STUDIES ON THERMOSTABILITY OF NEWCASTLE DISEASE VIRUS (LOCAL ISOLATE) FOR PREPARATION OF VACCINE

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VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

By
DEBDYUTI CHAKRABORTY
B.V.Sc. & A.H.

DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

FACULTY OF VETERINARY AND ANIMAL SCIENCES
37 & 68, KSHUDIRAM BOSE SARANI, KOLKATA- 700 037
WEST BENGAL

2013


Dedicated
To my
Beloved
parents, Teachers
& Well wishers

WEST BENGAL UNIVERSITY OF ANIMAL AND FISHERY SCIENCES
DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PRVENTIVE MEDICINE
FACULTY OF VETERINARY AND ANIMAL SCIENCES
37 & 68, KSHUDIRAM BOSE SARANI, KOLKATA- 700 037
WEST BENGAL

Prof. Chanchal Guha
M.V.Sc., Ph.D.

No: .....
Date: .....

CERTIFICATE

*This is to certify that the thesis entitled " Studies on Thermostability of Newcastle disease virus (local isolate) for preparation of vaccine" submitted by,Debdyuti Chakraborty in partial fulfillments of the requirements for the '**Degree of Master of Veterinary Science 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 24th Day of Oct. 2013

Chanchal Guha

(Prof. Chanchal Guha)
Chairman,
Advisory Committee

APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF MASTER IN VETERINARY SCIENCE IN VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

We, the undersigned, having been satisfied with the performance of Dr. Debdyuti Chakraborty, in the Viva-Voce Examination, conducted today, the 23rd December. ------,2013, recommended that the thesis be accepted for the award of the Degree of Master in Veterinary Science in Veterinary Epidemiology and Preventive Medicine.

Name	Signature
1. Prof. Chanchal Guha Chairman, Advisory Committee	23/12/2013
2. Dr. S. K. Maiti External Examiner	23.12.13
3. Dr. Ujjwal Biswas Member, Advisory Committee	Ujjwal Biswas
4. Dr. Partha Sarathi Jana Member, Advisory Committee	23/12/13
5. Dr. Amaresh Chatterjee Member, Advisory Committee	23/12/13

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Debdyuti Chakraborty.

(Debdyuti Chakraborty

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ABBREVIATION

APMV	Avian Paramyxovirus
AS	Alsever's solution
&	And
B.O.D	Biological Oxygen Demand
CMI	Cell Mediated Immunity
Conc.	Concentration
°C	Degree Celcius
EID_{50}	Embryo Infective Dose fifty
et al.	*et alli* (and others)
FAO	Food and Agricultural Organization
gm	gram
HA	Haemagglutination
HAU	Haemagglutination Unit
HI	Haemagglutination Inhibition
hr	Hour
i.e.	id est (that is)
IAH & VB	Institute of Animal Health & Veterinary Biologicals
$I_g A$	Immunoglobulin A.
Log	Logarithm
mg	milligram
ml	milliliter
M	Molar
min	minutes
µm	microgram
µl	microliter
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NSS	Normal saline solution
Non sp.	Non specific
OIE	Office of International des Epizooties

PMV	Paramyxovirus
PBS	Phosphate Buffer Saline
PVP	Polyvinyl Pyrollidone
PPLO	Pleuropneumonia like organism
%	percentage
rpm	rotation per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RD	Ranikhet Disease
SPF	Specific Pathogen Free
sp.	species
viz.	videlicet (namely)
v/v	volume/volume
WBUAFS	West Bengal University of Animal and Fishery Sciences

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Chapter I

Introduction

Introduction

Poultry Industry in India has now become full-fledged business and is recognized as one of the best industry in animal sector because of its enormous potential to bring rapid growth. The poultry component occupies the top most position as compared to other livestock industry in India. India ranks 3rd and 5th in the world with the annual growth rate of 8% and 15% in respect of egg and poultry meat production (Mohapatra, 2005). The broiler industry recorded faster growth than egg industry (Mohapatra, 2005) which has transformed itself from backyard farming into dynamic agro based industry. It has not merely developed in size but also in productivity and quality. India has one of the world largest and fastest growing poultry industries Real Gross Domestic Product (GDP) grew 6.4 per cent annually during 2000-2008, making India the second fastest growing major economy in the world (Ministry of food processing industry, 2008). The organized sector of poultry industry is contributing nearly 70% of the total output and the rest 30% in unorganized sector, India produces 3.6% of global egg production i.e., 61 million tons (National Meat and Poultry processing Board,2009).

A new chapter began with the integrated poultry operations throughout the country.

- Indian Poultry Industry is booming which is emerging as the world's 2nd largest market.
- Growing at the phenomenal rate of 12 to 15% every year (National Meat and Poultry Processing Board, 2009).
- Poultry Industry in India is constantly on the rise with modern techniques and changing from live bird to fresh chilled and frozen product market.

Family poultry are still very important in developing countries (Branckaert and Guèye 2000; Guèye 2000). Through using available natural resources efficiently, family poultry constitute an important component of the agricultural and household economy in low developing countries, a contribution that goes beyond direct food production for the fast growing human population as well as employment and income generation for resource-poor small farmers, especially women (Guèye 2002a). Furthermore, they are closely linked to the religious and socio-cultural lives of several million resource-poor farmers for whom poultry ownership ensures varying degrees of sustainable farming and economic stability. Therefore, the overall contribution provided by family poultry at household, community and country levels is generally underestimated since the multitude of roles played by poultry in developing countries are generally ignored, in part because they are extremely difficult to assess (Guèye 2002b).

ND or Ranikhet disease or avian pneumoencephalitis or pseudo fowl pest constitutes the most serious epizootic poultry disease throughout the world, particularly in developing countries (Chu and Rizk 1972; Aini *et al.*1990; Demey 1990; Spradbrow 1994, 1996; Bell 1996; Guèye 1998 2002b; Branckaert and Guèye 2000).

It is a highly contagious disease of the respiratory and nervous systems, mostly affecting chicken, but sometimes also affecting other poultry species, such as guinea fowls, ducks, turkeys, etc. It often devastates unvaccinated family poultry flocks in periodic outbreaks. The disease is caused by avian paramyxovirus type-I, with the most virulent strains isolated in Africa (Bell 1996; Spradbrow 1996; Verwoerd 1996).

ND is the one disease which is well known by most family poultry-keeping farmers. Family poultry-keeping farmers are at least usually well

aware of the virulence of ND, especially in chicken; the disease frequently leaves no survivors in unvaccinated flocks.

According to most farmers, ducks and geese and, to a smaller extent, guinea fowls are more resistant to the disease. Moreover, the season has an effect on the severity of ND, as it seems to flare up in village chicken flocks during the rainy seasons in two East African countries, i.e. Kenya (Anonymous 1997/1998) and Ethiopia (Sonaiya *et al*. 1999), while in West and Central Africa major outbreaks seem to occur generally during the dry seasons (Grundler *et al*. 1988; Mukiibi Muka 1992; Yongolo 1996; Bonfoh 1997; Guèye 1998).

No progress has been made in controlling ND in free-ranging village flocks, which represent more than 80 percent of the total poultry population in developing countries (Branckaert and Guèye 2000). In most developing countries, ND occurs every year and kills on average 70 to 80 percent of the unvaccinated rural family poultry (Branckaert and Guèye 2000). Therefore, ND control can appropriately be used as an entry-point for developing the family poultry sub-sector as a whole (Guèye 2002b).

Vaccination is the most effective means of controlling ND and has been used throughout the world since 1940 (Beard and Hanson, 1984). Now-a-days, various pharmaceutical private agencies and State Biological are producing ND vaccines in India, consisting of live attenuated LaSota strain, B1 strain, F-strain and ND VH strain. These are lentogenic strains of ND vaccine. 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 some problems like i) Heat labiality of vaccine strain of viruses, ii) Large dose presentation, iii) Affordability, iv) Cold chain for effective administration of the vaccine and v) 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 even in vaccinated flocks. Therefore, to prevent such type of economic loss by sudden outbreak of ND, it is very important to develop a simple but absolutely effective vaccine and easy to administration against the disease. Live vaccines are easy to apply and relatively inexpensive, and give moderately good immunity. Vaccinal reactions to them vary according to the vaccine strain. Among the live vaccines, the thermostable vaccines require less stringent transport requirements in the field.

The ACIAR sponsored project for vaccination against ND by using Australian V_4 and I_2 strains (ND asymptomatic pathotypes) in thirty countries in SEA, Africa and Austria. The protection in the birds is satisfactory but not desired level (Biswas *et al.* 1996).In this context, thermostable strains should be better for local field and use for vaccine preparation that may be more effective against Newcastle disease.

The Australian Centre for International Agricultural Research (ACIAR) has supported projects on the vaccination of village chicken since 1984. The original concept was the production of a thermostable strain of Newcastle disease virus, developed by artificial selection.

Hofstad and Yoder (1963) had suggested that robust Newcastle disease vaccines might be produced by seeking strains of virus that posses superior thermostability. Heat-resistant strains of Newcastle disease virus had actually been produced in the 1950s for use as phenotypic markers in genetic experiments (Goldman and Hanson, 1955).

Thermostability is a relative term. It has been unfortunate that thermostable vaccines have sometimes been considered as another basic commodity, like a sack of rice that needs no special treatment.

Thermostable vaccines have some viability away from the cold chain, but transport conditions should still be as cool as possible. When the cold chain ends, thermostable vaccines should be transported in wet cloth, preferably in an open weave basket. Beside the base of water pot is often a cool storage position in a hut.

In view of the above facts the present programme was conducted on the basis of the following objective:-

1. To test the thermostability of ND virus (local isolate) in different temperatures.

The following technical programme was undertaken to meet the objectives:

i. Determination of HA titre of virus isolate following serial passaging in embryonated SPF fowl eggs as per OIE, 2009.

ii. Determination of EID_{50} of the virus having highest HA titre before thermal exposure.

iii. Determination of HA titre and EID_{50} of virus in small aliquots (1 ml) following first thermal exposure at 25^0C for 36 days in B.O.D. incubator at 2 days interval.

iv. Determination of HA titre and EID_{50} of the residual virus after the end of the first cycle following serial passaging in embryonated SPF fowl eggs as per OIE, 2009.

v. Determination of HA titre and EID_{50} of virus in small aliquots (1 ml) following second thermal exposure at 37^0C for 29 days in B.O.D incubator at 2 days interval.

vi. Determination of HA titre and EID_{50} of the residual virus after the end of the second cycle following serial passaging in embryonated SPF fowl eggs as per OIE, 2009.

vii. Determination of HA titre and $EID_{50}$of virus in small aliquots (1 ml) following third thermal exposure in B.O.D incubator at 56^0C after 5 mins,10mins,15 mins,30 mins,1hr,2hrs,4 hrs and 8 hrs.

viii. Determination of HA titre and EID_{50} of the residual virus after the end of the third cycle following serial passaging in embryonated SPF fowl eggs as per OIE 2009.

Chapter II

Review of Literature

Review of Literature

2.1. Prevalence of Newcastle Disease:

Eidson *et al.* (1982) reported Newcastle disease (ND) as an important avian disease that had an economic impact on the poultry industry.

Bennejean (1988) observed that Newcastle disease had affected the international trading of poultry and poultry products around the world.

Kaleta and Baldauf (1988) observed that several epizootiological studies pointed to these avian species and village chicken as important factors in transmission and enzootic maintenance of NDV in various localities.

Spradbrow (1990) reported the Newcastle disease as almost worldwide in distribution except Australia and Scandinavian countries like Denmark, Norway, Sweden and Finland.

Alexander (1991) reported that the pathogenicity of the NDV isolates and strains varies markedly with the host.

Alexander and Gough (2003) stated that Newcastle disease is caused by a virus i.e. avian paramyxovirus type-1 (APMV-1) of the genus Avulavirus belonging to the family Paramyxoviridae and also reported that Newcastle disease (ND) as one of the most important viral diseases of poultry worldwide which may causes 100% mortality in susceptible chicken.

Alexander and Senne (2008) designated ND virus (NDV) as APMV-1 and grouped into five pathotypes i.e. Viscerotropic velogenic, Neurotropic velogenic, Mesogenic, Lentogenic and Asymptomatic on the basis of the clinical signs observed in infected chicken.

Miller *et al.* (2010) reported that the paramyxoviruses isolated from avian species had been classified by serological testing and phylogenetic analysis into ten subtypes designated APMV-1 to APMV-10.

2.2. Control of Newcastle Disease (ND):

Simi *et al.* (1970) stated that the vaccine stability is defined by the length of time the vaccine retains an infectivity titer sufficient to induce a protective immune response at a particular temperature.

Allan *et al.* (1973) stated that the stability of the ND virus is crucial to its value as a vaccine strain.

Hanson and Spalatin (1978) reported that a minimum of 70% of flocks in high risk areas must be included in sanitary and combined vaccination programs if control is to be effective.

Meulemans (1988) reported that effective control of Newcastle disease requires good sanitation, management, quarantine, an appropriate vaccination program, and monitoring system, including serotyping and pathogenicity testing of isolated virus he also reported that vaccination is the most important method of disease control particularly to decrease mortality from ND and vaccination results in a quite significant increase in chick survival from 30% to 60%.

Aini *et al.* (1990a) reported that the conventional vaccines are not heat stable; therefore, require complex cold- chains to link the vaccine producers and users.

Alexander (1997) reported that in some countries, the Newcastle disease can be controlled; nevertheless, it can cause economic loss due to the expense of vaccination and disease surveillance.

Chen and Wang (2002) reported that the commercial conventional vaccines available for the control of the disease are heat labile and hence cannot be used in the rural areas as to provide cold-chain facilities is practically impossible, coupled with the behavior of the rural scavenging chicken. Among the live vaccines, the heat resistant vaccines require less stringent transport requirements in the field, and they have also been widely used in villages.

Alders (2002) carried out a study on the Control of Newcastle Disease in Village Chicken Using Thermotolerant Vaccines.

Usman (2002) reported that the conventional vaccines are not heat stable; therefore, require complex cold- chains to link the vaccine producers and users.

Young *et al.* (2002) stated that the vaccine stability is defined by the length of time the vaccine retains an infectivity titre sufficient to induce a protective immune response at a particular temperature.

Adwar and Lukesova (2008) reported that the commercial conventional vaccines available for the control of the disease are heat labile and hence cannot be used in the rural areas since the provision of cold-chain facilities is practically impossible, coupled with the behavior of the rural scavenging chicken. Among the live vaccines, the heat resistant vaccines require less stringent transport requirements in the field, and they have also been widely used in villages.

Spradbrow (2011) reported that thermostable/heat stable Newcastle disease vaccines, suitably applied in village chicken of rural and peri-urban sector have proved effective in many trials under laboratory conditions and in villages.

2.3. Thermostable live Newcastle disease vaccines:

Hanson *et al.* (1949) concluded from detailed thermostability studies on heamagglutinins from NDV strain of different virulence that at 56^0C heamagglutinins of lentogenic strain proved to be heat labile whereas those mesogenic and velogenic strains maintain their activity for at least 30 minutes.

Turner and Kovesdy (1974) reported that surviving chicken showed no respiratory illness during 14 days of observation after vaccination with thermostable vaccine and yet exhibited a marked rise in antibody titres following challenge.

Lomniczi (1975) studied the thermostability of various strains of Newcastle disease virus (NDV) and observed that most of the strains lost their infectivity on exposure to 50–55^0C for 30 minutes.

Turner *et al.* (1976) reported that the mucosal immune system in addition to humoral immune responses was thought to be involved in the immunity and protection induced by NDV_4 vaccine.

Allan *et al.* (1978) showed that V_4HR-ND vaccine was prepared by chicken embryo propagation following standard method of vaccine production of FAO.

Kim and Spradbrow (1978) described that Australian V_4 strain thermostable vaccine of Newcastle disease virus had an inherent degree of thermostability.

Robyn Schalkoort (1979) reported that Australian V_4 strain thermostable vaccine of Newcastle disease virus responded to selection for heat-resistance.

Schalkoort and Spradbrow (1980) reported that under experimental conditions V_4 strain thermostable vaccine has been effective as an aerosol.

Spradbrow *et al.* (1980) reported that surviving chicken showed no respiratory illness during 14 days of observation after vaccination with thermostable vaccine and yet exhibited a marked rise in antibody titres following challenge.

Ibrahim *et al.* (1981) reported that preliminary work with food-coated V4 virus vaccine administered to chicken showed that it was possible to deliver the vaccine virus to chicken on food.

Ibrahim *et al.* (1981) reported that surviving chicken showed no respiratory illness during 14 days of observation after vaccination with thermostable vaccine and yet exhibited a marked rise in antibody titres following challenge.

Westbury (1981) reported that V_4HR strain was suitable as vaccine strain which produced an adequate serological response following mass administration to commercial meat chicken.

Westbury *et al.* (1984) assessed that among the strains V_4, Hitchner B1 and La Sota, V_4 was most affected by circulating maternal in chicken with

maternal antibody to NDV antibody. They found out that strain V_4 was less immunogenic than Hitchner B1 and LaSota when used.

They also reported that surviving chicken showed no respiratory illness during 14 days of observation after vaccination with thermostable vaccine and yet exhibited a marked rise in antibody titres following challenge.

Claxton and Leonard (1987) reported that V_4 virus lost less than 1 $\log_{10}$ unit after storage for 1 hour at 50^0C and less than 3 $\log_{10}$ units after 6 hours.

Ideris *et al.* (1987) reported that NDV4-HR vaccine is a living vaccine with the following characteristics – it is thermostable, retaining its activity for 12 weeks at a temperature of 28°C in freeze-dried form.

Ideris (1989a) reported that the Malaysian thermostable variant of V_4 survived exposure at 56^0C for at least 9 hours.

Ideris (1989b) noted that polyvenyl-pyrollidone (PVP) was more effective than skim milk, gelatine, methylcellulose or carboxy-methylcellulose for long-term protection (3 weeks) of Malaysian thermostable variant of V_4 strain at 20-25^0C.

Aini *et al.*(1990b) reported that chicken vaccinated under laboratory conditions with food-based thermostable vaccine in Malaysia showed better than 90% protection.

Bell *et al.* (1991) reported that V_4HR strain was suitable as vaccine strain which produced an adequate serological response following mass administration to commercial meat chicken

Health *et al.* (1991) reported that commercial vaccine was stable in freeze-dried form for 3 months at 18-22^{0}C, and lost only 0.3 $\log_{10}$ on storage for a further 3 months.

Jagne *et al.* (1991) reported that chicken receiving the oral thermostable vaccines produced antibodies suggestive of protection, while there was no antibody response in control chicken.

Anon (1991) reported that the biological safety of NDV_4HR vaccine is superior to that of other living ND vaccine strains such as B1 or LaSota.

Aini *et al.* (1992) reported that under stimulated village conditions and under real village conditions with thermostable vaccine delivered by farmers, protection rates were about 60%.

Spradbrow (1992a) reported from several studies that it is possible to select virus subpopulations with heat resistance for production of a robust vaccine that can be taken into the field with minimum dependence on cold chains.

Spradbrow (1992b) reported that extreme heat selection gives rapid results and it is assumed that virions that survive at 56^{0}C will certainly for 10 passages.

Alders *et al.* (1994) observed that the vaccine strain can be transmitted by contact from vaccinated to non-vaccinated birds.

Jayawardane and Spradbrow (1995a) demonstrated cell-mediated immunity developed in chicken vaccinated with V_4 vaccine.

Jayawardane and Spradbrow (1995b) reported that oral vaccination of chicken with V_4 vaccine induced the production of IgA antibody and an associated mucosal immunity

Uruakpa (1997) observed that in favourable diluents (with lactose, sodium glutamate and bovine serum albumin) I_2 lost only 0.3 $\log_{10}$ units on storage for 1 month at 22^0C.

Olabode (1998) reported that thermostable V_4 strain vaccine remained viable for over 3 months on storage at 20^0C.

Tran Dinh Tu *et al.* (1998) reported that the I_2 vaccine was superior than La Sota vaccine for village use because of its ready spread by contact.

Tu *et al.* (1998) reported that the thermostable variants of Newcastle disease virus strains V_4 and I_2, spread rapidly from vaccinated chicken to village chicken. He also reported that freeze-dried I_2 vaccine lost about 1 $\log_{10}$ unit of infectivity when stored for 6 days at 26-32^0C.

Bensink and Spradbrow (1999) reported that I_2 strain vaccine can be produced and stored in liquid form, and suitably diluted in a protective solution such as 1 % gelatin in which the vaccine will maintain its activity for at least 12 weeks at 22°C before use.

Iroegbu and Nchinda (1999) reported that although the carrier food need not be nutritious for it to be effective in conveying the thermostable vaccine virus, it ought to be palatable and desirable to the chicken.

Alders and Spradbrow (2000) stated that the development of thermostable vaccine might offer an opportunity to improve ND vaccination strategy for poultry in tropical countries.

Kinde *et al.* (2000) observed that the progenies derived from passing ND virus strains through thermal cycles show greater heat stability than the parent virus.

Nssien and Adene (2000) carried out a study to determine the stability of HA titres of reconstituted form of Hitchner – B1 (B1), LaSota (L) and Komarov (K) strains of Newcastle Disease Vaccine (NDV) at 36^0C. They observed that there is declining the HA titre on the basis of a two-step (2 $\log_2$) titer as evidence of loss of stability of HA titres (LST), the LST therefore, occurred at 50^{th}, 24^{th} and 95^{th} hour for BI, L and K strains, respectively, post – temperature exposure.

Wambura *et al.* (2000) reported that in Tanzania, I_2 strain vaccine has given protection for at least two months after vaccination.

Alders and Spradbrow (2001) stated that the thermostable vaccine enables distributors and users to reduce the problems associated with inadequate cold chains in the field, so it is essential that users understand that a thermostable vaccine must still be treated with some of the respect due to a biological product, that is the vaccine cannot expose to sunlight and frequent shifts in temperature and still expect it to remain active.They reported that thermostable I_2 strain vaccine underwent laboratory tests in several countries and proved to be protective against local virulent strains of the ND virus. They also reported that field records in Mozambique indicated I_2 ND vaccine provided approximately 80 % protection in the field in the face of an outbreak, when given every four months via eye-drop.

King (2001) reported that 38% of field ND virus isolates were more thermostable than the NDV I/O and NDVL strains.

Chamling Rai *et al.* (2002) reported that the I_2 ND vaccine has been registered as the ND vaccine for village chicken, following successful local laboratory and village trials in Bhutan.

Kafi (2003) reported that the V_4 ND virus spread readily among chicken, causes no disease and protect against challenge with virulent strains of NDV.

Rahman *et al* .(2003) carried out a study with thirty days old chicks of cobb-100 breed with the history of vaccination of parent stock against Newcastle disease and they equally divided the chicks into two groups i.e. group A and B. At seven days of age, birds of group A were vaccinated with experimentally prepared vaccine V_4HR-ND vaccine via eye drop @ 10^8 EID_{50}/bird to determine the efficacy of the vaccine while those of group B were kept as unvaccinated control. The result showed that V_4HR-ND vaccine conferred 60% protection of vaccinated birds against challenge infection.

Azzam (2007) reported that *Dongola* strain could provoke comparable immunogenicity and replace LaSota strain in vaccination of chicken. Unfortunately the strain was found thermolabile.

Echeonwu *et al.* (2007) observed the efficacy of treated broken millet grains as a carrier for delivery of thermostable Newcastle disease (ND) vaccine HRV_4 to free-range chicken in three locations and it was assessed by haemagglutination inhibition (HI) test and challenge experiment. Out of 256 birds fed with first dose of the vaccine, 130 (50.8%) produced detectable HI antibody but only 16 (6.3%) attained serum antibody level of log 2 to log 3 adjudged protective. From the locations, Igumale (74 birds), Kuru (88 birds), Riyom (94 birds), only 1 (1.4%), 8 (9.1%) and 7 (7.4%) attained log 2 titre to log 3 titre respectively.

Ibu *et al.* (2009) carried out a study at 56^0C temperature with 12 field virus isolates and 5 vaccine virus strains to assess the Haemagglitinin thermostability of Newcastle disease virus isolates obtained from wild birds in three climatically distinct states in central Nigeria. The result showed that 3 field isolates were inactivated in 5 minutes, 3 in 10 minutes and 1 in 15 minute. The most thermostable of the field isolates was inactivated in 40 minute. For the vaccine strains, NDV (I/O) B1 and NDV (K) were inactivated in 20 minutes while NDV (L) was inactivated in 40 minute. Thermostable strains NDV_4 and NDI_2 were inactivated in 90 minutes each.

Islam and Abdellatif (2011) carried out a series of selection procedure at 56^0C to enhance heat resistance and observed that extreme heat selection was better since infectivity titre of the 10^{th} passage decreased by 1 logarithmic order within 15min of incubation at 56°C, while the titre decreased by 2 logarithmic orders in stepwise method.

2.4. Routes of administration and dose of thermostable ND vaccine:

Ibrahim *et al.* (1981) reported that it was possible to deliver the V_4 strain vaccine to chicken on food-coated.

Saglid *et al.* (1982) reported that heat stable avirulent strain V_4 and I_2 strains applied through eye drop, drinking water, food or feed particles had been found to be a suitable oral vaccine for village chicken. They also reported that application of thermostable vaccine by mouth drop has also proved effective.

Spradbrow *et al.* (1988) demonstrated that birds that received a higher oral dose of I_2 strain vaccine generated a higher immune response similar to that of NDV_4-HR vaccine when confined in cages with wire floors.

Aini *et al.* (1990) reported that 60% protection rate was recorded on challenge of village chicken with virulent NDV strain in Malaysia and farmers have been benefited by oral food vaccination in their flocks with NDV_4HR.

Anon (1991) reported that NDV_4HR vaccine can be administered via eye-drop (intraocular), nose-drop (intranasal), oral drench, or drinking water; mixed with certain feeds or by injection and is an avirulent strain and can be safely administered to chicken of any age from day-old chick to adult birds and its biological safety was superior to that of other living ND vaccine strains such as B1 or LaSota.

Cumming (1992) suggested that short boiling; washing and coarse cracking of the grain might significantly extend the survival of the virus on the grain.

Darminto and Daniels (1992) in Indonesia reported that application of thermostable vaccine by mouth drop has also proved effective.

Samuel *et al.* (1992) found that uncooked grains were not entirely satisfactory as vaccine carriers and showed that vaccine, washed off immediately after addition to grains, had lost at least 90% of its initial virus titer.

Tantaswasdi *et al.* (1992) reported that application of thermostable vaccine through the drinking water usually gave good result, sometimes similar to those obtained with eye drop vaccines

Spradbrow (1993) reported that NDV_4HR vaccine can be administered via eye-drop (intraocular), nose-drop (intranasal), oral drench, or drinking water; mixed with certain feeds or by injection and an avirulent strain can be safely administered to chicken of any age from day-old chick to adult birds and its biological safety was superior to that of other living ND vaccine strains such

as B1 or LaSota. He also suggested that an ideal vaccine carrier food should be cheap, readily available in the target locality and should not contain substances that would inactivate the vaccine virus.

Bell *et al.* (1995) found that a single eye drop administration of NDV_4HR vaccine provided acceptable protection of village chicken in Cameroon.

Rehmani *et al.* (1995) described experimental lactose-based pellets that were effective as a food carrier for thermostable vaccine.

Foster *et al.* (1996) observed that thermostable V_4 strain vaccine supplied on bolied sorghum to village chicken gave only low levels of protection in central Tanzania.

Anonymous (1997) reported that Newcastle disease was largely controlled in Malaysia villages while V_4 strain vaccine was supplied on food (pellets and later wheat).

Salum *et al.* (1997) reported that cassava granules were found to be an adequate food carrier for thermostable V_4 strain vaccine in Southern Tanazania.

Anonymous (1998) observed that the most effective way to administer thermostable Newcastle disease vaccines was by eye drop.

Nasser (1998) reported that oral application of NDV-I_2 was also shown to be effective with barley as vaccine carrier, if barley was pre treated by parboiling.

Tu *et al.* (1998) reported that application of thermostable vaccine through the drinking water usually gave good result, sometimes similar to those obtained with eye drop vaccines.

Foster *et al.* (1999) carried out a study with groups of chicken in rural villages received V_4 vaccine by introduction to the conjunctival sac, in drinking water or on food. Control chicken received no vaccine. Results were judged by serology and survival after artificial challenge. After conjunctival vaccination 55 of 70 chicken had levels of antibody indicative of protection and 8 of 11 survived challenge. The respective figures for water vaccination were 45 of 64 and 8 of 11. For control chicken only 5 of 56 had levels of antibody presumed to be protective and 0 of 10 survived challenge.

Foster *et al.* (1999) reported that application of thermostable vaccine through the drinking water usually gave good result, sometimes similar to those obtained with eye drop vaccines.

Alders and Spradbrow (2001) reported that field records in Mozambique indicated that I_2 ND vaccine provided approximately 80% protection in the field in the face of an outbreak, when given every four months via eye-drop .

Alders (2005) observed that I_2 strain vaccination by eye-drop resulted in the highest titer development. The mean protection that could be reached in the field was 80%.

Chapter III

Materials and Methods

Materials and Methods

3.1. MATERIALS

3.1.1. Glass wares

The glass wares used for the study were procured from M/s. Borosil, India Pvt. Ltd. that underwent standard sterilization procedures prior to their use.

3.1.2. Plastic wares

Ready to use, polypacked, ultraviolet irradiated, plastic wares were procured from M/s. Tarson India Pvt. Ltd. for the study.

3.1.3. Biologicals

3.1.3.1. Source of the virus for the vaccine production

Viruses used in this study for thermostability testing were isolated, characterised and provided by the Department of Veterinary Epidemiology and Preventive Medicine, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata-37.

3.1.3.2. Embryonated Specific Pathogen Free (SPF) fowl eggs

Embryonated SPF fowl eggs were procured from Venky's (India) Ltd., SPF Eggs Division, Pune for propagation of virus as recommended by OIE, 2010. The embryonated SPF fowl eggs were temporarily stored in the BOD incubator at 15°C with a relative humidity of 60 percent prior to their incubation.

3.2. METHODS

3.2.1. STERILIZATION

3.2.1.1. Sterilization of Glass wares

The glass wares were first washed with the detergent solution followed by rinsing with tap water several times. Then washed glass wares were rinsed with triple glass distilled water. After drying the mouth of glass wares was wrapped with aluminium foil and sterilized in the hot air oven at 180°C for 30 minutes.

3.2.1.2. Sterilization of the laboratory

Day prior to any experimental work, the laboratory was cleaned with the disinfectant solution followed by fumigation overnight. Special asepsis was maintained throughout the study period by ultraviolet irradiation of the laboratory one hour prior to any experimental work.

3.2.2. SEROLOGICAL STUDY

The allantoic fluid collected from each serial passage was tested for haemagglutination activity using methods as per the standard protocol mandated by OIE, 2010.

- Spot/slide Haemagglutination test
- Standard plate Haemagglutination test (HA)

3.2.2.1. Preparation of 0.5% chicken RBC

i) 2 ml blood was drawn aseptically from unvaccinated SPF layer bird, known to be free from antibody of NDV, and mixed with equal volume of Alsever's solution.

ii) Blood was centrifuged at 1500 rpm for 10 minutes and the supernatant fluid was discarded.

iii) The cells were suspended with double volume of PBS (0.1 Molar, pH-7.2), washed and centrifuged thrice at 1500 rpm for 10 minutes.

iv) The packed RBCs were resuspended in PBS to have a 0.5% v/v suspension.

3.2.2.2. Spot/slide agglutination test

One drop of allantoic fluid, collected during harvesting of virus, was dropped on a grease free glass slide. Freshly prepared 0.5% chick RBC suspension was added to the allantoic fluid in 1:1 ratio (50% v/v). Both the suspensions were mixed thoroughly by rotating the slide gently. The slide was examined by diffused light to see any haemagglutination.

3.2.2.3. Standard Plate Haemagglutination Test (HA) as per OIE (2009)

a) 25µl of PBS (0.1 M, pH-7.2) was dispensed into each well of a plastic V-bottomed microtitration plate.

b) 25µl of infective allantoic fluid (virus suspension) was placed in the first well.

c) 2 fold serial dilution of 25µl volume were made across the plate up to 11^{th} well. 12^{th} well was kept as control.

d) 25µl of 0.5% (v/v) chicken RBC was dispensed to each well.

e) The solution was mixed by gentle tapping of the plate and RBC's were allowed to settle for about 40 minutes at room temperature (20°C-26°C).

f) Haemagglutination was determined by tilting the plate and observing the presence of tear shaped streaming of RBC's.

g) The highest dilution showing complete haemagglutination represented one haemagglutination unit (1HAU)

3.2.3 . Determination of Embryo Infective Dose fifty (EID_{50}) of the virus as per Reed and Muench (1938) and FAO (2002)

Embryo infective dose fifty (EID_{50}) was performed to measure the concentration of the virus isolate (live attenuated virus) in a suspension. The concentration of the live virus in a suspension was expressed as the infectivity titre. The infectivity titer was established by carrying out a titration as per the method of FAO, 2002.

To determine the EID_{50} of the virus isolate, a series of tenfold dilutions were carried out. Five embryonated SPF fowl eggs were inoculated each with 0.1 ml of each dilution from 10^{-6} to 10^{-10}. After incubation for 8 days, the allantoic fluids were harvested and the HA activity was assessed to determine whether or not the virus had infected and multiplied in each of the inoculated egg. On the basis of HA test, for each dilution, it was recorded that number of eggs infected (HA +ve) and the number of eggs not infected (HA –ve) by the inoculums.

The Reed and Muench (1938) mathematical technique was used to calculate the end point from the results of the HA test on each of the inoculated egg. A formula was used to calculate an index (proportionate distance) that was applied to appropriate distance. The infectivity titre was expressed as EID_{50}/ml.

3.2.4. Thermostability testing of the virus isolate as per Aini Ideris (1992)

i) In the first cycle, vials containing virus were placed in B.O.D incubator at 25^0C for 36 days. The HA test was performed at every 2 days interval.

ii) At the end of the first cycle the virus was passaged in embryonated SPF eggs. In the second cycle, the virus was kept at 37^0C for 29 days and HA activity was checked at every 2 days interval. After

end of the second cycle the virus again inoculated in embryonated SPF eggs.

iii) In the third cycle, the virus was exposed to 56^0C for different time period i.e. 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours and HA activity was performed.

iv) At the end of the third cycle the virus was passaged in the embryonated SPF egg and HA activity was checked and virus was stored at -70^0c for further use.

3.2.4.1. Determination of Embryo Infective Dose fifty (EID_{50}) of the virus after thermostability testing

The Embryo Infective Dose fifty (EID_{50}) was determined as per the FAO, 2002 and Reed and Muench, 1938.

Chapter IV

Results and Discussion

Results and Discussion

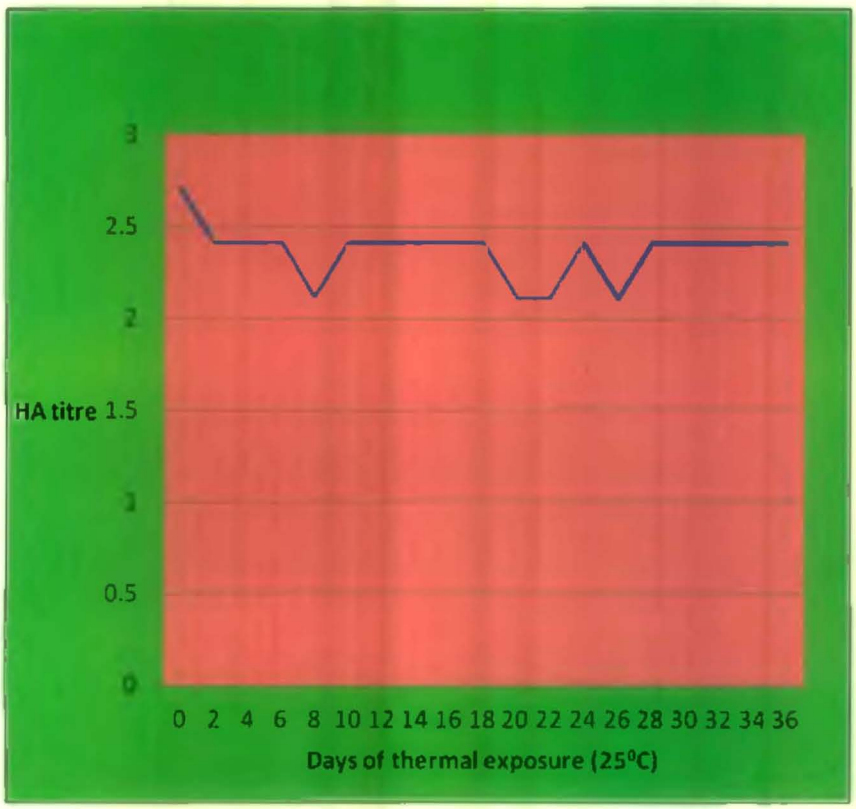
4.1. *The HA titre of the virus in the first thermal cycle:*

The virus was exposed as 1 ml aliquot in 20 vials at 25^{0}C for 36 days. The HA titre was determined at every 2 days interval from 1 ml aliquot as per OIE (2009).The results were presented in table 1 and figure 1.

Table 1. Showing HA titre of the virus in the first thermal cycle at 25^{0}C for 36 days

Serial no.	Period of thermal exposure(in days)	HA titre	Log value of HA titre
1.	0(before thermal exposure)	2^9	2.71
2.	2	2^8	2.41
3.	4	2^8	2.41
4.	6	2^8	2.41
5.	8	2^7	2.11
6.	10	2^8	2.41
7.	12	2^8	2.41
8.	14	2^8	2.41
9.	16	2^8	2.41
10.	18	2^8	2.41
11.	20	2^7	2.11
12.	22	2^7	2.11
13.	24	2^8	2.41
14.	26	2^7	2.11
15.	28	2^8	2.41
16.	30	2^8	2.41
17.	32	2^8	2.41
18.	34	2^8	2.41
19.	36	2^8	2.41

Fig 1: Showing HA titre (log_2 basis) of the virus up to 36 days of thermal exposure at 25^0C



From the above table it was evident that the pre-thermal exposed Heamagglutination (HA) titre of the virus was very good i.e. 2^9 (log value2.71). Similar observations expressed by Biswas (2006), Chhetri (2010), Biswas (2011) and Sharma (2012).During thermal exposure in the first cycle the HA titre was screened every 2 days interval and observed that the HA titre was slightly decreased i.e.2^8 (log value 2.41) or sometimes 2^7(log value) which were remained in satisfactory level.

Literature regarding HA titre of thermal exposed virus at 25^0C for 36 days was scanty. Therefore, from the result it was concluded that the virus (local isolate) withstand the 25^0C for long time (36 days) without losing its viability/infectivity.

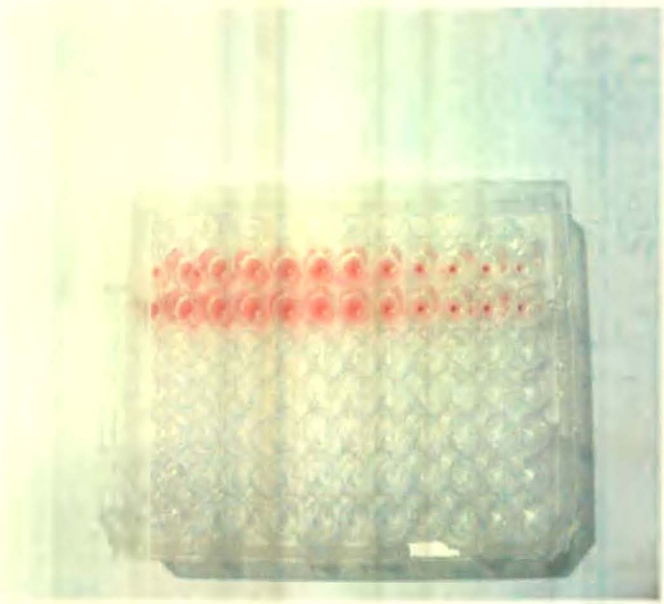


Fig 2. Showing HA titre of the virus in the first thermal cycle

4.2. *EID_{50} of the first thermal cycle:*

The virus was exposed as 1 ml aliquot in 20 vials at 25^0C for 36 days.The EID_{50} was determined before thermal exposure and subsequently every 2 days interval of thermal exposure from one aliquot as per Reed and Muench(1938) and FAO (2002).The results were presented in table 2 ,3 ,4 and 5 and in figure 3.

Table 2. Estimation of EID_{50} of the virus before thermal exposure in the first cycle

Dilution of virus (inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ve)	No.of eggs not infected (HA, -ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	4(1nonsp. death)	3	0	8	0	8	8/8	100
10^{-7}	4(1nonsp. death)	3	0	5	0	5	5/5	100
10^{-8}	4	2	2	2	2	4	2/4	50
10^{-9}	4	0	4	0	6	6	0/6	0
10^{-10}	4	0	4	0	0	10	0/10	0

From the above table it was clear that the calculation of EID_{50} was made on the basis of infected (HA positive) and not infected (HA negative) embryos.

The accumulated numbers for infected embryos were calculated on the assumption that, if the host system was affected at particular dilution, it would also be affected at the next lowest dilution, which had a greater concentration of virus and vice-versa was true for not infected embryos.

From the above table it was seen that 50% end point was at 10^{-8} dilution.

Therefore, the 50% end point dilution = 10^{-8}

The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titer of the virus was $10^{8}EID_{50}$/ 0.1 ml or $10^{9}EID_{50}$/ml.

From the above calculation it was evident that the EID_{50} (i.e. total number of viable virus per ml) of the virus before thermal exposure to the first cycle was $10^{9}EID_{50}$/ml. Similar observation expressed by Biswas (2006), Chettri (2010), Biswas (2011) and Sharma (2012).

From the result it was observed that the EID_{50} of unexposed virus was $10^9 EID_{50}$/ml which was satisfactory.

Table 3. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus after 2 days thermal exposure at 25^0C of first cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ ve)	No.of eggs not infected (HA, - ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	3(1non sp. death)	2	0	6	0	6	6/6	100
10^{-7}	3	2	1	4	1	5	4/5	80
10^{-8}	3	1	2	2	3	5	2/5	40
10^{-9}	3	1	2	1	5	6	1/6	16.67
10^{-10}	3	0	3	0	8	8	0/8	0

From the above table it was seen that 50% end point was somewhere in between 10^{-7} (80%) and 10^{-8} (40%)) dilution. By using the following formula the proportionate distance (PD) between 10^{-7} to 10^{-8} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 80\text{-}50/80\text{-}40$$

$$= 30/40$$

$$= 0.75$$

The end point dilution was expressed as EID_{50} per dose of inoculum. Here, the dose was 0.1 ml and therefore, the infectivity titer of the virus was $10^{7.75}$ EID_{50}/ 0.1 ml or $10^{8.75}$ EID_{50}/ml.

Literature was scanty in relation to the 2 days thermal exposure of virus at 25^0C in the first cycle.

Table 4. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus in the first cycle after 36 days of thermal exposure at 25^0C

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA,+ ve)	No.of eggs not infected (HA, -ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	3	2	1	6	1	7	6/7	85.7
10^{-7}	3	2	1	4	2	6	4/6	66.67
10^{-8}	3	1	2	2	4	6	2/6	33.33
10^{-9}	2	1	1	1	5	6	1/6	16.67
10^{-10}	2	0	2	0	7	7	0/7	0

From the above table it was seen that 50% end point was somewhere between 10^{-7} (66.67%) and 10^{-8} (33.33%) dilution. By using the following formula the proportionate distance (PD) between 10^{-7} to 10^{-8} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 66.67\text{-}50/66.67\text{-}33.33$$

$$= 16.67/33.33$$

$$= 0.50$$

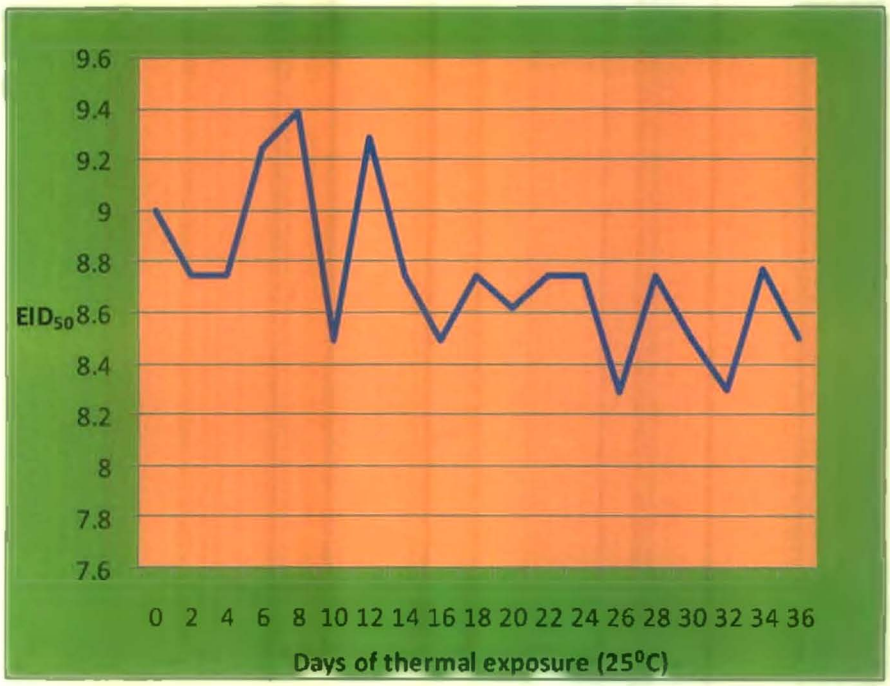
The end point dilution is expressed as EID_{50} per dose of inoculum. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titer of the virus was $10^{7.50}$ EID_{50}/ 0.1 ml or $10^{8.50}$ EID_{50}/ml.

Literature in this aspect was scanty in relation to the 36 days thermal exposure of virus at 25^0C in the first cycle.

Table 5. Showing EID_{50} of the virus in the first thermal cycle at 25^0C for 36 days

Serial no.	Period of thermal exposure (in days)	EID_{50}	Log value of EID_{50}
1.	0(before thermal exposure)	10^9	9.00
2.	2	$10^{8.75}$	8.75
3.	4	$10^{8.75}$	8.75
4.	6	$10^{9.25}$	9.25
5.	8	$10^{9.39}$	9.39
6.	10	$10^{8.49}$	8.49
7.	12	$10^{9.29}$	9.29
8.	14	$10^{8.75}$	8.75
9.	16	$10^{8.49}$	8.49
10.	18	$10^{8.75}$	8.75
11.	20	$10^{8.62}$	8.62
12.	22	$10^{8.75}$	8.75
13.	24	$10^{8.75}$	8.75
14.	26	$10^{8.29}$	8.29
15.	28	$10^{8.75}$	8.75
16.	30	$10^{8.50}$	8.50
17.	32	$10^{8.30}$	8.30
18.	34	$10^{8.77}$	8.77
19.	36	$10^{8.50}$	8.50

Fig 3: Showing EID_{50} (Log_{10} basis) of the virus up to 36 days of thermal exposure at 25^0C in the first cycle



From the table 5 and figure 2 it was observed that the pre-thermal exposure value of EID_{50} of the virus was 10^9 EID_{50}/ml (log value 9.00). During thermal exposure at 25^0C for 36 days the EID_{50} value initially decreased and then increased above the pre-thermal exposure value. After 14 days of thermal exposure the EID_{50} value slightly decreased and retained in satisfactory level i.e.$10^{8.50}$$EID_{50}$/ml at the end of the cycle.

Literature was scanty in relation to 25^0C thermal exposure for 36 days. From the result of HA titre and EID_{50} it may be concluded that at initial stage of thermal exposure the viability of the virus slightly decreased.With the advancement of time of exposure tolerancy of the virus increased and multiplied in the inoculated embryonated eggs. Therefore, the concentration of the live virus increased and the viability of the virus retained in satisfactory level at the end of the first cycle.

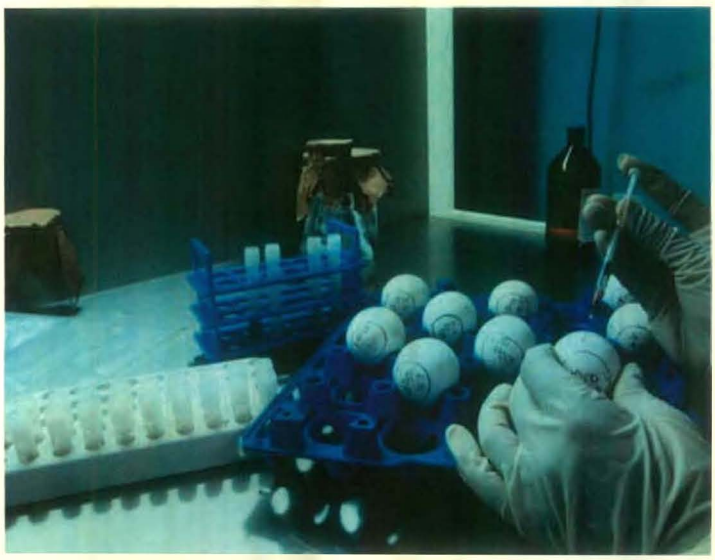


Fig 4: Showing inoculation of virus in embryonated SPF eggs for determination of EID_{50}

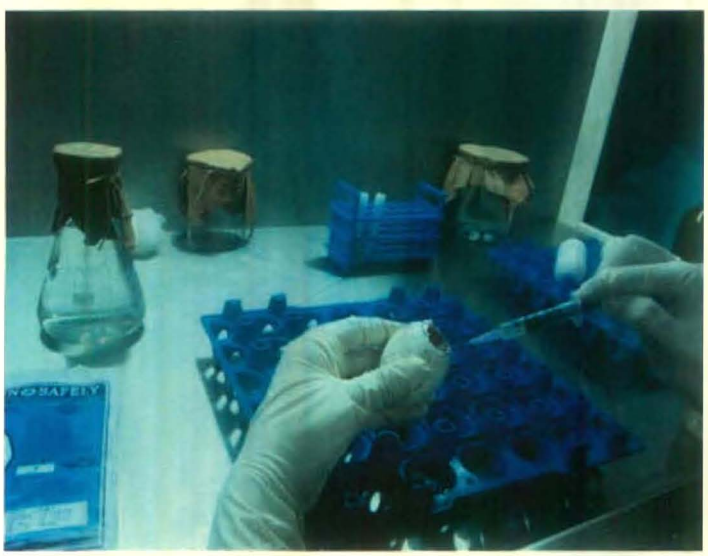


Fig 5: Showing harvesting of allantoic fluid from inoculated eggs

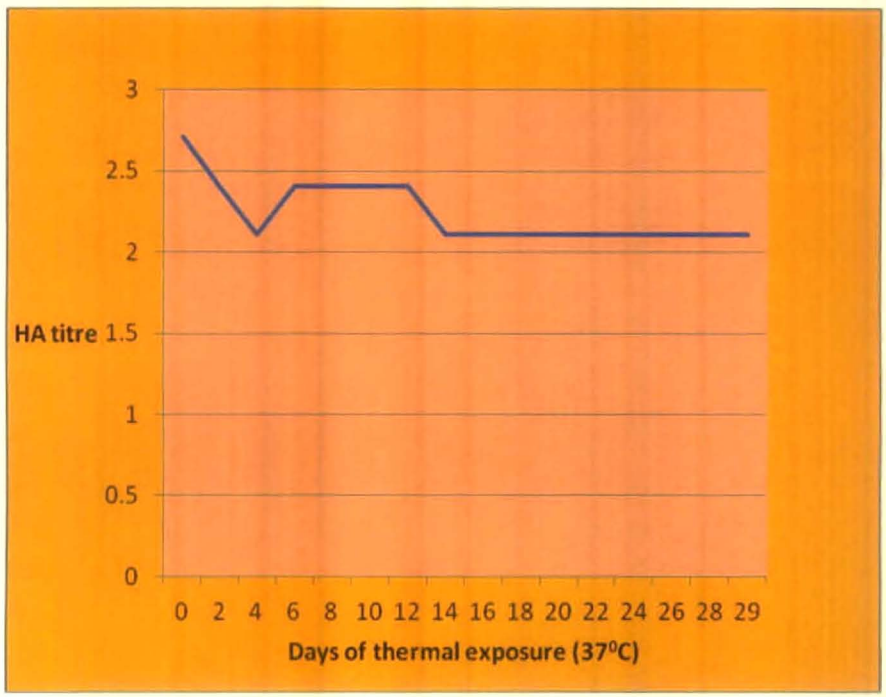
4.3. *The HA titre of the virus in the second thermal cycle:*

The virus was exposed as 1 ml aliquot in 16 vials at 37^0C for 29 days.The HA titre was determined at every 2 days interval from 1 ml aliquot as per OIE (2009).The result were presented in table 6 and figure 6.

Table 6. Showing HA Titre of the virus in the second thermal cycle at 37^0C for 29 days

SL. no.	Period of thermal exposure (in days)	HA titre	Log Value of HA titre
1.	0 (before thermal exposure)	2^9	2.71
2.	2	2^8	2.41
3.	4	2^7	2.11
4.	6	2^8	2.41
5.	8	2^8	2.41
6.	10	2^8	2.41
7.	12	2^8	2.41
8.	14	2^7	2.11
9.	16	2^7	2.11
10.	18	2^7	2.11
11.	20	2^7	2.11
12.	22	2^7	2.11
13.	24	2^7	2.11
14.	26	2^7	2.11
15.	28	2^7	2.11
16.	29	2^7	2.11

Fig-6: Showing HA titre (log_2 basis) of the virus up to 29 days of thermal exposure at 37^0C



From the above table it was evident that the pre-thermal exposed Heamagglutination (HA) titre of the virus was very good i.e. 2^9 (log value 2.71). During thermal exposure in the second cycle the HA titre was screened every 2 days interval and observed that the HA titre was slightly decreased on 4^{th} post thermal exposure day i.e. 2^7 (log value 2.11). Then the HA titre increased on the next observation and retained up to 12^{th} days of post thermal exposure i.e. 2^8 (log value 2.41). Then the titer decreased one log and remained as such 2^7 (log value 2.11) at the end of the second cycle.

Literature regarding HA titre of thermal exposure virus at 37^0C for 29 days was scanty.

Nassien and Adene (2002) reported that Hitchner-B_1 strain, K strain completely lost the titre at 72 hours and 95 hours respectively of 36^0C temperature post thermal exposure. Therefore, from the result it was concluded that the virus (local isolate) withstand the 37^0C for long time (29days) without losing its viability/infectivity.

4.4. *EID_{50} of the second thermal cycle:*

The virus was exposed as 1 ml aliquot in 16 vials at 37^0C for 29 days. The EID_{50} was determined before thermal exposure and subsequently every 2 days interval of thermal exposure from 1 aliquot as per Reed and Muench (1938) and FAO (2002).The results were presented in table 7, 8, 9 and 10 and in figure 7.

Table 7. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus before thermal exposure in the second cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No.of eggs infected (HA,+ ve)	No.of eggs not infected (HA, - ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	5(1non sp. death)	4	0	16	0	16	16/16	100
10^{-7}	5	4	1	12	1	13	12/13	92.30
10^{-8}	5	5	0	8	1	9	8/9	88.89
10^{-9}	4	3	1	3	2	5	3/5	60
10^{-10}	3	0	3	0	5	5	0/5	0

From the above table it was seen that 50% end point was somewhere between 10^{-9}(60%) and 10^{-10}(0%) dilution. By using the following formula the proportionate distance (PD) between 10^{-9} to 10^{-10} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= \frac{60\text{-}50}{60\text{-}0}$$

$$= 10/60$$

$$= 0.16$$

The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titer of the virus was $10^{9\,16}$ EID_{50}/ 0.1 ml or $10^{10\,16}$ EID_{50}/ml.

From the above calculation it was evident that the EID_{50} (i.e. total number of viable virus per ml) of the virus before thermal exposure to the second cycle was $10^{10\,16}EID_{50}$/ml. Literature regarding EID_{50} of thermal exposure virus at 37^0C for 29 days was scanty. From the result it was observed that the EID_{50} of unexposed virus was $10^{10\,16}EID_{50}$/ml which is satisfactory.

Table 8. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus after 2 days thermal exposure at 37^0C of second cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ve)	No.of eggs not infected (HA, -ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	5	4	1	12	1	13	12/13	92.30
10^{-7}	5	3	2	8	3	11	8/11	72.72
10^{-8}	5	3	2	5	5	10	5/10	50
10^{-9}	4	2	2	2	7	9	2/9	22.23
10^{-10}	4	0	4	0	11	11	0/11	0

From the above table it was seen that 50% end point was at 10^{-8} dilution.

Therefore, the 50% end point dilution = 10^{-8}

The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore the infectivity titre of the virus was $10^8 EID_{50}$/ 0.1 ml or 10^9 EID_{50}/ml.

Literature is scanty in relation to the two days thermal exposure at 37^0C in the second cycle. But the value of the EID_{50} is remained in satisfactory level.

Table 9. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus after 29 days thermal exposure at 37^0C of second cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA,+ ve)	No.of eggs not infected (HA, -ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	5	4	1	11	1	12	11/12	91.66
10^{-7}	5	3	2	7	3	10	7/10	70
10^{-8}	5	3	2	4	5	9	4/9	44.45
10^{-9}	5(2non sp. death)	1	1	1	6	7	1/7	14.29
10^{-10}	5(1non sp. death)	0	0	0	6	6	0/6	0

From the above table it was seen that 50% end point was somewhere between 10^{-7}(70%) and10^{-8} (44.44%) dilution. By using the following formula the proportionate distance (PD) between 10^{-7} to 10^{-8} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 70\text{-}50/70\text{-}44.45$$

$$= 20/25.56$$

$$= 0.78$$

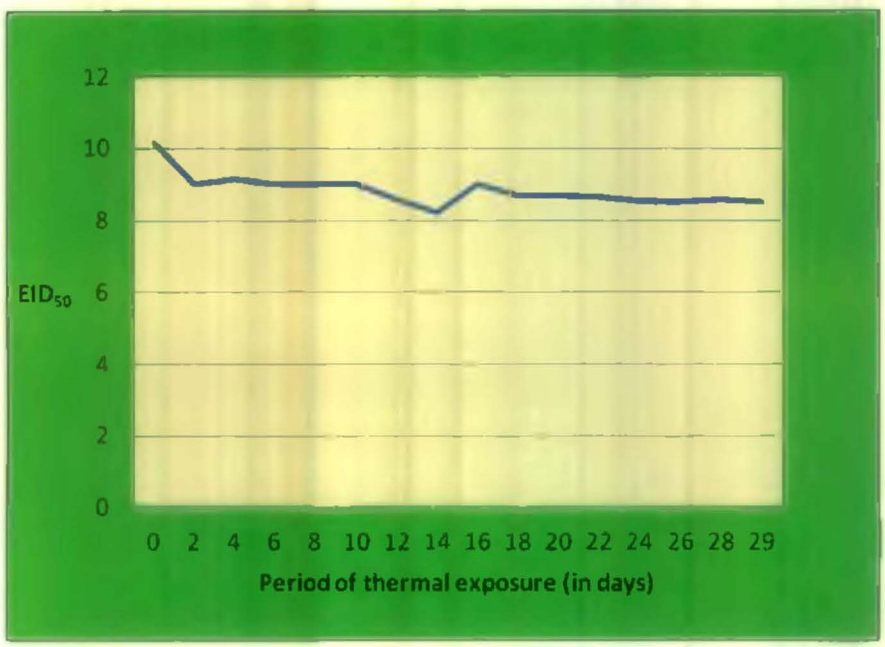
The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titre of the virus was $10^{7.78}$ EID_{50} / 0.1 ml or $10^{8.78}$ EID_{50} /ml.

Literature in this aspect is scanty in relation to the 29 days thermal exposure at 37^0C in the second cycle. But the value of EID_{50} i.e. $10^{8.78}EID_{50}$/ml after 29 days thermal exposure at 37^0C was considered as satisfactory.

Table 10. Showing EID_{50} of the virus in the second thermal cycle at 37^0C for 29 days

Serial no.	Period of thermal exposure (in days)	EID_{50}	Log value of EID_{50}
1.	0 (before thermal exposure)	$10^{10.16}$	10.16
2.	2	10^{9}	9.00
3.	4	$10^{9.16}$	9.16
4.	6	10^{9}	9.00
5.	8	10^{9}	9.00
6.	10	10^{9}	9.00
7.	12	$10^{8.57}$	8.57
8.	14	$10^{8.22}$	8.22
9.	16	10^{9}	9.00
10.	18	$10^{8.67}$	8.67
11.	20	$10^{8.70}$	8.70
12.	22	$10^{8.64}$	8.64
13.	24	$10^{8.53}$	8.53
14.	26	$10^{8.50}$	8.50
15.	28	$10^{8.59}$	8.58
16.	29	$10^{8.50}$	8.50

Fig 7: Showing EID_{50} (Log_{10} basis) of the virus up to 29 days of thermal exposure at 37^0C in the second cycle



From the table 10 and figure 4 it was observed that the pre-thermal exposure value of EID_{50} of the virus was $10^{10.16}$ EID_{50}/ml (log value, 10.16) which was very good. During thermal exposure in the second cycle the EID_{50} value was screened every 2 days interval and observed that the EID_{50} value was slightly decreased but retained in good satisfactory level i.e. $10^{8.50}EID_{50}$/ml at the end of the cycle.

Literature was scanty in relation to 37^0C thermal exposure for 29 days.

From the result of HA titre and EID_{50} it may be observed that after thermal exposure the viability of the virus slightly decreased and with the advancement of exposure in time the viability of the virus retained in good satisfactory level at the end of the second cycle.

From the result it may be concluded that the virus (local isolate) is considered to stable at 37^0C for one month. Therefore, the isolate can be used as vaccine to protect the chicken against Newcastle disease at high ambient temperature.

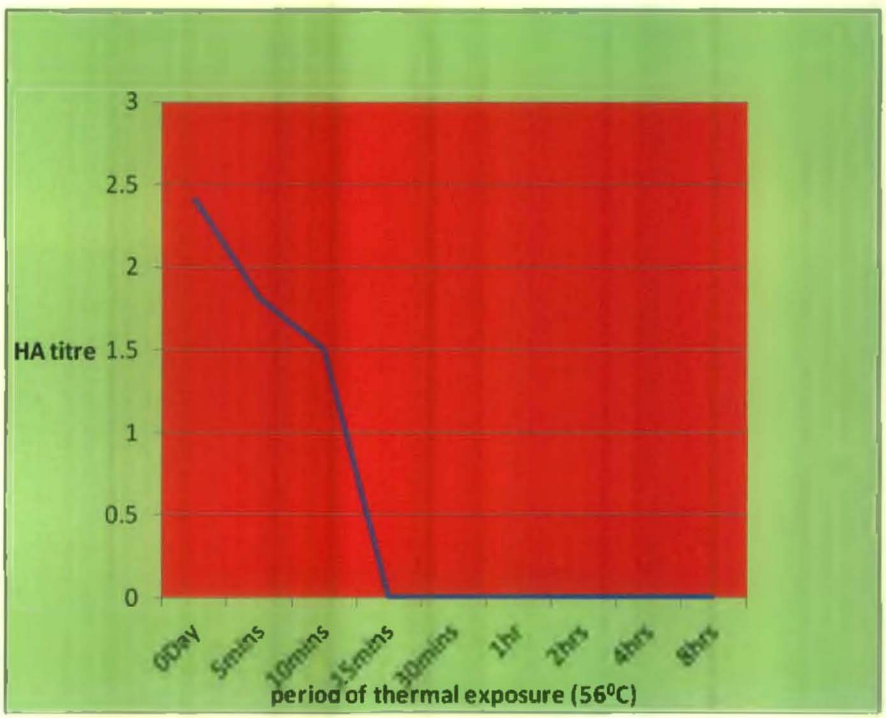
4.5. *The HA titre of the third thermal cycle:*

The virus was exposed as 1 ml aliquot in 9 vials at 56^0C within 5 minutes to 8 hours. The HA titre was determined at 5 min,10 min,15 min, 30 min,1hr, 2 hrs, 4 hrs and 8 hrs interval from 1 ml aliquot as per OIE (2009).The results were presented in table 11 and figure 8.

Table 11. Showing HA titre of the virus in the third thermal cycle at 56^0C within 5 minutes to 8 hours

Serial no.	Period of thermal exposure	HA titre	Log value of HA titre
1.	0 min	2^8	2.41
2.	5 min	2^6	1.81
3.	10 min	2^5	1.51
4.	15 min	nil	nil
5.	30 min	nil	nil
6.	1 hr	nil	nil
7.	2 hrs	nil	nil
8.	4 hrs	nil	nil
9.	8 hrs	nil	nil

Fig 8: Showing HA titre (log_2 basis) of the virus within 5 minutes to 8 hours of thermal exposure at 56^0C



From the above table it was evident that the pre-thermal exposed Heamagglutination (HA) titre of the virus was good i.e. 2^8 (log value2.41).

During thermal exposure in the third cycle the HA titres were screened within 5 minutes to 8 hours. It was observed that the HA titre was decreased i.e.2^6 at 5 minutes of thermal exposure, 2^5 at 10 minutes of thermal exposure. The titre was absolutely nil at 15 min, 30 min, 1 hr, 2hrs, 4 hrs and 8 hrs of thermal exposure.

Similar observation was repeated by King (2001) who declared that extreme selection at 56^0C of few longest-surviving virus particles was an effective way of obtaining the seed for the next generation.

Similar finding was recorded by Azzam (2007) that the thermostability testing showed the HA activity of the virus was decreased by about 2log10, following exposure at 56^0C for 15minutes.

Ibu *et al.* (2010) reported that out of 12 field virus isolates 3 isolates were inactivated in 5 minutes, 3 were inactivated in 10 minutes, 1 in 15 minutes,1 in 20 minutes,2 in 25 minutes and 3 in 30 minutes at 56^0C .They also reported the thermostable V_4 strain and I_2 strain were also inactivated in 115 and in 90 minutes respectively. Lasota strain & B_1 strain and Kamorov strain got inactivated in 30 and 25 minutes respectively.

4.6. *EID_{50} of the third thermal cycle:*

The virus was exposed as 1 ml aliquot in 8 vials at 56^0C for 5 minutes to 8 hours. The EID_{50} was determined before thermal exposure and subsequently for 5 min,10 min,15 min,30 min,1 hr,2 hrs,4 hrs and 8 hours of thermal exposure from 1 aliquot as per Reed and Muench (1938) and FAO (2002).The results were presented in table 12, 13, 14 and 15 and in figure 9.

Table 12. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus before thermal exposure in the third cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ ve)	No.of eggs not infected (HA, - ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	5	4	1	11	1	12	11/12	91.67
10^{-7}	5	3	2	7	3	10	7/10	70
10^{-8}	5	3	2	4	5	9	4/9	44.45
10^{-9}	5(2non sp. death)	1	1	1	6	7	1/7	14.29
10^{-10}	5(1non sp. death)	0	0	0	6	6	0/6	0

From the above table it was seen that 50% end point was somewhere in between 10^{-7}(70%) and10^{-8} (44.44%) dilution. By using the following formula the proportionate distance (PD) between 10^{-7} to 10^{-8} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 70-50/70-44.45$$

$$= 20/25.55$$

$$= 0.78$$

The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore the infectivity titer of the virus was $10^{7.78}$ EID_{50} / 0.1 ml or $10^{8.78}$ EID_{50} /ml.

From the above calculation it was evident that the EID_{50} (i.e. total number of viable virus per ml) of the virus before thermal exposure to the third cycle was $10^{8.78}EID_{50}$/ml.

From the result it was observed that the EID_{50} of thermal unexposed virus was $10^{8.78}EID_{50}$/ml which is satisfactory.

Table 13. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus after 5 minutes of thermal exposure at 56^0C in the third cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ve)	No.of eggs not infected (HA, -ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	4(1non sp. death)	2	1	4	1	5	4/5	80
10^{-7}	3	1	2	2	3	5	2/5	40
10^{-8}	3	0	3	1	6	7	1/7	14.28
10^{-9}	3(1non sp. death)	1	1	1	7	8	1/8	12.5
10^{-10}	3	0	3	0	10	10	0/10	0

From the above table it was seen that 50% end point was somewhere in between 10^{-6} (80%) and 10^{-7} (40%) dilution. By using the following formula the proportionate distance (PD) between 10^{-6} to 10^{-7} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 80\text{-}50/80\text{-}40$$

$$= 30/40$$

$$= 0.75$$

The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titer of the virus was $10^{6.75}$ EID_{50} / 0.1 ml or $10^{7.75}$ EID_{50} /ml.

Literature regarding EID_{50} was scanty in relation to the 5 minutes thermal exposure at 56^0C in the third cycle.

Table 14. Estimation of Embryo Infective Dose fifty (EID_{50}) of the virus after 10 minutes of thermal exposure at 56^0C in the third cycle

Dilution of virus (Inoculum)	Total no. of eggs inoculated	Embryos		Accumulated no.			Proportion infected (A/A+B)	% of dead
		No. of eggs infected (HA ,+ ve)	No.of eggs not infected (HA, - ve)	Infected (A)	Not infected (B)	Total no. tested (A+B)		
10^{-6}	4	1	3	4	3	7	4/7	57.14
10^{-7}	3	1	2	3	5	8	3/8	37.50
10^{-8}	3	2	1	2	6	8	2/8	25.00
10^{-9}	3	0	3	0	9	9	0/9	0
10^{-10}	3(1non sp. death)	0	2	0	11	11	0/11	0

From the above table it was seen that 50% end point was somewhere in between 10^{-6} (57.14%) and 10^{-7} (37.50%) dilution. By using the following formula the proportionate distance (PD) between 10^{-6} to 10^{-7} was calculated.

$$PD = \frac{\text{Distance a}}{\text{Distance b}}$$

$$= \frac{\text{Percentage infected at dilution next above 50\% - 50\%}}{\text{Percentage infected at dilution next above 50\% - Percentage infected at dilution next below 50\%}}$$

$$= 57.14\text{-}50/57.14\text{-}37.50$$

$$= 7.14/19.64$$

$$= 0.36$$

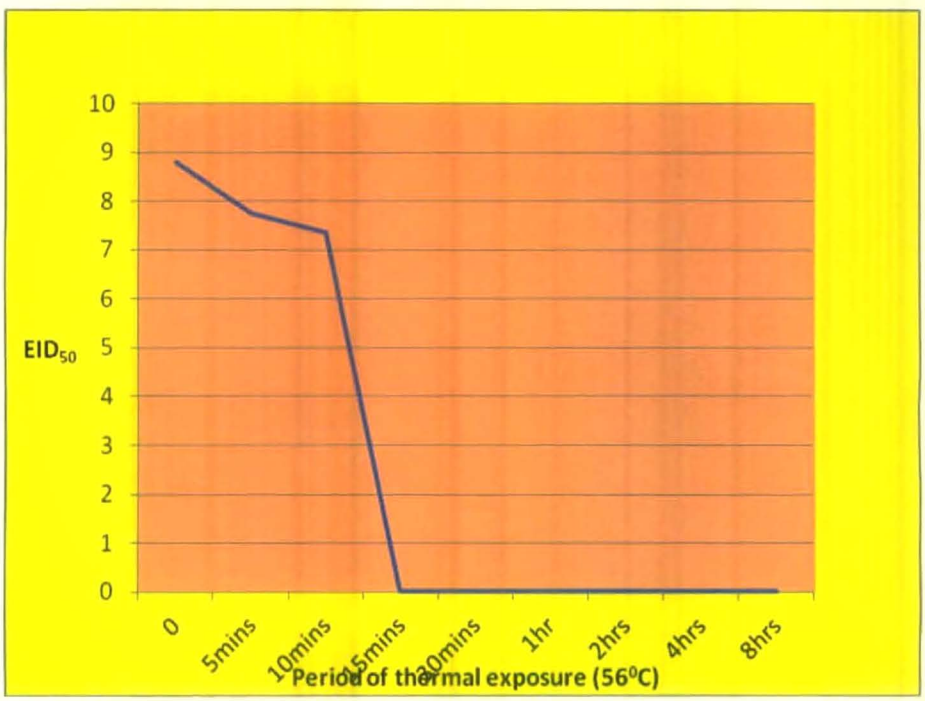
The end point dilution is expressed as EID_{50} per dose. Here, the dose of inoculum was 0.1 ml and therefore, the infectivity titer of the virus was $10^{6.36}$ EID_{50} / 0.1 ml or $10^{7.36}$ EID_{50} /ml.

Literature regarding EID_{50} was scanty in relation to the 10 minutes thermal exposure at 56^0C in the third cycle.

Table 15. Showing EID_{50} of the virus in the third thermal cycle at 56^0C within 5 minutes to 8 hours

Serial no.	Period of thermal exposure	EID_{50}	Log value of EID_{50}
1.	0 min	$10^{8.78}$	8.78
2.	5 min	$10^{7.75}$	7.75
3.	10 min	$10^{7.36}$	7.36
4.	15 min	nil	nil
5.	30 min	nil	nil
6.	1 hr	nil	nil
7.	2 hrs	nil	nil
8.	4 hrs	nil	nil
9.	8 hrs	nil	nil

Fig 9: Showing EID_{50} (Log_{10} basis) of the virus from 5 minutes to 8 hours of thermal exposure at 56^0C in the third cycle



From the table 15 and figure 6 it was observed that the pre-thermal exposed value of EID_{50} of the virus was $10^{8.78}$ EID_{50}/ml (log value, 8.78) which is satisfactory. During thermal exposure in the third cycle the EID_{50} value was screened from 5 minutes to 8 hours and observed that the EID_{50} value was decreased at 5 minutes of thermal exposure i.e. $10^{7.75}$ and at 10 minutes of thermal exposure i.e. $10^{7.36}$ with the advancement of time exposure the EID_{50} value was nil at 15 min, 30 min, 1 hr, 2 hrs, 4 hrs and 8 hrs.

From the result of HA titre and EID_{50} it may be concluded that after thermal exposure the viability of the virus decreased on 5 minutes HA titre 2^6, EID_{50} $10^{7.75}$and on 10 minutes HA titre 2^5, EID_{50} $10^{7.36}$ with the advancement of exposure in time the viability of the virus became nil in 15 minutes and onward. Literature is scanty in relation to EID_{50} at 56^0C thermal exposure within 5 minutes to 8 hours

Islam and Abdellatif (2011) reported that titer of Dongola strain decreased 2 logarithmic order within 15 minutes of incubation at 56^0C and EID_{50}/ml decreased from $10^{10.0}EID_{50}$/ml of unheated to $10^{8.0}$/ml of heated virus.

From the above result of HA titre and EID_{50} it may be concluded that the field isolate (virus) unable to survive at high temperature i.e.56^0C for long time. The survivality of the virus (local isolate) is comparatively less than the Lasota, B_1 and Kamorov strains. On the other comparative studies by Chhetri (2010), Biswas (2011) and Sharma (2012) it was observed that the efficacy of the virus (local isolate) was superior than Lasota,B_1 and NDVH strain by intranasal/drinking water administration. Therefore, the virus (local isolate) can be used as vaccine at 37^0C environmental temperature.

Chapter V

Summary and Conclusion

Summary and Conclusion

The present study was conducted to test the thermostability of a virus sample which was isolated, characterised and provided by the Department of Veterinary Epidemiology and Preventive Medicine, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata-37.

Before carrying out of the thermostability test, the titer of the virus was increased by following serial passages in embryonated SPF fowl eggs. The infectivity titer of the virus was checked by determination of Embryo Infective Dose fifty (EID_{50}). The thermostability test procedure consisted of three cycles in different temperature and time duration.

In the first thermal cycle 20 vials each containing 1 ml virus (infected allantoic fluid) were kept in B.O.D. incubator at 25^0C for 36 days. The HA titer and the EID_{50} of the virus were checked using 1 vial at every two days interval. At the end of the first thermal cycle, the HA titre i.e. 2^9 and EID_{50} i.e. 10^9EID_{50}/ml of the heat unexposed virus decreased to 2^8 and $10^{8.5}EID_{50}$/ml respectively.

At the end of the first thermal cycle the concentration of virus increased by 3 serial passages in embryonated SPF fowl eggs and the HA titre and EID_{50}/ml was detected before using it in the second thermal cycle.

In the second thermal cycle 16 vials each containing 1 ml virus (infected allantoic fluid) were kept in B.O.D. incubator at 37^0C for 29 days. The HA titre and the EID_{50} of the virus were checked using 1 vial at every two days interval. At the end of the second thermal cycle, the HA titre i.e. 2^9 and EID_{50} i.e.

$10^{10.16}EID_{50}$/ml of the heat unexposed virus decreased to 2^7 and $10^{8.50}EID_{50}$/ml respectively.

At the end of the second thermal cycle the concentration of the virus again increased by 3 serial passages in embryonated SPF fowl eggs and the HA titre and EID_{50}/ml were detected before using it in the third thermal cycle.

In the third thermal cycle 9 vials each containing 1 ml virus (infected allantoic fluid) were kept in B.O.D. incubator at 56^0C for 8 hours. The HA titre and the EID_{50} of the virus were checked using 1 vial at 5 min, 10 min, 15 min, 30 min, 1 hr, 2 hrs, 4 hrs and 8 hrs interval. In the third cycle, the HA titre i.e. 2^8 and EID_{50} i.e. $10^{8.78}EID_{50}$/ml of the heat unexposed virus decreased to 2^5 and $10^{7.36}EID_{50}$/ml respectively in 10 minutes of thermal exposure and the HA titre and EID_{50} value became undetectable from 15 minutes and onward till the end of the third thermal cycle.

From the results of the present study, it can be concluded that the lentogenic strain ND virus (local isolate) could withstand the thermal exposure of 25^0C for 36 days, 37^0C for 29 days and 56^0C for 10 minutes without losing its viability. Therefore, the virus can be used as vaccine strain which will be able to withstand the environmental temperature up to 37^0C for a month without losing its viability and infectivity.

Chapter VI

Future Scope of Research

Future Scope of Research

Newcastle disease is a great threat to the poultry industry and has become a real challenge to the veterinarians to combat it. Adoption of suitable biosecurity measures along with use of potent vaccine can help limiting the disease and subsequently its eradication. Yet such measures appear not to be effective in many cases as frequent ND outbreaks even in vaccinated flocks are reported. Therefore, to prevent such type of economic loss by sudden outbreak of ND, it is very important to develop a cheap but absolutely effective vaccine against the disease. Live vaccines are easy to apply and relatively inexpensive, and give moderately good immunity. Among the live vaccines, the thermostable vaccines require less stringent transport requirements in the field conditions. Therefore, it is needed to develop a most potent and effective thermostable vaccine. Since the period allotted for this study was limited, all the aspects could not be observed properly. Therefore, the following aspects may be explored further in relation to this work.

1. Shelf life of the virus (local isolate) at 37^0C temperature may be tested.
2. Shelf life and viability of the virus (local isolate) at immediate higher temperature up to 56^0C may be detected
3. Thermostable virus at higher temperature may be tested as vaccine in laboratory conditions followed by field trial.

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Appendix

APPENDIX

1. Normal saline solution (NSS)

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

Autoclaved at 15 lbs pressure for 15 minutes.

2. Phosphate Buffer Saline (PBS), (0.1 Molar, 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

Autoclaved at 15 lbs pressure for 15 minutes

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

Autoclaved at 15 lbs pressure for 15 minutes.

4. Nutrient broth:

Peptic digest of animal tissue	5.00 g
Beef extract	1.50 g
Sodium chloride	5.00g
Yeast extract	1.50g

Final pH 7.3±0.2 (at 25°C)

Suspend 13 grams to 1000 ml of distilled water, boil to dissolve, autoclave at 15 lbs pressure for 15 minutes.

5. Nutrient Agar:

Peptic digest of animal tissue	5.00 g
Beef extract	3.00 g
Sodium chloride	8.00g
Agar agar(agrose)	15.00g

Final pH 7.3±0.2. (at 25°C)

Suspend 65.00 grams in 1000 ml distilled water, boil to dissolve, autoclave at 15 lbs pressure for 15 minutes.

4. PPLO (Mycoplasma agar) Agar:

Hiveg infusion	6.00 g
Hiveg peptone	10.00 g
Sodium chloride	5.00g
Agar agar(agrose)	15.00g

Final pH 7.8±0.2 (at 25°C)

Suspend 36 grams in 700 ml of distilled water, heat to dissolve, autoclave at 15 lbs pressure for 15 minutes.