Molecular Characterization of Thermostable Newcastle disease virus Isolated from Pigeon

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Abstract The HN and L gene sequences of an Indian isolate of Newcastle disease virus was analyzed prior to and after exposure to 56 °C at tenth passage and fifteenth passage to study the variations at molecular level. In the HN gene sequence of progenitor and thermostable strain, substitution of K373I, F374L, M516R, D517V were considered to contribute to the increase in the stability of the protein. In the L gene of the thermostable strain, variations were observed at many positions and among these the substitutions at position P675H K677R, K893D, R1132K, had charged amino acids, and at L656A, F657V, F869L, T886I, M899I, G1131V, V1675L, had hydrophobic amino acids that could be related to increased stability of L protein at high temperatures. The changes in amino acid sequence in HN and L gene of the thermostable strain might render structural variations that might have contributed to the stability of the strain at higher temperature.

Keywords Newcastle disease virus-thermostable · HN and L gene- sequencing · Sequence variation analysis

Introduction

Newcastle disease (ND) is one of the most important avian viral diseases caused by avian *paramyxovirus*-1 which has been classified under *Avulavirus* [19]. ND causes devastating losses in both commercial and village chickens [14]. The

genome of Newcastle disease virus (NDV) is approximately 15,186 nucleotide long encoding six proteins which includes hemagglutinin-neuraminidase protein (HN), fusion protein (F), nucleocapsid protein (NP), matrix protein (M), phosphoprotein (P), and RNA-directed RNA polymerase or large protein (L) [27]. Although control of ND by vaccination is widely practiced, vaccines used in commercial flocks are generally unsuitable for use in village chickens in developing countries [1, 3]. Since maintaining cold chain under village conditions is really challenging. Thermostable ND vaccines will solve many of the managerial problems associated with ND vaccination faced at farm/field level. Thermostable ND vaccines can make an important contribution in the control of ND in areas where the cold chain is weak or non-existent [4, 9].

In this scenario, several attempts are being made to develop a potent thermostable ND vaccine incorporating a locally available thermostable NDV isolate. The process of selecting a thermostable isolate has always been challenging. Physically, the thermostability of an isolate can be assessed by the stability of haemagglutinins and the infectivity following exposure to higher temperatures. Nevertheless, assessing the molecular changes of a thermostable virus will give better information in respect of a thermostable and thermolabile isolate. And in some way the observed difference in genotypic level is correlated with the phenotypic characteristic of increased viral thermostability without losing its essential viral property [12]. Molecular characterization of thermostable variants will provide new insights in our understanding of their adaptability, and give better knowledge on thermostable enhancement without altering their immunogenicity. Le Long et al. [16] hypothesized that changes in the HN may reflect immunological selection pressures and reported that the immune system of birds recognizes the HN protein in preference to the F protein.

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RNA polymerase is required for transcription of mRNAs and full genome-length positive sense RNA. If the virion is exposed to environmental heat, the RNA polymerase contained within it may be rendered inactive and although attachment and entry of the virus particle may still occur following infection of a host, transcription would not occur and virus replication would be terminated. It is possible that changes to the structure or configuration of the RNA polymerase allow this protein to retain activity when the virion is subjected to heat. This would then allow a complete cycle of virus replication to occur following infection of a host. Analyzing the nucleotide/protein sequence variations of NDV genes particularly the haemagglutination neuraminidase gene (HN) and RNA-directed RNA polymerase gene (L) for heat exposed thermostable variants would reveal the possible molecular adaptation of the virus.

Materials and Methods

Propagation and Identification of NDV

Newcastle disease virus (NDV) of pigeon origin (NDV-2K3) classified as genotype IV [24] available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai-7, India which (Progenitor virus) had a thermostability of 60 min was used in this study. The virus was propagated in 9 day old embryonated chicken eggs through the allantoic cavity route. The identity of the isolate was confirmed by haemagglutination inhibition test using NDV polyclonal Serum and chicken erythrocytes.

Assessing the Thermostability of the Isolate

NDV-2K3 isolate was exposed to 56 °C in a water bath for various time points, namely 30, 60 and 90 min and inoculated into embryonated chicken eggs to assess the infectivity following exposure to 56 °C. The Amino allantoic fluid (AAF) was collected from dead or chilled embryos and checked for HA activity, which was then used for subsequent passages in embryos after exposure to 56 °C every time. Likewise the virus was subjected to 15 passages (thermostable variant) and its haemagglutinin stability and infectivity were assessed by HA test and mean death time [3] respectively.

Primers

Ten sets of primers described earlier [13] were used to amplify the entire Large (L) protein gene. Haemagglutinin neuraminidase (HN) gene was amplified with the following primers (FP 5' CGC ATA TGA TGG ACA GCG CAG

TTA GCC AAG 3'; RP 5' GGT AGC CCA GTT AAT TTC CA 3').

RNA Isolation, cDNA Synthesis and Sequencing

After tenth and fifteenth passages, RNA was isolated using Trizol (Invitrogen, USA) and cDNA synthesis was done by using Superscript RT enzyme as per manufacturer instructions (Invitrogen). PCR reactions were done using Long PCR Kit (Genei, Bangalore, India), and the PCR products were purified using PureLink™ gel extraction kit (Invitrogen) according to the manufacturer's instructions. Purified DNA was then sequenced using the Big Dye terminator sequencing system (ABI, USA). DNA fragments were sequenced in both directions to generate contig sequences. DNA sequences were aligned using GeneTool and analysed using Bioedit program. Aligned sequences were translated with translate viewer programme to assess the amino acid sequence and the variations between the thermostable variant and the progenitor sequence.

Results

Stability of Haemagglutinins and Infectivity Following Exposure at 56 °C

Progenitor NDV-2K3 isolate was exposed to 56 °C for various time duration and inoculated into embryonated chicken eggs to assess its infectivity following exposure at 56 °C. The AAF was harvested following embryonic death and checked for HA activity, which was then used for subsequent passages after exposure at 56 °C every time. The results of stability of haemagglutinins and infectivity following exposure to 56 °C are presented in Table 1. It is evident from the results that the haemagglutinins of the isolate were stable even after 90 min exposure at 56 °C irrespective of the number of passages. Similarly the infectivity was also found to be stable after 90 min exposure at 56 °C. However, as the passage level increased there was an increase in the time taken to kill the embryos as evident from the increased mean death time.

Molecular Characterization

HN and RNA dependent RNA polymerase (L) gene sequences were RT-PCR amplified and sequenced at tenth and fifteenth passage after heat exposure. The sequence analysis revealed a number of observable changes in both HN and L gene predicted protein sequences. No sequence difference was observed after tenth and fifteenth passage following heat exposure.

Table 1 Results of infectivity and haemagglutinin stability of Newcastle disease virus isolate 2K3 after exposure to 56 °C for 90 min in water bath and inoculation into 9 days old embryonated chicken egg for thermostable strain development

Passage number	MDT (h)	HA Titre ($\log^2$)
1	60	2 ⁸
2	60	2 ⁶
3	55	2 ⁸
4	58	2 ⁸
5	55	2 ⁹
6	60	2 ¹⁰
7	60	2 ⁸
8	74	2 ⁸
9	76	2 ⁸
10	82	2 ⁹
11	78	2 ¹⁰
12	76	2 ⁹
13	84	2 ⁹
14	84	2 ⁸
15	84	2 ⁸

Analysis of HN Gene Sequence

RT-PCR resulted in the amplification of 2,000 bp HN gene (Fig. 1b). The HN gene sequence positional variation of thermostable 2K3 HN protein sequence is shown in Table 2. The thermostable variant 2K3 had amino acid substitutions at position H201P, N203Y, R326S, V357S, K373I, F374L, L375S, M516R, D517V, G519S, Q520G, I521S, and A522T with its counterpart progenitor 2K3 sequence.

Analysis of L Gene Sequence

RT-PCR amplicons of various portions of L gene are shown in Fig. 1a. The 6,779 bp long L gene sequence

positional variations of thermostable 2K3 is shown in Table 2. Predicted protein sequence revealed that the thermostable variant had amino acid substitutions at position R417M, N535S, V654N, K655Q, L656A, F657V, A658P, A660P, P675H, K677R, K679M, R859N, F869L, T886I, T889N, K893D, R896Q, M899I, T954N, G1131V, R1132K, F1476S, R1665G, V1675L, F1684C with its counterpart progenitor sequence.

Discussion

Though there are good number of vaccines available, Newcastle disease continues to plague the poultry industry. Failure to maintain proper cold chain could be one of the reasons for vaccine failures especially in tropical countries. Use of thermostable strains in live vaccines should address this problem to a very great extent. Over the years, several techniques have been used to select heat resistant strains of NDV. Lomnizi, in 1975 [17] has used a stepwise procedure to gradually increase the propagation temperatures with ultimate selection at 56 °C.

In the present study, we selected a NDV isolate which was fairly stable at 56 °C and exposed the isolate repeatedly to 56 °C until 15th passage. Exposure of AAF to 56 °C after each passage showed retention of infectivity and stable haemagglutinins even after 15 passages. This was evident from the ability of the heat exposed virus to infect the embryos during subsequent passages. We analyzed the sequences of HN and L genes of NDV 2k3 prior and tenth, fifteenth passage of after exposure to 56 °C for assessing possible variations.

It is reported that thermostability might be attributed to the alterations within the HN gene, specifically a deletion of amino acid 403 [23, 26]. However, a later attempt by Kattenbelt et al. [13] to find sequence variations for a thermostable phenotype found no such deletion in the

Fig. 1 a Agarose gel (1 %) electrophoresis of RT-PCR amplified L gene fragments showing various amplicons. Lane 1, 100 bp ladder; lane 2, 578 bp; lane 3, 755 bp; lane 4, 264 bp; lane 5, 708 bp; lane 6, 792 bp; lane 7, 794 bp; lane 8, 1,031 bp; lane 9, 715 bp; lane 10,

613 bp; lane 11, 2,261 bp b Agarose gel (1 %) electrophoresis of RT-PCR amplified HN gene showing 2,000 bp product. Lane 1 and 2, 2,000 bp HN gene product; lane 3, 1 Kb ladder

Table 2 Results of amino acid positional variations between Newcastle disease virus isolate 2K3 progenitor and thermostable strain 2K3

Gene	S. no.	Amino acid position	Progenitor 2K3 strain	Thermostable 2K3 strain
HN Gene	1	201	H	P
	2	203	N	Y
	3	326	R	S
	4	357	V	S
	5	373	K	I
	6	374	F	L
	7	375	L	S
	8	516	M	R
	9	517	D	V
	10	519	G	S
	11	520	Q	G
	12	521	I	S
	13	522	A	T
L Gene	14	417	R	M
	15	535	N	S
	16	654	V	N
	17	655	K	Q
	18	656	L	A
	19	657	F	V
	20	658	A	P
	21	660	A	P
	22	675	P	H
	23	677	K	R
	24	679	K	M
	25	859	R	N
	26	869	F	L
	27	886	T	I
	28	889	T	N
	29	893	K	D
	30	896	R	Q
	31	899	M	I
	32	954	T	N
	33	1,131	G	V
	34	1,132	R	K
	35	1,146	F	S
	36	1,165	R	G
	37	1,675	V	L
	38	1,684	F	C

Variations were derived by translating primary nucleotide sequences obtained by direct sequencing of RT-PCR fragments from the HN and L protein gene

thermostable strain I-2. In the present study too no such deletion was found at position 403. However other differences in the protein sequence were found between 2K3 progenitor and its thermostable variant strain sequences.

The observed differences in HN gene amino acid position H201P, N203Y, R326S, V357S, L375S, G519S, Q520G, I521S and A522T between progenitor and thermostable variant sequences had no significant influence in the thermostability of the isolate. Ser and Thr are known as the best residues for interacting with the water surrounding

protein structure [18]. Since the water, which could be interacted with Ser (usually mediated by hydrogen bonding), would be released at high temperature, the protein structure around water-binding site like Ser or Thr could be changed to be unstable enough to evoke protein instability [8]. Systematic analyses showed that the thermophilic proteins have very low frequency of Ser compared with mesophilic proteins [5, 15]. Gly is known as the residue for making void volume or cavity in the inner part of protein structure [7]. However, other systematic analysis showed

that there is no typical pattern of GLY occurrence in thermophilic proteins. Pro is known as the residue for making rigid conformation or turn conformation in protein structure [25].

The other observable changes in HN sequence of progenitor and thermostable variant sequences were K373I, F374L, M516R, and D517V. Among them the aliphatic amino acids Ile, Leu, and Val would contribute to the hydrophobic interaction, which is the main force for maintaining conformational stability in inner part of protein [20]. Sheehan et al. [21] found a strict correlation between the presence of cysteine at amino acid position 123 and reducing agent sensitive dimerization of the HN protein. This is important, since reducing agents eliminate viral infectivity as well as other biological functions associated with each glycoprotein. The presence of cysteine at amino acid position 123 correlates with the existence of disulfide-linked HN in virions, implicating this cysteine in intermolecular disulfide bonding between HN monomers [10]. In our study, the cysteine residue at 123 was found to be conserved in the HN protein.

Apart from the obvious variations seen in the length of the HN protein, the amino acids responsible for disulphide bonds, glycosylation sites and predicted active or catalytic sites were conserved in the 2k3 isolate even after exposure to 56 °C. The R416 essential for receptor binding, and neuraminidase (NA) and haemagglutination (HA) activity was likewise conserved even after exposure to 56 °C, as were the receptor binding site involving E401, R416 and Y526 [6, 11].

Being an important protein molecule in the replication complex of NDV, the L protein is as an important gene to look for variation that may confer thermostability of the virus. The L protein of thermostable strain of 2K3 when aligned with its 2K3 progenitor sequence revealed amino acid substitutions at twenty-five positions. The positional substitutions at R417M, N535S, V654N, K655Q, A658P, A660P, K679M, R859N, T889N, R896Q, T954N, F1476S, R1665G, F1684C considered less important in conferring increased protein stability at high temperatures. Whereas Asn and Gln are known as thermolabile amino acids due to their tendency to undergo deamination at high temperature [20] and Ser or Thr could be changed to be unstable enough to evoke protein instability at high temperatures. And also the Cys and Met are known as thermolabile amino acids due to their tendency to undergo oxidation at high temperature [20]. Charged amino acids such as Arg, Asp, His, Glu, and Lys would contribute to the electrostatic interaction, which is an important force for maintaining conformational stability in outer part of protein. The observed substitutions at position P675H, K677R, K893D, R1132K, had charged amino acids which would increase the stability of the L protein at high temperatures.

The aliphatic amino acids (Ala, Ile, Leu, and Val) contribute to the hydrophobic interaction, which is the main force for maintaining conformational stability in inner part of protein [2, 7, 22]. The observable difference at L656A, F657V, F869L, T886I, M899I, G1131V, and V1675L, may stabilize the protein core through hydrophobic interactions that would confer for increased stability of L protein at high temperature.

Natural selection shapes the sequence, structure and biophysical properties of proteins to fit their environment. In the present study HN protein variations with its progenitor sequence revealed that it has not had much implication in terms of biophysical properties other than the position changes at, K373I, F374L, and D517V. These three observed changes involved in hydrophobic interactions might have contributed to increased stability at higher temperature. The positional changes observed in L protein gene of thermostable strain might have conferred the biochemical aspects of thermostability. However, further studies on structural variation may throw more light on the stability of the strain. It is concluded, that the changes in amino acids of HN and L gene of the thermostable strain, might contribute to the stability of the strain at high temperature.

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