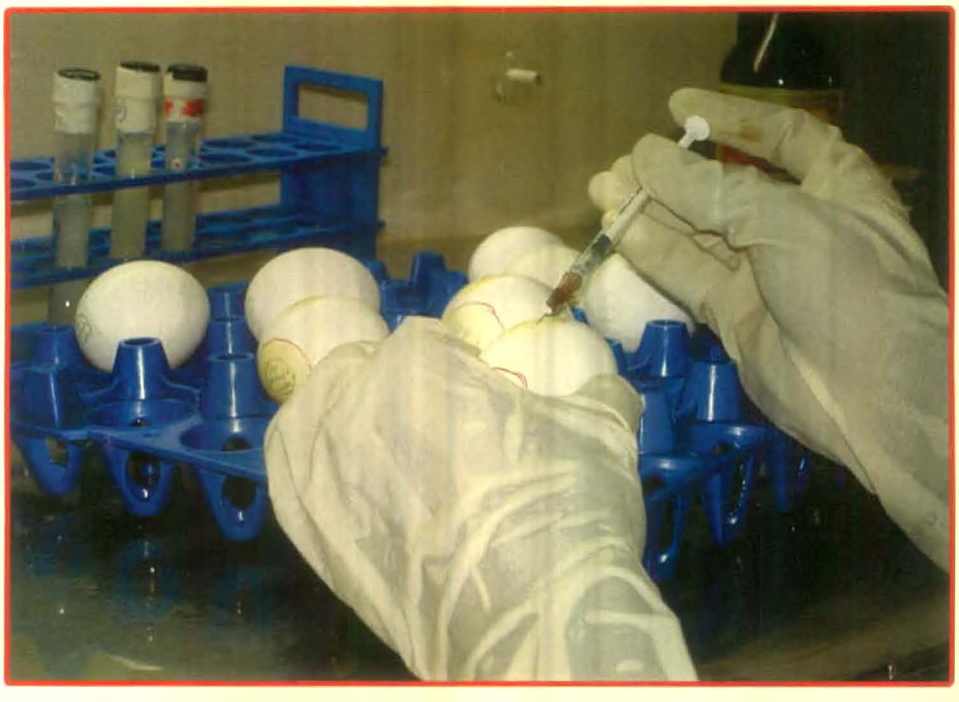

Fig. 2 Showing virus inoculation in embryonated SPF eggs for calculation of MDT.

Universitia, 10 - 35020, LEGNARO (PD), Italy for confirmation and characterization.

3.6. Determination of Embryo Infectious dose (EID_{50})

EID_{50} of the virus isolates were determined using 5th passages virus as test samples in 9th to 11th day old embryonated SPF chicken eggs (Fig. 4) as per the standard method (Reed and Muench, 1938).

3.7. Preparation of vaccine

3.7.1. Selection of seed for preparation of live attenuated vaccine.

Selection of virus (isolates) for preparation of live attenuated vaccine as per the recommendation of Commission of the European Communities (1993) and OIE (2000).

3.7.2. Preparation of modified (attenuated) live virus by serial passages from selected virus isolates.

All the isolates were serially passaged in the embryonated SPF eggs upto 15th passages and then allantoic fluids from the 15th passage harvest were given as nasal drop infection (Fig.5) with 10^6 EID_{50} of virus to 10 SPF chicks 5 days old and observed for 21 days for any clinical sign of illness. In case of any illness or death, they were further passaged upto 20th passages till it was found to cause no illness in any of the 10 chicks. The allantoic fluid of respective passage were checked for sterility, infectivity, safety, and potency and finally stored at -20°c after lyophilisaion.

3.7.2.1. Sterility

For confirmation of sterility of the isolated viruses, following tests were conducted.

Fig.3 Showing Intra-cerebral inoculation of virus for calculation of ICPI.

Fig. 4 Showing virus inoculation in embryonated SPF eggs for determination of EID_{50}

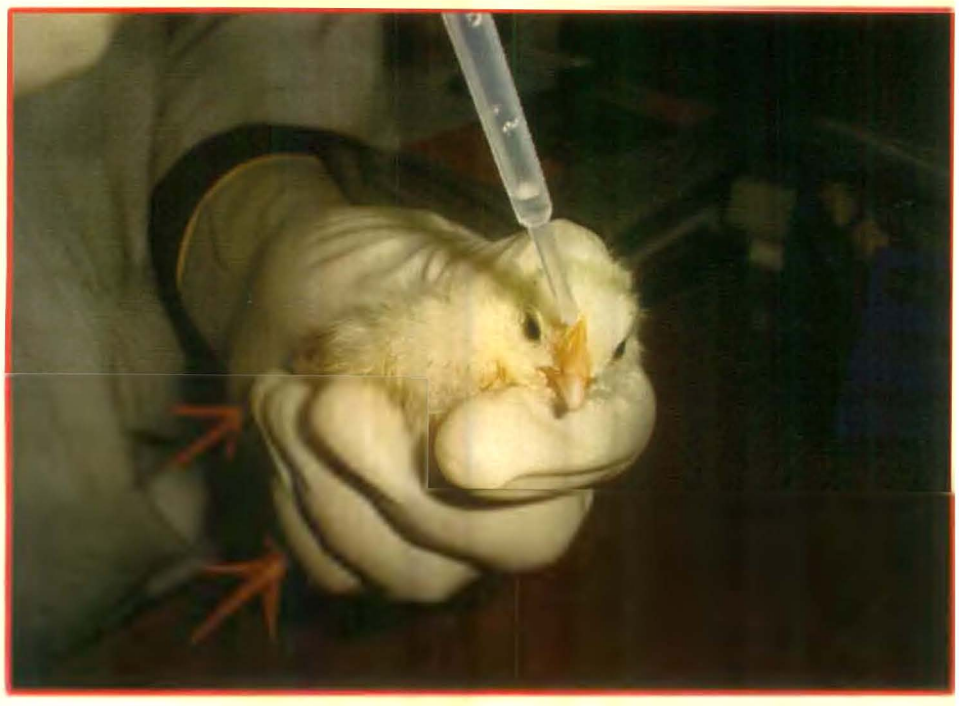

Fig. 5 Showing Intra-nasal inoculation of virus in 5 days old SPF Chicken.

3.7.2.1.1. Bacterial culture.

All the selected allantoic fluid containing virus were individually cultured in nutrient broth (Himedia laboratory, India) and incubated for 48 hours. Then transferred in nutrient agar aseptically and incubated for 24 hours at 37°C and examined for any bacterial growth

3.7.2.1.2. Fungal Culture

All the selected allantoic fluid containing virus cultured in Sabourauds dextrose agar and incubated at 37°C for 21 days and observed for any growth.

3.7.2.1.3. Mycoplasma culture

All the selected allantoic fluid containing virus were inoculated separately in PPLO agar (Himedia laboratory, India) and incubated at 37°C for 7 days for growth of any colony.

3.7.2.2. Infectivity

Infectivity of every isolates of virus was determined by haemagglutination activity i.e. standered haemagglutination test method as described earlier and the EID_{50} calculated (Reed and Muench, 1938), by inoculating serial tenfold dilutions of attenuated virus in 9-11 days old embryonated SPF chicken eggs.

3.7.2.3. Safety

Ten 5 days old Newcastle disease antibody free chicken (from SPF chicken eggs) were intranasally instillated with 10^7 EID_{50} of virus to chicken and observed for 21 days for any clinical disease or mortality as per the standered method (OIE, 2004 and Seang, 1987).

3.7.2.4. Potency

Ten, 5 days old Newcastle disease antibody free chicken were inoculated with 10^6 EID_{50} of each selected virus. After 21 days post inoculation, the antibody titre was determined. The chickens were

further inoculated with the same dose of virus and after 21 days of 2nd post- inoculation the antibody titre was once again determined.

At the same time, a control group of 10 chicks were reared separately as control. The antibody titre against Newcastle disease virus was determined at 26th and 47th days of age.

All 47th days old chicks (test and control group) were challenged through intramuscular route with 10^6 EID_{50} of velogenic virus per bird, (isolated from field cases, Barman, 2002). All the birds were kept under observation for next 21 days for detection of clinical disease if any.

Chapter – 4

Results and Discussion

RESULTS AND DISCUSSION

4.1. District wise outbreak of Newcastle Disease

District wise distribution of Newcastle disease outbreak was presented in Table 5 and Fig. 6 & 7 (Broiler) and Table 6 and Fig. 8 & 9 (Free range rearing birds). From the table it was evident that highest morbidity (56.74%) and mortality (38.00%) of broiler birds occurred of Murshidabad and Uttar Dinajpur Districts respectively and lowest morbidity (27.25%) and mortality (8.91) in Bankura and 24 Parganas (North) Districts respectively. The case fatality rate was highest (74.50%) in Dakhin Dinajpur and lowest (26.97%) in 24 Parganas (North) districts. The overall average morbidity, mortality and case fatality rates were 37.64%, 16.28% and 43.26% respectively in Newcastle disease affected district of West Bengal.

The highest morbidity (80.00%), mortality (64.00%) in the free range rearing birds was found in Nadia District and lowest morbidity (37.50%) in Hoogly Districts. The lowest mortality (33.33%) was found in three Districts viz. 24 Parganas (south), Hoogly and Bankura. The case fatality rate was highest (95.35%) in 24 Parganas (North) and lowest (83.33%) in Bankura District in the free range rearing birds. The average morbidity, mortality and case fatality rates were 55.39%, 47.37% and 85.52% respectively in all the district of West Bengal.

From the table-5 & 6 it was also found that the morbidity, mortality and case fatality rates were more in free range rearing flocks than the broiler flock; this was in accordance with Martin (1992), who reported that velogenic Newcastle disease virus was responsible for

Table 5. Showing district wise morbidity and mortality pattern of Newcastle disease out break of broiler birds.

Name of the District	Population at risk	Affected	death	CFR (%)	Morbidity (%)	Mortality (%)
Uttar Dinajpur	2450	1330	931	70.00	54.29	38.00
Dakshin Dinajpur	2800	1400	1043	74.50	50.00	37.25
Malda	8400	4396	2541	57.80	50.24	29.04
Murshidabad	6650	3773	1960	51.92	56.74	29.47
Nadia	35,700	10,122	3710	36.65	28.85	10.39
24- Parganas (N)	31,500	10,409	2807	26.97	30.04	8.91
24-Parganas(S)	9100	4620	2149	46.52	50.77	23.62
Howrah	1750	742	385	51.89	42.40	22.00
Hoogly	7000	2933	1498	51.07	41.90	21.40
Midnapur (east)	700	301	203	67.44	43.00	29.00
Bankura	2800	1043	441	42.28	27.25	15.75
Burdwan	3150	1267	630	49.72	40.22	20.00
Birbhum	1400	483	224	46.38	34.50	16.00
Total	**1,13,750**	**42,819**	**18,522**	**43.26**	**37.64**	**16.28**

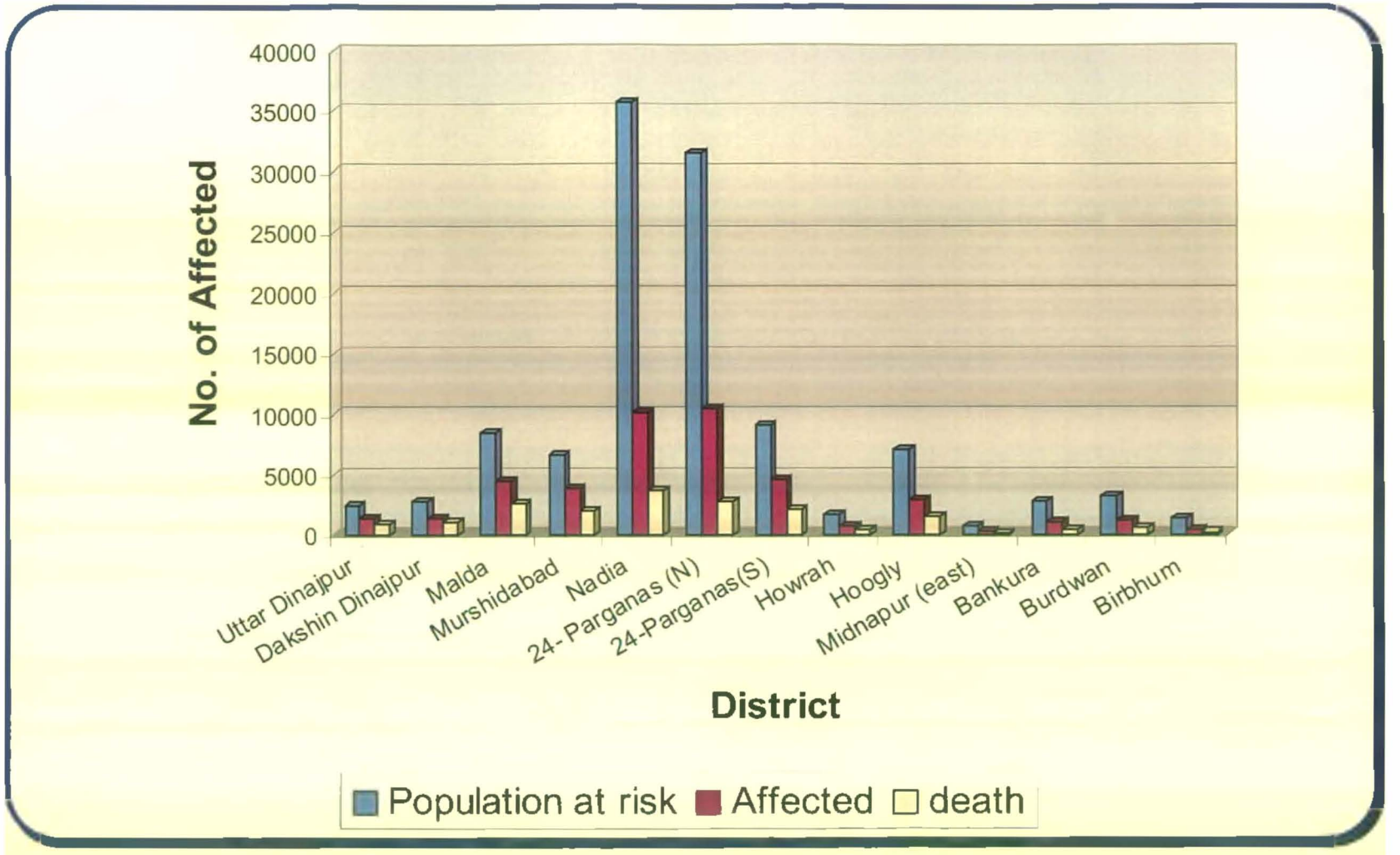

Fig. 6 Showing district-wise outbreak of New Castle Disease of broiler birds in West Bengal

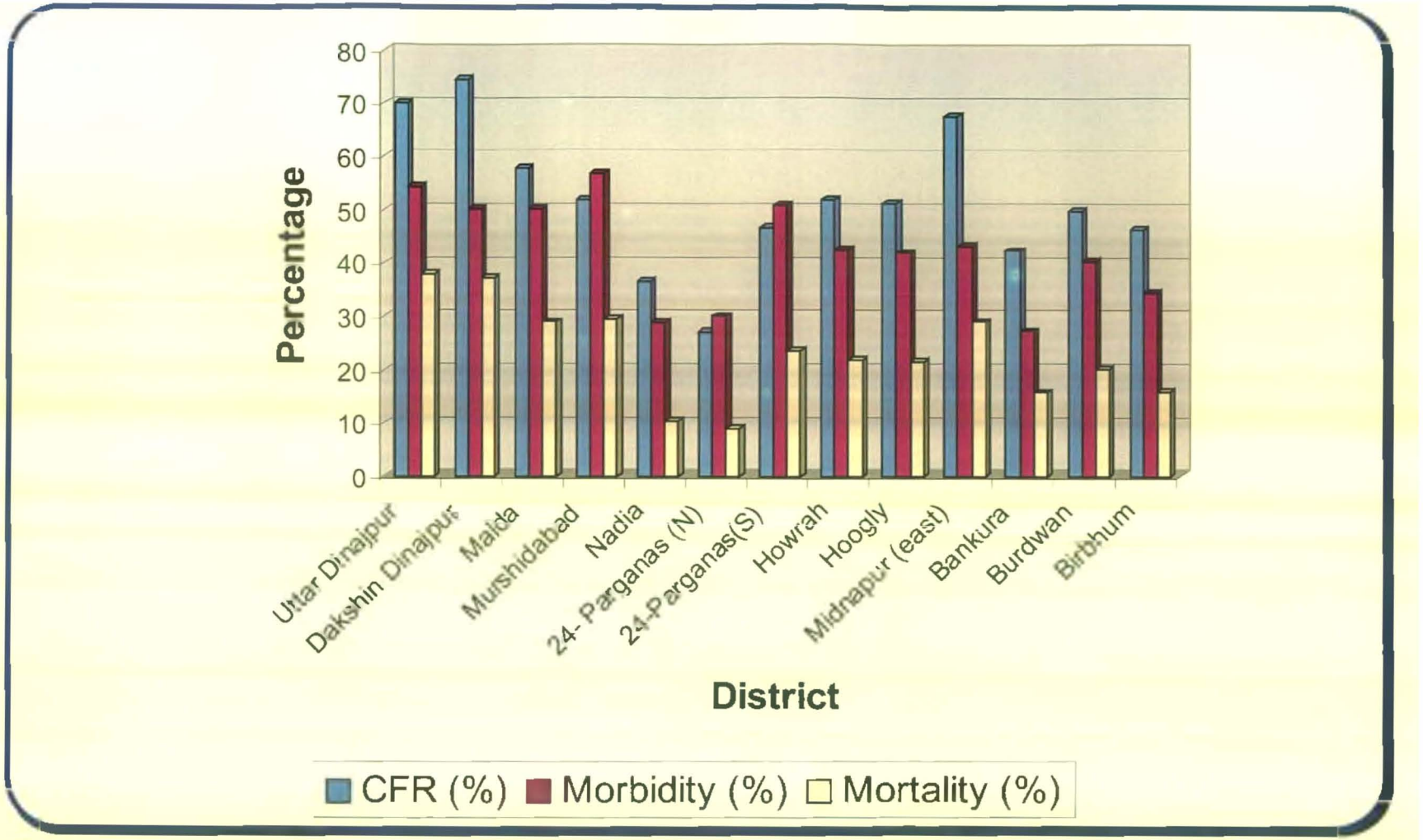

Fig. 7 Showing district-wise morbidity, mortality and Case Fatality Rate of New Castle Disease outbreak of broiler birds in West Bengal.

Table 6. Showing district wise morbidity and mortality pattern of Newcastle disease out break of free range rearing birds.

Name of the District	Population at risk	Affected	death	CFR (%)	Morbidity (%)	Mortality (%)
Murshidabad	539	273	231	84.62	50.65	42.86
Nadia	700	560	448	80.00	80.00	64.00
24 Parganas (North)	483	301	287	95.35	62.32	59.42
24 Parganas (South)	315	119	105	88.24	37.78	33.33
Hoogly	336	126	112	88.89	37.50	33.33
Bankura	420	168	140	83.33	40.00	33.33
Total	**2793**	**1547**	**1323**	**85.52**	**55.39**	**47.37**

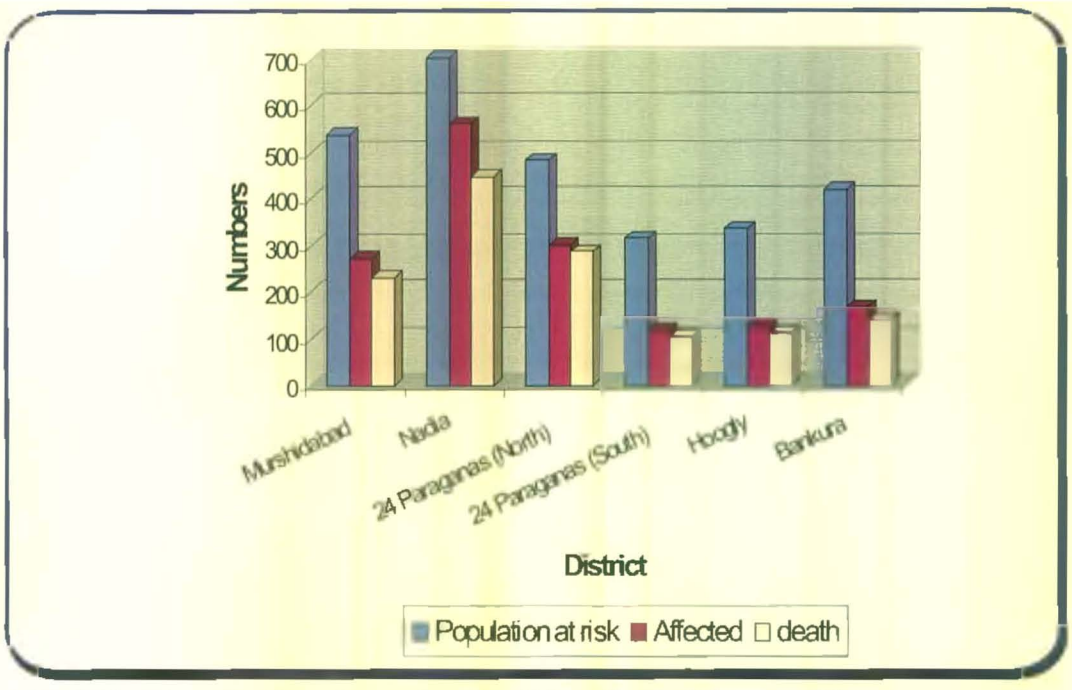

Fig. 8 Showing district-wise outbreak of New Castle Disease of free range rearing birds in West Bengal.

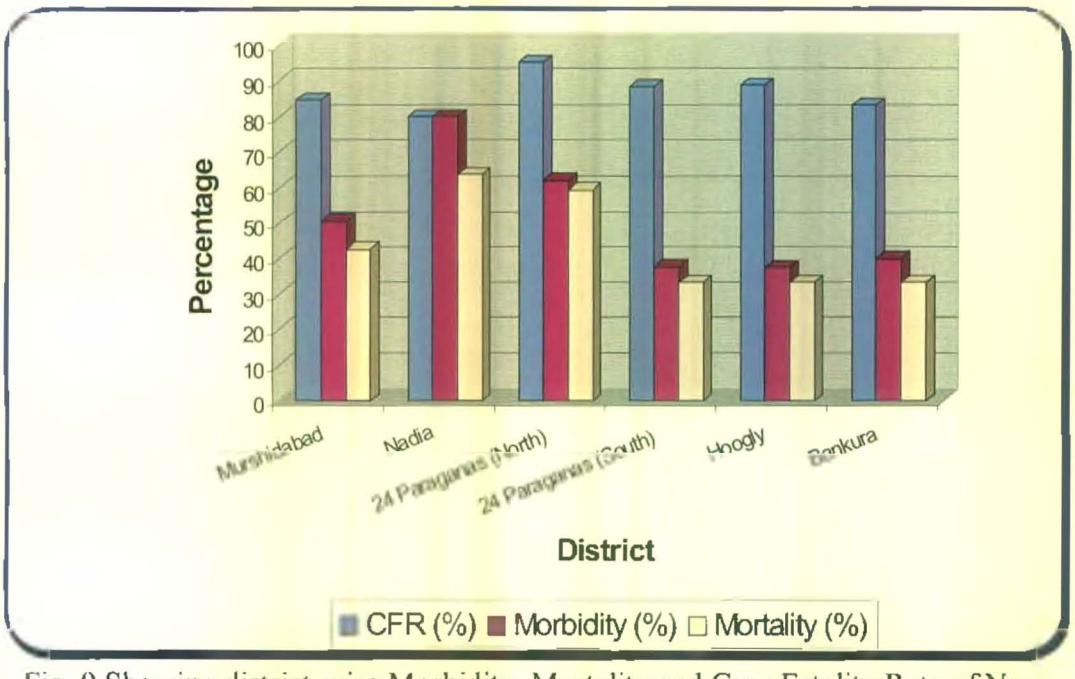

Fig. 9 Showing district-wise Morbidity, Mortality and Case Fatality Rate of New Castle Disease outbreak of free range rearing birds in West Bengal.

the majority of Newcastle disease outbreak in village poultry. Abu et al (1985) reported that velogenic strain of Newcastle disease viruses were prevalent in traditionally managed poultry. Chowdhury *et al.* (1982) reported that 80% of isolates in Bangladesh were found to be velogenic. As per the report of FAO, about half of the countries of Asia, there is high prevalence of velogenic strain of Newcastle disease virus.

During this study, out of 14 positive isolates from free range rearing flocks, 4 were characterized and found velogenic which were isolated from 24 Parganas(North) and Nadia districts. But from broiler chicken (70 isolates were positive), 20 isolates were characterized, out of which 8 were velogenic, 2 were mesogenic and 10 were lentogenic.

From the present study it was concluded that the morbidity, mortality, and case fatality rates were higher in free range rearing flocks compared to broiler flocks and which might be due to high prevalence of velogenic strain of Newcastle disease virus. It was also concluded that the morbidity and mortality were highest in Nadia District and case fatality rate was highest in 24 Parganas (North) district which might be due to more prevalence of velogenic strain in those districts.

4.2. Season wise Prevalence

In West Bengal, 3 prominent seasons i.e. summer, rainy and winter prevail throughout the year. Month wise distribution of each season as follows:

Season	**Month**
Winter	: November, December, January and February
Summer	: March, April, May and June
Rainy	: July, August, September and October

Season wise distribution of Newcastle disease outbreak was presented in table 7 and Fig. 10 & 11. From the table it was evident that Newcastle disease outbreak was highest 105 (40.54%) in winter

Table 7. Showing season wise distribution of Newcastle disease outbreak in both Broiler and Free range rearing Flocks.

Season	Type of Birds			
	Broiler		Free range rearing flock	
	Total no of flock	Percentage (%)	Total no of flock	Percentage (%)
Summer	70	27.03	21	16.67
Rainy	84	32.43	42	33.33
Winter	105	40.54	63	50.00
Total	259	100	126	100

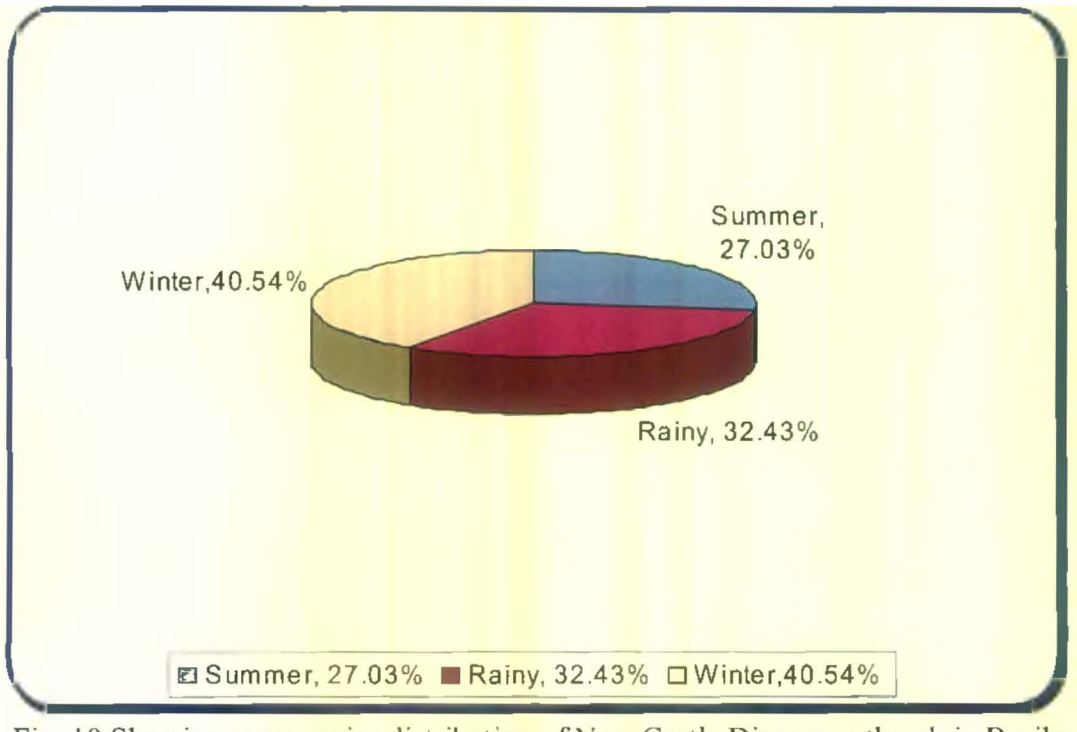

Fig. 10 Showing season wise distribution of New Castle Disease outbreak in Broiler Flocks in West Bengal

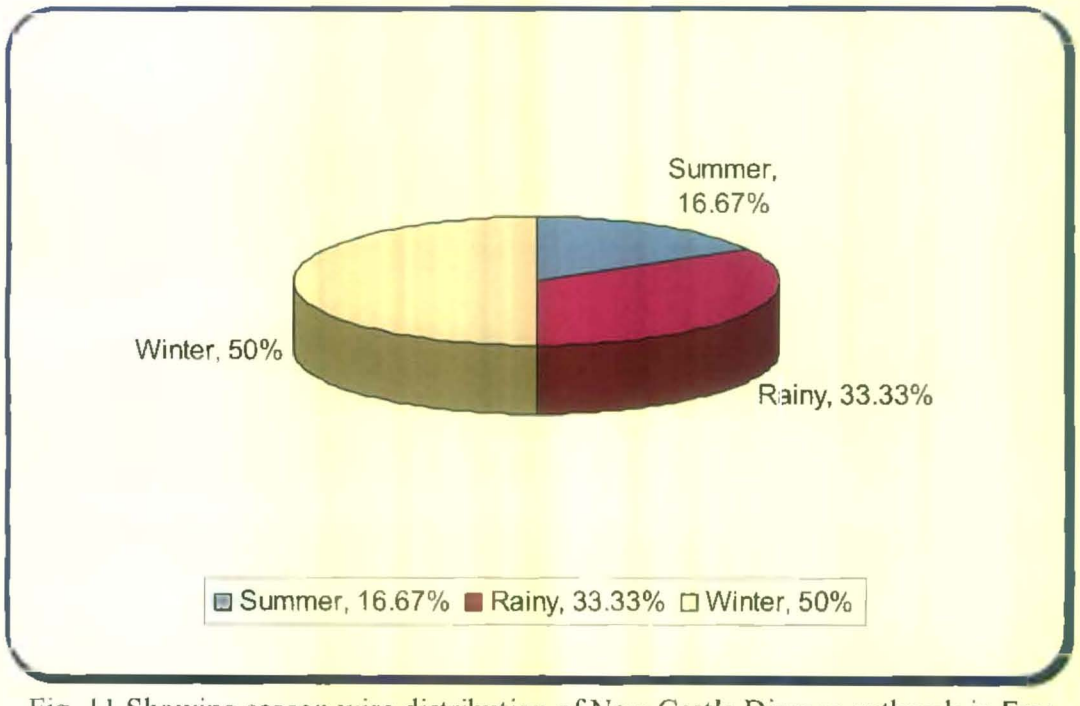

Fig. 11 Showing season wise distribution of New Castle Disease outbreak in Free Range Rearing Flocks in West Bengal

season and the lowest 70 (27.03%) in summer in broiler flocks. In rainy season 84 (32.43%) outbreaks were recorded.

Similarly 63(50.00%) of the highest outbreaks were recorded in winter season in free range rearing flocks. The lowest i.e. 21 (16.67%) outbreaks were observed in summer season and 40 (33.33%) recorded in rainy season.

Nguyen (1992) reported that Newcastle disease outbreak appeared most commonly in the winter (Nov-Feb) season in Vietnam which is similar to the present finding. Asadullah (1992) reported that outbreak occurred throughout the year, with a peak during the winter (Nov- Feb) in Bangladesh. Similar observations have also been reported by Musiime (1992) in Kenya.

The highest number of Newcastle disease outbreaks during winter compared to the summer and rainy season might be due to the fact that the birds were exposed to cold stress which predisposed them to infection. The other contributory factor could be the low temperature during the winter influence survival of Newcastle disease virus in the atmosphere and cold and dry winds cause rapid dissemination of Newcastle disease virus among the susceptible population.

4.3. Age wise Distribution

Age wise distribution of Newcastle disease outbreak in broiler and free range rearing flocks was presented in table 8 and Fig. 12 & 13. From the table it was evident that the highest number of outbreaks i.e.182 (70.27%) occurred in the age group of 22-42 days and the lowest number of outbreaks i.e.14 (5.41%) in 43 and above day's age group of broiler birds. Sixty three out break (24.32%) was recorded in 0-21 days of age group of broiler flocks.

Similarly, greater number of outbreak i.e. 98 (77.785) occurred in 43 and above days age group and 28 (22.22%) occurred in 22-42 days age group of free range rearing flocks. But no outbreak was

Table 8. Showing age wise distribution of Newcastle disease outbreak in broiler and free range rearing birds.

Age Group	Type of birds							
	Broiler				Free range rearing birds			
	Out break		Population at risk		Out break		Population at risk	
	Total Number of flock	Percentage (%)	Flock strength	Percentage (%)	Total Number of flock	Percentage (%)	Flock strength	Percentage (%)
0-21days	63	24.32	14,000	12.31	-	-	-	-
22-42days	182	70.27	96,550	84.88	28	22.22	595	21.30
43 and above	14	5.41	3200	2.81	98	77.78	2198	78.70
Total	259	100	113750	100	126	100	2793	100

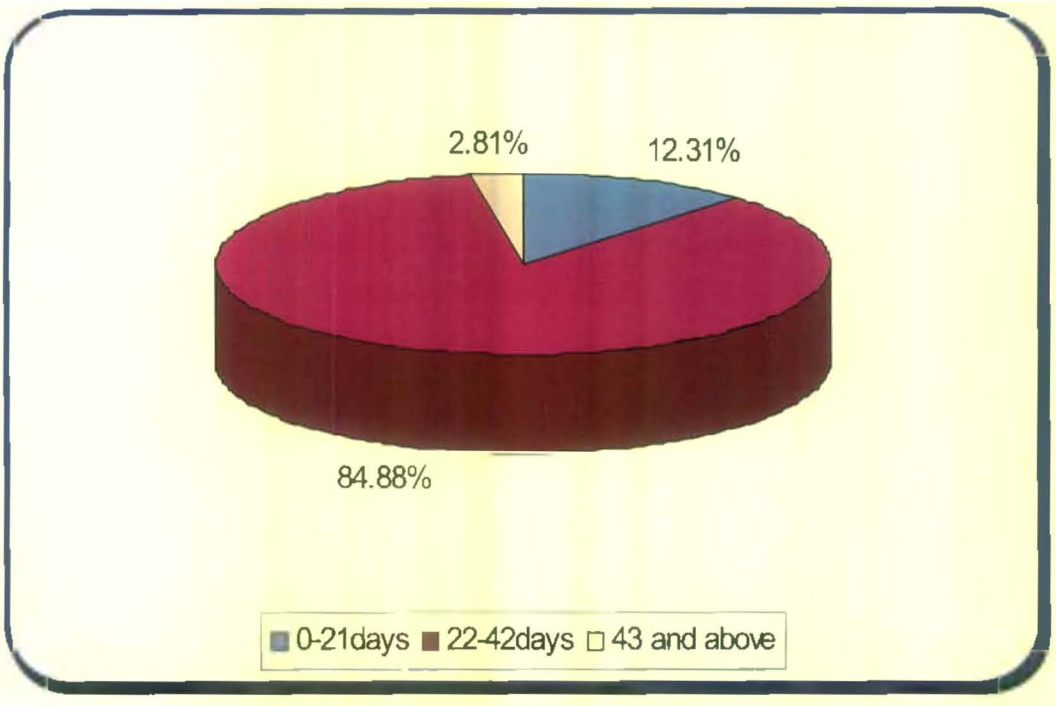

Fig. 12 Showing age wise distribution of New Castle Disease outbreak in Broiler Flocks in West Bengal

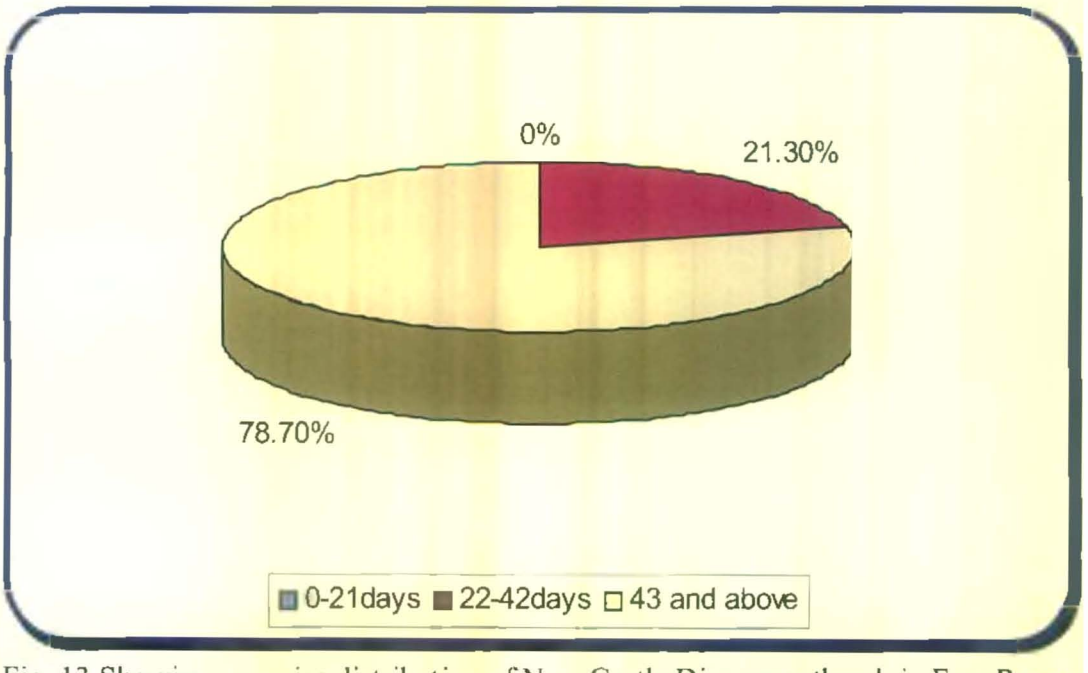

Fig. 13 Showing age wise distribution of New Castle Disease outbreak in Free Range Rearing Flocks in West Bengal

found in 0-21 day's age group of free range rearing birds during the 2 years periods of observation.

Similar type of findings had been also observed by Barman (2002).

The present finding was in agreement with a study made by Ezeokoli *et al.* (1984) who found that in backyard management system birds around 16-24 weeks of age had highest risk of Newcastle disease infection.

Schmidt and Shimidt (1955) reported that the HI titre 32 and above to be protective against Newcastle disease. Thitissak et al (1989) explained that the mean HI titre for Newcastle disease was higher in newly hatched chicks and maternal antibodies disappeared at about 90 days, Chowdhury *et al* (1982) also reported that the maternal antibodies were higher in young age group of chicken. But we were not confirmed whether backyard birds were vaccinated or not. Spradbrow (1993-94) reported that overall11.1% of the village farmer regularly vaccinated their birds, 13.9% occasionally vaccinated and 75.0% of farmers did not use vaccine against Newcastle disease in backyard poultry mainly due to lack of proper knowledge, multiage of their flocks, lack of cold chain facilities and poverty. Ronohardjo (1984) observed higher antibodies titre was in 58% of 2 days old chicks and 42% had no detectable antibodies and undetectable at the 4 week of age in all chickens though parent flocks were under intensive vaccination programme.

From the present study it may be concluded that higher level of antibodies might protect the initial stage of life and thereafter they became susceptible and were infected when maternal antibodies declined below protective level. Proportions of chicks in large flocks (broiler) were always susceptible to infection perhaps due to inadequate maternal antibodies (Ronohardjo, 1984). Higher number of outbreaks in 3-6 week age group might be due to inadequate protective titre either due to no vaccination or a single vaccination in first week of life where the protective titre declined from 4 week of age

(Shakour et al 1971). The lifespan of commercial broiler was 6- 7 weeks, therefore after 6 weeks of life, rearing broiler population was scanty, which was favourable for least chance of outbreak.

4.4. District wise seroprevalence of Newcastle disease

One thousand two hundred ninety five serum samples from New- castle disease infected flocks (vaccinated) and five hundred sixty serum samples from healthy vaccinated flocks were tested for presence of antibodies against ND virus by Haemagglutination Inhibition test (OIE, 2004).The result has been presented in the table 9.

From the table 9, it was evident that 100% (1295) samples collected from affected flocks had shown the antibody titre (≤ 1:16) which was below the protective level (1:32). But out of 560 samples collected from non-affected flocks, 455 (81.25%) samples had shown protective titre (1:32) and 105 (18.75%) samples had a very good protective titre (≥1:64).

Similar observation had also been reported by Biswas *et al* (2006) and R.D. Immune status Studies Laboratory, IAH&VB, Directorate of Animal Resources and Animal Health, Govt. of West Bengal. From the study it was concluded that the vaccinated birds having the protective titre could resist the infection.

4.5. Isolation of Newcastle disease virus.

For isolation of Newcastle disease virus, a total 385 suspected samples, 259 from commercial broiler flocks and 126 from free range rearing flock were collected and inoculated through allantoic cavity in 9^{th} to 11^{th} days old embryonated SPF chicken eggs for isolation of Newcastle disease virus. The embryos mortality were presented in the table10.

Table 9. Showing district-wise seroprevalence of Newcastle disease in affected and non-affected flocks.

District	No of flocks	No. of sample tested	Affected/ Non affected birds	HI titre		
				≥ 1:64 (2^6)	1:32 (2^5)	≤1:16 (2^4)
Uttar Dinajpur	20	100	Affected	-	-	100
Dakshin Dinajpur	8	40	Affected	-	-	40
Malda	26	130	Affected	-	-	130
	14	70	Non-affected	12	58	-
Murshidabad	23	115	Affected	-	-	115
	13	65	Non-affected	-	65	-
Nadia	49	245	Affected	-	-	245
	14	70	Non-affected	12	58	-
24 Parganas (N)	39	195	Affected	-	-	195
	21	105	Non-affected	38	67	-
24 Parganas (S)	31	155	Affected	-	-	155
	21	105	Non-affected	23	82	-
Howrah	5	35	Affected	-	-	35
Hooghly	19	95	Affected	-	-	95
	15	75	Non-affected	-	75	-
Purba Medinipur	8	40	Affected	-	-	40
Bankura	9	45	Affected	-	-	45
Burdwan	15	75	Affected	-	-	75
Birbhum	7	35	Affected	-	-	35
Total			Affected	-	-	1295 (100%)
			Non-affected	105 (18.75%)	455 (81.25%)	-

Table 10. Showing embryonic-death of inoculated samples into SPF chicken eggs

Type of birds	No of sample inoculated	No of sample causes death of embryo	Percentage(%) of sample causes death
Commercial Broiler	259	70	27.03
Free range rearing flock	126	14	11.11
Total	385	84	21.83

From the table 10 it was evident that out of 385 inoculated samples, 84(21.83%) could cause death of the embryos within 7 days of post inoculation. Accordingly to the type of birds 70 (27.03%) samples were collected from 259 different commercial broiler flocks/farms and 14 (11.11%) samples from 126 different free range rearing flocks, could cause death of the embryos of the inoculated SPF eggs.

All the dead embryonated eggs were chilled at 4^0C for atleast 4 hrs and allantoic fluids were harvested aseptically and stored individually at -20^0C before performing haemagglutination test.

4.6. Identification of Newcastle disease virus.

The allantoic fluids of 84 isolates were tested for haemagglutination (HA) activity with1% washed chicken RBC and all 84 isolates were positive and identified as WB_1 to WB_{84} (table11). The HA titre varied from 2^4 to 2^8 which were inhibited by known Newcastle disease positive antiserum by haemagglutination inhibition (HI) test, which was the confirmatory test for identification of Newcastle disease virus (OIE, 2004).

The positive isolates of Newcastle disease along with their HA titre has been presented in the table 11.

Table 11. Showing HA titre of the positive isolates of Newcastle disease

SL. No of the Isolates	Identification number of the isolates	Isolated from	HA titre	SL. No of the Isolates	Identification number of the isolates	Isolated from	HA titre
1	WB_1	Broiler	2^4	29	WB_{29}	Broiler	2^4
2	WB_2	flocks	2^5	30	WB_{30}	flocks	2^7
3	WB_3		2^4	31	WB_{31}		2^4
4	WB_4		2^4	32	WB_{32}		2^5
5	WB_5		2^5	33	WB_{33}		2^4
6	WB_6		2^4	34	WB_{34}		2^5
7	WB_7		2^7	35	WB_{35}		2^7
8	WB_8		2^8	36	WB_{36}		2^5
9	WB_9		2^7	37	WB_{37}		2^8
10	WB_{10}		2^4	38	WB_{38}		2^7
11	WB_{11}		2^7	39	WB_{39}		2^6
12	WB_{12}		2^4	40	WB_{40}		2^7
13	WB_{13}		2^5	41	WB_{41}		2^8
14	WB_{14}		2^8	42	WB_{42}		2^7
15	WB_{15}		2^7	43	WB_{43}		2^6
16	WB_{16}		2^4	44	WB_{44}		2^7
17	WB_{17}		2^5	45	WB_{45}		2^4
18	WB_{18}		2^7	46	WB_{46}		2^6
19	WB_{19}		2^4	47	WB_{47}		2^7
20	WB_{20}		2^6	48	WB_{48}		2^8
21	WB_{21}		2^6	49	WB_{49}		2^4
22	WB_{22}		2^7	50	WB_{50}		2^5
23	WB_{23}		2^4	51	WB_{51}		2^6
24	WB_{24}		2^8	52	WB_{52}		2^5
25	WB_{25}		2^7	53	WB_{53}		2^4
26	WB_{26}		2^6	54	WB_{54}		2^4
27	WB_{27}		2^4	55	WB_{55}		2^4
28	WB_{28}		2^5	56	WB_{56}		2^7

SL. No of the Isolates	Identification number of the isolates	Isolated from	HA titre	SL. No of the Isolates	Identification number of the isolates	Isolated from	HA titre
57	WB_{57}	Broiler	2^4	71	WB_{71}	Free	2^7
58	WB_{58}	Flock	2^5	72	WB_{72}	range	2^6
59	WB_{59}		2^6	73	WB_{73}	rearing	2^5
60	WB_{60}		2^8	74	WB_{74}	flock	2^6
61	WB_{61}		2^7	75	WB_{75}		2^7
62	WB_{62}		2^8	76	WB_{76}		2^7
63	WB_{63}		2^6	77	WB_{77}		2^8
64	WB_{64}		2^5	78	WB_{78}		2^8
65	WB_{65}		2^5	79	WB_{79}		2^5
66	WB_{66}		2^6	80	WB_{80}		2^5
67	WB_{67}		2^8	81	WB_{81}		2^6
68	WB_{68}		2^8	82	WB_{82}		2^7
69	WB_{69}		2^7	83	WB_{83}		2^6
70	WB_{70}		2^8	84	WB_{84}		2^7

From the table 11 it was also evident that the HA titre of the isolates (WB_{71} to WB_{84}) isolated from free range rearing flocks were comparatively higher than the HA titre of broiler flocks (WB_1 to WB_{70}).

Similar findings had also been reported by Barman (2002) and Chowdhury *et al.* (1982) in Bangladesh and Danials *et al.* (1987) in Indonesia.

4.7. Serotyping of the isolated virus.

Out 84 isolates of Newcastle disease virus, a total of 24 isolates (28.57%) were taken for serotyping (5^{th} passage allantoic fluid) and out of 24 isolates, 20 (83.33%) were from broiler flocks (identified as WB_1 to WB_{20}) and 4 (16.67%) were from free range rearing flock (identified as $WB_{71}$to WB_{74}). Serotyping of 24 isolates were done by following standarad protocols i.e. mean death time (MDT), intracerebral

pathogenecity index (ICPI) and intravenous pathogenecity index (IVPI) prescribed by OIE, 2004 and results presented in the table 12.

Table 12. Showing MDT, ICPI, IVPI along with serotypes of isolates

Identification number of the isolates	HA titre	Isolated from	MDT (hours)	ICPI	IVPI	Serotype
WB_1	2^6	Commercial broiler flocks	106.40	0.14	0.0	lentogenic
WB_2	2^6		102.40	0.05	0.0	lentogenic
WB_3	2^4		98	0.06	0.0	lentogenic
WB_4	2^7		106.20	0.00	0.0	lentogenic
WB_5	2^{10}		99.20	0.4	0.0	lentogenic
WB_6	2^7		77.20	1.50	0.0	mesogenic
WB_7	2^7		52	1.91	2.54	velogenic
WB_8	2^9		54.40	1.88	2.54	velogenic
WB_9	2^9		54	1.84	2.56	velogenic
WB_{10}	2^{10}		58	1.75	2.52	velogenic
WB_{11}	2^9		44.40	1.67	2.46	velogenic
WB_{12}	2^7		44.20	1.85	2.44	velogenic
WB_{13}	2^9		96.40	0.46	0.0	lentogenic
WB_{14}	2^7		99	0.48	0.0	lentogenic
WB_{15}	2^9		94.40	0.51	0.0	lentogenic
WB_{16}	2^{10}		42	1.83	2.50	velogenic
WB_{17}	2^7		106.20	0.60	0.0	lentogenic
WB_{18}	2^7		103.40	0.42	0.0	lentogenic
WB_{19}	2^9		54.40	1.77	2.21	velogenic
WB_{20}	2^8		76.40	1.40	0.0	mesogenic
WB_{71}	2^8	Free range rearing flocks	44.40	1.87	2.46	velogenic
WB_{72}	2^7		44.20	1.75	2.44	velogenic
WB_{73}	2^6		52.40	1.90	2.46	velogenic
WB_{74}	2^6		54.20	1.85	2.44	velogenic

From the table 12, it was evident that out of 24 serotyped Newcastle disease virus isolates, 12 (50%) were velogenic; 2(10%) were mesogenic and 10 (40.00%) were lentogenic pathotype. Two mesogenic and 10 lentogenic serotypes were isolated from broiler flocks and out of 12 velogenic pathotype 8 were isolated from broiler flocks and 4 were from free range rearing flocks.

From the present study it was also evident that velogenic, mesogenic and lentogenic serotypes were 40%, 10% and 50% respectively prevalent in broiler flocks but in free range rearing flocks only velogenic (100%) serotype was predominant which could be the cause of high morbidity and mortality in free range rearing flocks.

Similar observation had also been reported by Abu *et al* (1985), Bell and Moulodi (1988), Chowdhury *et al* (1982) and Asadullah (1992), Alexander (1989) and Daniels *et al.* (1987).

Twelve isolates were sent to OIE, FAO and National Reference Laboratory for Newcastle disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell' Universitia, 10 – 35020 LEGNARO (PD), Italy for confirmation and characterization. The authorities have informed that the samples are under process and a preliminary report has been sent which is enclosed in appendix-II.

4.8. Prevalence of pathotypes of Newcastle disease virus

Table13. Showing district-wise prevalence of pathotypes of Newcastle disease virus

Pathotypes	Type of bird affected	Morbidity (%)	Mortality (%)	CFR (%)	Prevalence area (Districts)
Velogenic	Broiler	59.85	40.31	67.35	Nadia, Hoogly, 24 Pgs (N), 24 Pgs (S)
Mesogenic	Broiler	40.00	26.00	65.00	24 Pgs (S)
Lentogenic	Broiler	24.02	5.80	24.12	Nadia, 24 Pgs (N)
Velogenic	Free range rearing flock	97.44	94.87	97.37	Nadia, 24 Pgs (N)

In the present study Newcastle disease virus was isolated from 84 outbreaks. Out of 84 isolates, 24 isolates, 20 from broiler flocks and 4 from free range rearing flocks were characterised. Out of 24 isolates from broiler flocks 10, 2, and 8 isolates were lentogenic, mesogenic and velogenic respectively. All 4 isolates obtained from free range rearing flocks were found velogenic.

From the table 13 and Fig. 14, 15, 16 & 17, it is evident that the morbidity, mortality and case fatality were 59.85%, 40.31%, and 67.35% respectively, due to velogenic pathotype infection.

From the table it is also evident that morbidity due to the velogenic, mesogenic and lentogenic pathotype infection were 59.85%, 40.00% and 24.02% respectively. Similarly mortality due to velogenic, mesogenic and lentogenic pathotype affected flock were 40.31%, 26.00%, and 5.80% respectively and case fatality due to velogenic, mesogenic, and lentogenic pathotype in infected flock were 67.35%, 65.00% and 24.12% respectively. The morbidity, mortality and case fatality of free range rearing flocks affected with velogenic pathotype were 97.44%, 94.87% and 97.37% respectively. From the present study it is also evident that velogenic pathotypes were predominant in Nadia, Hoogly, 24 Parganas (N) and 24 Parganas (S) whereas the mesogenic pathotype was predominant in 24 Parganas (S) and lentogenic pathotype was predominant in Hoogly, Nadia and 24 Parganas (N) in broiler flocks. But the velogenic pathotype in free range rearing flocks was predominant in Nadia and 24 Parganas (N).

Similar observation was made by Martin (1992) and Abu *et al.* (1985) in Nigeria, Chowdhury *et al.* (1982) in Bangladesh and Denials *et al.* (1987) in Indonesia.

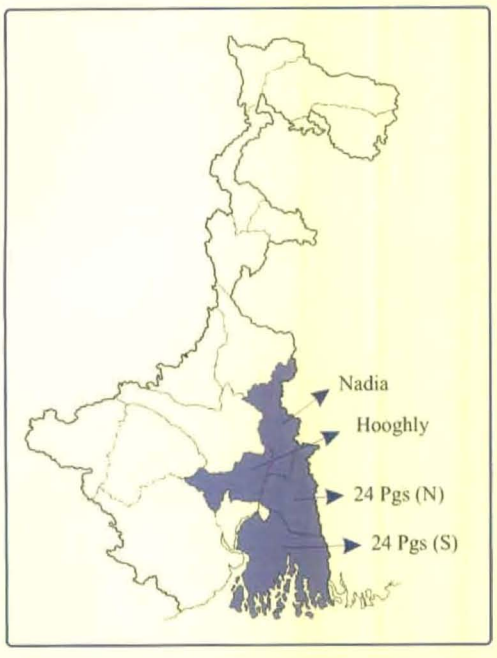

Fig. 14 Showing Prevalence of Velogenic pathotype in broiler birds.

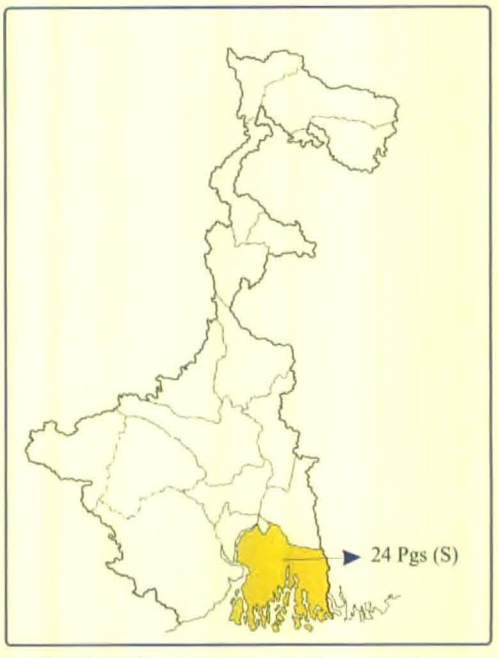

Fig. 15 Showing Prevalence of Mesogenic pathotype in broiler birds.

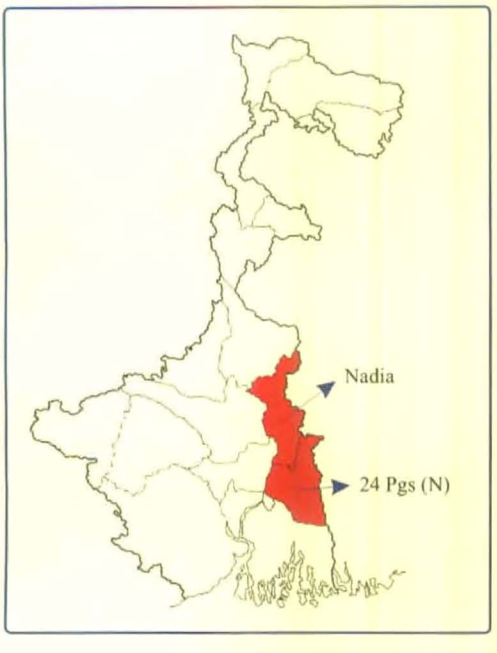

Fig. 16 Showing Prevalence of Lentogenic pathotype in broiler birds.

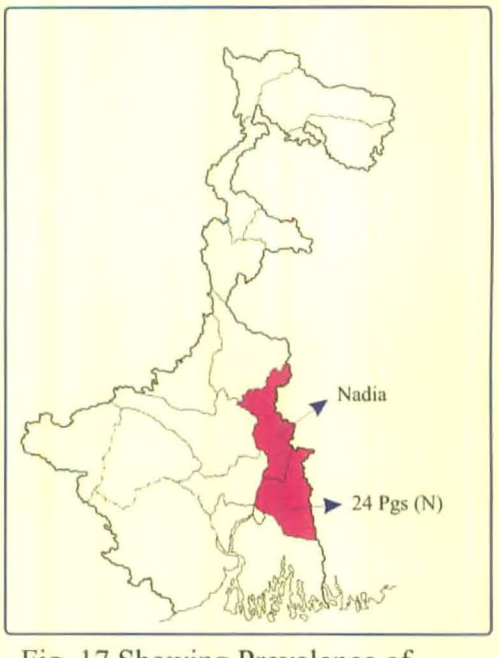

Fig. 17 Showing Prevalence of Velogenic pathotype in free range rearing flocks.

Table 14. Showing mortality pattern in broiler and free range flocks by different pathotypes of Newcastle disease virus (Mean ± SE).

Type of bird	Pathotype of isolates	Mortality pattern	
		Pick (hours)	Cessation of mortality (hours)
Broiler	Velogenic	66 ± 0.02	96 ± 0.03
	Mesogenic	96 ±0.03	168 ± 0.03
	Lentogenic	96 ± 0.02	174 ± 0.03
Free range rearing	Velogenic	48 ± 0.01	78 ± 0.02

In the present study, the mortality was high (40.31%) with velogenic pathotypes. Maximum mortality occurred within 66 hours manifestation of clinical symptoms which was continued upto 96 hours. Similarly the mortality was 26% and 5.80% in case of mesogenic and lentogenic pathotypes respectively in broiler flocks. The maximum mortality was at 96 hours both with the mesogenic and lentogenic pathotypes and that continued upto 168 hours and 174 hours respectively. Whereas, the mortality was too high (94.87%) by velogenic pathotype in free range rearing flocks where the maximum mortality was observed within 48 hours of the onset of clinical manifestation and continued upto 78 hours. From the observation it was concluded that the mortality pattern was very rapid by velogenic pathotype in comparison to the mesogenic and lentogenic pathotypes of Newcastle disease virus.

4.9. Determination of Embryo Infectious Dose (EID_{50})

EID_{50} of each 24 characterised pathotype viruses identification numbers WB_1 to WB_{20} and WB_{71} to WB_{74}, was determined using 5^{th} passages virus as test sample. For this purpose 0.1ml of virus was inoculated in 5^{th}, 9^{th} to 11^{th} days old embryonated SPF chicken eggs by allantoic cavity of dilutions (10^{-4} to 10^{-10}). Embryos mortality was recorded from 2^{nd} to 8^{th} days of post- inoculation in different dilution.

The EID_{50} was calculated using the standered method of Reed and Muench (1938).

The results of EID_{50} of 24 characterised viruses (12 velogenic, 2 mesogenic and 10 lentogenic serotypes) were presented in table 15.

Table 15. Showing pathotype-wise EID_{50} Of the isolates

Identification number of the isolates	Pathotype	EID_{50}/ml	Name of the isolates	Pathotype	EID_{50}/ml
WB_1	lentogenic	$10^{8.5}$	WB_{13}	lentogenic	$10^{8.56}$
WB_2	lentogenic	$10^{8.5}$	WB_{14}	lentogenic	$10^{8.5}$
WB_3	lentogenic	$10^{8.5}$	WB_{15}	lentogenic	$10^{8.54}$
WB_4	lentogenic	$10^{8.5}$	WB_{16}	velogenic	$10^{8.5}$
WB_5	lentogenic	$10^{8.53}$	WB_{17}	lentogenic	$10^{8.54}$
WB_6	mesogenic	$10^{8.95}$	WB_{18}	lentogenic	$10^{8.5}$
WB_7	velogenic	$10^{8.5}$	WB_{19}	velogenic	$10^{8.5}$
WB_8	velogenic	$10^{8.5}$	WB_{20}	mesogenic	$10^{9.0}$
WB_9	velogenic	$10^{8.5}$	WB_{71}	velogenic	$10^{8.5}$
WB_{10}	velogenic	$10^{8.5}$	WB_{72}	velogenic	$10^{8.5}$
WB_{11}	velogenic	$10^{8.5}$	WB_{73}	velogenic	$10^{8.5}$
WB_{12}	velogenic	$10^{8.5}$	WB_{74}	velogenic	$10^{8.5}$

From the table, it was evident that the EID_{50} of lentogenic pathotype varied from $10^{8.5}$ to $10^{8.56}$ and for mesogenic pathotype the EID_{50} were $10^{8.95}$ to 10^{9} which were higher than the lentogenic pathtype (varied from $10^{8.5}$ to $10^{8.56}$).

But the EID_{50} of all velogenic pathotype were $10^{8.5}$ which were lesser than mesogenic pathtypes and some lentogenic pathotypes which was justified although supporting literature on this aspect was scanty.

4.10. Preparation of modified/attenuated virus.

Five lentogenic Newcastle disease virus i.e. WB_1 WB_2 WB_3 WB_4 and WB_5 were randomly selected on the basis of ICPI values and EID_{50} of the virus as per the recommendation of Commission of the European Communities (1993) and OIE (2000) for attenuation were presented in the table 16.

Table 16. Showing the selected isolates along with ICPI and EID_{50} for attenuation/ modification

Identification number of the isolates	ICPI	EID_{50}/ml
WB_1	0.14	$10^{8.5}$
WB_2	0.05	$10^{8.5}$
WB_3	0.06	$10^{8.5}$
WB_4	0.00	$10^{8.5}$
WB_5	0.40	$10^{8.53}$

All the five isolates were passaged individually to 5, 9^{th} to 11^{th} days old embryonated SPF chicken eggs up to 15^{th} passages. The 10^6 EID_{50} of 15^{th} passages virus was inoculated in 10, 5 days old SPF chicken intranasally and observed for any clinical disease or mortality. The selected lentogenic virus isolates were passaged in SPF eggs and allantoic fluid harvest was inoculated intranasally in 10^6 EID_{50} dose to ten, 5 days old SPF chickens till was no mortality was detected in the inoculated chicks. The effect of inoculated virus of various passages into SPF chickens (10 chickens were inoculated intranasally by each passage of virus) was presented in the following table17.

Table 17. Showing effect of inoculated virus at different passage level into SPF chicken for attenuation / modification

Identification number of the Isolates	No. of dead chicks after inoculation					
	15th passage	16th passage	17th passage	18th passage	19th passage	20th passage
WB_1	10	10	10	10	10	10
WB_2	1	0	-	-	-	-
WB_3	10	10	0	-	-	-
WB_4	10	10	10	10	10	10
WB_5	10	10	10	10	5	0

From the table it was evident that all the inoculated SPF chickens (antibody free chicken) died up to 20th serial passages by two isolates viz. WB_1 and WB_4. All the dead chickens were examined for necroscopy and haemorrhagic lesion were detected in proventriculous. Viruses were re-isolated from those very dead chickens by inoculation of necroscopic materials into 9th to 11th day old embryonated SPF chicken eggs. This confirmed that the WB_1 and WB_4 isolates retained their virulence and were not attenuated by 20th serial passages in embryonated SPF chicken eggs.

But incase of WB_2 only one inoculated chicken died on 15th passages and no mortality occurred on 16th passages.

In case of WB3 isolate all the inoculated chickens died with the 16th passage allantoic fluid but mortality was nil with the 17th passage allantoic fluid.

Out of 10 inoculated chickens, 5 died with the 19th passage allantoic fluid but no mortality occurred with the 20th passage allantoic fluid in case of WB_5 isolates.

Thus it was concluded that viruses of those 3 isolates viz. WB_2, WB_3, and WB_5 might be attenuated by 16th, 17th and 20th serial passages in to embryonated SPF chicken eggs respectively.

Further it was concluded that the 2 isolates viz. WB_1 and WB_4 might require more passages for attenuation and the viruses of 3 isolates namely WB_2, WB_3 and WB_5 of 16th, 17th and 20th serial passages respectively could be used as master seed for preparation of live attenuated vaccine.

Literature regarding attenuated/modified virus is scanty. Therefore it would be concluded that if the 16th,17th and 20th passaged viruses of the isolates WB_2, WB_3, and WB_5 respectively could meet the criteria of control test i.e. sterility, infectivity, safety and potency. Then only they might be used for preparation of live attenuated vaccine.

4.10.1. Control Test

4.10.1.1. Sterility

For sterility test, the 16th, 17thand 20th passaged viruses of the isolates namely WB_2, WB_3, and WB_5 respectively were inoculated individually in aseptic condition, into nutrient broth (for bacterial growth), Sabouraud's Dextrose Agar (for fungal growth) and in PPLO agar (for culture of Mycoplasma) and incubated. But no growth or characteristic colony was detected in the growth media after the due incubation period.

From the result it was concluded that all the 3 viruses of 16th, 17th and 20th passages viz. WB_2, WB_3, and WB_5 respectively were sterilized for any exogenous contamination.

4.10.1.2. Infectivity

For assessing the infectivity of the modified viruses of the 3 isolates (16th, 17th, and 20th passaged viruses of the isolates namely WB_2, WB_3, and WB_5 respectively), two test were performed i.e. haemagglutination test (OIE, 2004) and detection of EID_{50} (Reed and Muench, 1938).

The results of both the test of the 3 modified viruses were presented in the table 18.

Table 18. Showing HA titre and EID_{50}/ml of attenuated/modified isolates

Attenuated virus	HA titre	EID_{50}/ml
WB_2 (16th passaged virus)	2^7	$10^{8.5}$
WB_3 (17^{th} passaged virus)	2^8	$10^{8.5}$
WB_5 (20^{th} passaged virus)	2^7	$10^{8.5}$

From the table it was evident that the EID_{50} of all the modified viruses were same i.e. $10^{8.5}$ per ml. It was also found that the EID_{50} per ml had no change or a little change between the modified viruses and 5^{th} passaged viruses i.e. $10^{8.5}$, $10^{8.5}$, and $10^{8.53}$ of WB_2, WB_3, and WB_5 isolates respectively. On the other hand, the HA titre of the modified viruses were 2^7, 2^8, and 2^7 which were higher than the titres of the 5^{th} passaged viruses i.e. 2^6, 2^4, and 2^6 respectively of the isolates namely WB_1, WB_2, and WB_5.

Literature on this aspect was scanty. But from the results it may be concluded that the live virus in optimum concentrated were present in the 16^{th}, 17^{th} and 20^{th} passaged allantoic fluids of the isolates viz. WB_2, WB_3 and WB_5 respectively but had no ability to produce disease with no detectable antibody level. From the observation it was concluded that after 1^{st} and 2^{nd} inoculation by WB_2 (16^{th} passaged), WB_3 (17^{th} passaged) and WB_5 (20^{th} passaged) viruses, inoculated in chickens acquired protective immunity against highly virulent Newcastle disease virus. It was also concluded that the 16^{th} passage of WB_2, 17^{th} passage of WB_3 and 20^{th} passage of WB_5 viruses were modified/attenuated and had fulfilled the criterias as per the recommendation of OIE, 2004 and might be used as master seed for preparation of live attenuated Newcastle disease vaccine.

4.10.1.3. Safety

Ten, 5 days old ND antibody free chicken were inoculated with 10^7 EID_{50} of 16^{th} passaged virus of WB_2 isolates to each chicken, intranasally in batch-1, Similarly 17^{th} passaged virus of WB_3 isolate and 20^{th} passaged virus of WB_5 isolates were inoculated in 10 number of chicken with each virus i.e. batch-2 and batch-3 and observed for 21 days for mortality. The results were presented in table 19.

Table 19. Showing mortality pattern (upto 21 days observation) of attenuated/modified isolates

Identification No. of the test virus	No. of Chicks	Batch no	Observation (mortality)		
			7th days	14thdays	21days
WB_2 (16thpassaged virus)	10	Batch-1,	Nil	Nil	Nil
WB_3 (17^{th} passaged virus)	10	Batch-2	Nil	Nil	Nil
WB_5 (20^{th} passaged virus)	10	Batch-3	Nil	Nil	1

From the table, it was evident that there was no mortality within 21^{st} day post-inoculation when administered by 10 times more than single inoculation dose of virus to each chicken of WB_2 and WB_3 isolates. But only one mortality occurred on 21^{st} days in case of WB_5 isolates.

Similar recommendation was given by Council of Europe (1997), Seang (1987) and OIE (2004).

From the present observation it was concluded that 10 times higher dose of virus could be tolerated by chicken when the virulence of the virus has been reduced by serial passaging and that the virus had no capability to produce the disease.

4.10.1.4. Potency

Fourty, 5-days-old ND antibody free chicken were divided into equal four groups i.e. group-1, Group-2, Group-3 and group-4. Group-1, Group-2, Group-3 were inoculated with 10^6 EID_{50} of WB_2 (16^{th} passaged), WB_3 (17^{th} passaged) and WB_5 (20^{th} passage) virus intranasally and kept separately. Group-4 also kept separately as untreated control. HI titre were estimated of all the groups of chicken at the age of 26^{th} day and re-inoculated by the same dose of virus to the correspondence group of chicken. The HI titre were again tested from all the groups of chicken at the age of 47^{th} days of age (21days of post- 2^{nd} – inoculation) and all the chicken (40chicks) of all the four groups were challenged with 10^6EID_{50} virulent strain of virus, isolated from field cases, intramuscular route to each chick and observed for next 21 days up to 68 days of age for any mortality. The results were presented in table 20.

Table 20. Showing HI titre and mortality of post challenged of attenuated/modified isolates

Name of the isolates	Group	No. of chicken	HI titre after 21 days of 1st inoculation	HI titre after 21 days of 2nd inoculation	Number of dead-chicks of post challenged			
					7th days	14th days	21days/HI titre	
WB_2 (16^{th} passaged)	Gr-1	10	2^4-2^6	2^6-2^8	Nil	Nil	Nil	2^8-2^{11}
WB3 (17th passaged)	Gr-2	10	2^4-2^6	2^6-2^8	Nil	Nil	Nil	2^8-2^{11}
WB_5 (20^{th} passaged)	Gr-3	10	2^4-2^6	2^6-2^8	Nil	Nil	Nil	2^8-2^{11}
Control	Gr-4	10	Non detectable	Non detectable	8	2	-	-

From the table it was evident that HI titre were gradually increased after 1st and 2nd inoculation of all the 3 groups of test chicken (ranges from 2^6 to 2^8) and finally reached to 2^8 to 2^{11} after 21 days of post challenged without any single mortality. But all the chicken of control groups died within 14 days of post challenge.

Chapter – 5

Summary and Conclusion

SUMMARY AND CONCLUSION

Three eighty five outbreaks, clinically suspected of Newcastle disease from different districts of West Bengal were studied during the period from April, 2004 to March,2006. Necropsy materials from recently dead birds or moribund birds and oro-nasal swab from clinically sick, birds were collected from 385 farms/flocks. Out of 385 samples 259 were collected from 259 vaccinated broiler farm/flock and 126 samples from free range rearing flocks which were vaccinated or non-vaccinated was not clear. The samples were processed and stored at -20^{0}C for future use.

During epidemiological observation of Newcastle disease outbreak, it was found that the highest morbidity (56.74%) and mortality (38.00%) were detected in the Murshidabad and Uttar Dinajpur districts respectively and the lowest morbidity (28.35%) and mortality (8.91) were found in the Nadia and 24-Pargana (North) districts respectively whereas the overall morbidity and mortality were 37.22% and 16.28% in case of broiler farms/flocks.

But incase of free range rearing flocks, the highest morbidity (50.65%) was in Murshidabad district and the lowest (37.50%) was in Hoogly district whereas the highest mortality (64.00%) was in the Nadia and the lowest (33.33%) in the 24 Parganas (south), Hoogly and Bankura districts. But the average morbidity and mortality were 55.53% and 47.49% respectively.

The case fatality rate was highest (74.5%) in Dakshin Dinajpur and lowest in 24-Parganas (north) districts though the average case

fatality rate was 43.26% in broiler flocks but the case fatality rate in free range rearing flock was highest (95.35%) in 24- Parganas (North) and lowest (83.33%)in the Bankura districts, whereas the average case fatality rate was 86.52%.

The disease was more prevalent in winter in both broiler(40.54%) and free range rearing flocks (50.00%) and less number of out breaks occurred in summer in both the groups i.e.27.03% and 16.67% respectively.

The outbreaks were higher (70.27%) at the age of 22-42 days old in broiler birds with a high percentage (84.88%) of population at risk. But least occurrence (5.4%) of the disease with less number of populations at risk (2.81%) was observed at the age of 43 and above days old broiler birds.

But highest outbreak (77.78%) with a higher population at risk (78.70%) was observed in 43 days and above old age group of free range rearing flock and 22.22% outbreak with 21.30% population at risk observed in 22-42 days old age group and not a single outbreak was detected at the young age group (0-21 day's old), free range rearing flocks.

After processing of the collected sample as per the recommendation of OIE, 2004, the processed materials were inoculated in to allantoic cavity of 9^{th} -11^{th} days old embryonated SPF chicken eggs for virus isolation. After death of the embryos of the inoculated eggs, the allantoic fluids were tested by spot haemagglutination test for the presence of virus. Out of 385 samples, 84 samples had shown haemagglutination with 1% chick RBC by spot haemagglutination test and identified as WB_1 to WB_{84}. Then the titre of all the 84 isolates was determined by haemagglutination test which showed the HI titre ranging from 2^4 to 2^8. The isolates were identified /confirmed as Newcastle disease virus by haemagglutination inhibition test using Newcastle disease positive antiserum.

Out of 84 isolates of Newcastle disease virus, 24 were randomly selected for serotyping by. mean death time (MDT),intracerebral

pathogenecity index (ICPI) and intravenous pathogenecity index (IVPI) by using 5th passaged virus as test sample. Out of 24 isolates, 20 isolates (WB_1 to WB_{20}) were selected from broiler flocks and 4 isolates (WB_{71} to WB_{74}) from free range rearing flocks. After serotyping it was found that out of 20 isolates from broiler flock 8 (40%) were velogenic, 2 (10%) were mesogenic and 10 (50%) was lentogenic serotype. All (100%) 4 isolates from free range rearing flocks, were found velogenic serotype.

After serotyping EID_{50} of 24 characterized isolates was determined by using 5th passaged virus (allantoic fluid) as test samples and it was found that all the velogenic serotype (12 isolates) having the same EID_{50} ($10^{8.5}$). The EID_{50} of 10, lentogenic serotype isolates varied from $10^{8.5}$ to $10^{8.56}$ and 2 mesogenic serotype isolates showed the value $10^{8.95}$ and 10^9.

From the 10 lentogenic serotype isolates, seed virus for preparation of live attenuated vaccine was selected that 5 lentogenic isolates (WB_1 to WB_5) out of 10 lentogenic isolates having the ICPI value 0.4 with EID_{50} value more than 10^7.

Those 5 selected lentogenic isolates were serially passaged in to 9th to 11th day's old SPF chicken eggs up to 20th passages and tested in 5 days old SPF chicken for attenuation with15th passage allantoic fluid as inoculum. It was found that 16th passage allantoic fluid of WB_2 isolate, 17th passage of WB_3 isolate and 20th passage WB_5 isolate had lost their ability to kill the inoculated chicken. Those 3 isolates i.e. WB_2 (16th passage), WB_3 (17th passage), and WB_5 (20th passage) were then tested for sterility, infectivity, safety and potency for their confirmation for attenuation / modification.

For sterility test, all the 3 isolates were inoculated into nutrient broth and agar for bacterial culture, Sabourauds Dextrose Agar for fungal culture and PPLO agar for mycoplasma culture separately. But macroscopically/microscopically no organism was detected, which indicated that the isolates were free from contamination.

The haemagglutination (HA) activity and EID_{50} of the selected three isolates was determined (i.e. 16^{th} passage of WB_2 isolate, 17^{th} passage of WB_3 isolate and 20^{th} passage WB_5 isolate). The HA titres were 2^7, 2^8 and 2^7 and EID_{50} of all isolates were $10^{8.5}$ respectively.

Safety test was performed and it was found that all the chicken, inoculated with ten times more than the normal recommended doses of virus, survived and no single mortality occurred within the 21 days of observation periods.

Potency test was also performed and found that out of ten control challenged birds, 8 died within 7 days and the other 2 died within 14 days post challenge. But not a single mortality was detected within 21 days of post challenge observation period when the chickens were inoculated with 10^6 EID_{50} of WB_2 (16^{th} passage) and WB_3 (17^{th} passage) virus at the age of 5^{th} and 21^{st} day. But only single mortality was recorded out of 10 chickens, at 21 days of post observation period when the chickens (10) were inoculated with 10^6 EID_{50} of WB_5 (20^{th} passage) virus at the above mentioned age.

Conclusion

1. From the study it is clear that the mortality due to Newcastle disease in free range rearing flocks (farmers generally do not vaccinate their birds) is higher compared to the broiler flocks (broiler birds are generally given a single vaccine at the first week of life). The case fatality rate was also higher, almost double in free range flocks than the broiler flocks. Prevalence of velogenic strain in the field might be due to ill management or zero management in free range rearing flocks. Vaccination against Newcastle disease is not always effective due to problem of delivery of the vaccine in optimum conditions, problem of interference in the development of immunity by other organisms. Thus the farmers are becoming dishearted and reluctant for vaccinating their birds.

2. In the present study it was shown that in the age group of 22 to 42 days old vaccinated broiler birds were more susceptible to Newcastle disease. It might be due to gradual waning of the antibody after single lentogenic vaccine with the age. As the broiler flocks were given a single vaccine at the age of 5-7 days, show second dose of lentogenic strain vaccine is obligatory at three weeks age.

3. From the study, it has also been observed that 43 and above age old free range rearing flocks were more susceptible to Newcastle disease. As the free range birds had a greater exposure to velogenic type of Newcastle disease virus and they were also inconsistently vaccinated; on the top of that there was practically no management and the birds suffered from malnutrition due to heavy parasitic load. Therefore, in order to check mortality it is recommended they should be treated with anthelmintic drugs periodically and vaccination should be a compulsion between 6-8 weeks of age with mesogenic strain of vaccine.

4. Number of Newcastle disease outbreaks were higher during winter (November- February) compared to the summer and Rainy season. The low temperature during the winter season could influence

the disease occurrence i.e. the survival of Newcastle disease virus in the cold and humid environment is longer, compared to the other seasons. The carcass of the free range rearing birds were not disposed hygienically under rural condition which helped in the dissemination of the virus more so in the winter months, when the ambient temperature was low and the viability of the virus was high.

5. At the same time the prevalence of velogenic serotype of Newcastle disease virus was more in chicken which were irregularly vaccinated. Therefore, it is recommended that one vaccination with mesogenic strain should be introduced one month prior to winter season. Detailed studies considering the effects of all other concurrent diseases like parasitic, bacterial, viral, mycoplasmal or fungal etc. which are present in village chicken might influence the occurrence of Newcastle disease.

6. Regarding the prevalence of pathotypes of Newcastle disease virus, it was concluded that velogenic pathotype was highly predominant in free range rearing flocks.

7. From the study it was also shown that three lentogenic isolates from local field cases has been confirmed for its attenuation/modification in the laboratory trials. Further studies are needed in field in large scale before using as master seed for preparation of commercial live attenuated vaccine.

Chapter – 6

Future Scope of Research

FUTURE SCOPE OF RESEARCH

So far the literature is concerned; the control of the highly devastating Newcastle disease is only possible by identification of virus, surveillance and identification of prevalent serotypes along with judicial vaccination programme with effective vaccine. Therefore, it is need of the day to develop a simple, most potent and effective vaccine. The method of vaccination against Newcastle disease with the following programme.

1. Detailed serosurviellence study may be performed in different agro- climatic zone of West Bengal.
2. Continuous isolation of virus along with molecular characterization and strain identification is to be needed.
3. Extensive field trial is required with the isolated attenuated virus before using the same as master seed for preparation of commercial live attenuated vaccine.
4. The attenuated viruses will be subjected to exposure to ambient temperature to be used as oral thermostable vaccine in the form of feed pellet.

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APPENDIX-I

BUFFER AND SOLUTIONS

1. Normal saline solution (NSS)

Sodium chloride (NaCl)	0.85 g
Distilled water	100 ml.

2. Phosphate Buffer Saline (PBS), pH- 7.2

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.20 g
Distilled water	1000 ml

3. Alsever's Solution

D. glucose	2.050 g
Sodium citrate	0.800 g
Sodium chloride	0.420 g
Citric acid	0.055 g
Distilled water	100.00 ml

APPENDIX-II

OIE, FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza

Viale dell'Università, 10 – 35020 LEGNARO (PD)

DOC. NO.: 06RS/1468

Applicant: Prof. Chanchal Guha, Head of Deptt. Of Veterinary Epidemiology and Preventive Medicine. West Bengal University of animal and fishery sciences, Faculty of Veterinary & Animal Sciences.

SAMPLE	ID NO.	ANALYSIS	RESULT
chicken	1	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	5b
chicken	2	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	5b
chicken	3	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	5b
chicken	4	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	5b
chicken	5	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)

		Phylogenetic analysis: lineage**	5b
chicken	6	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	5b
chicken	7	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2
chicken	8	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2
chicken	9	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2
chicken	10	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2
chicken	11	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Negative
chicken	12	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Negative
pigeon	13	RRT-PCR for H9 subtype	Positive
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GRRQKR*F (Velogenic)
		Phylogenetic analysis: lineage**	4a
pigeon	14	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2

pigeon	15	RRT-PCR for H9 subtype	Negative
		V.I.	In progress
		RT-PCR for NDV	Positive
		SEQ.	GGRQGR*L (Lentogenic)
		Phylogenetic analysis: lineage**	2

ABBREVIATIONS

SEQ.= SEQUENCING OF THE CLEAVAGE SITE OF THE F GENE OF NDV
V. I.= VIRUS ISOLATION IN SPF EMBRYONATED EGGS
RRT-PCR = PCR REAL TIME
RT-PCR= REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

**We adopt genotyping method described by Aldous et al., 2003 (reference: Avian Pathology, June 2003, 32(3), 239-257).

Legnaro, Padua, Italy, 24th 2006

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