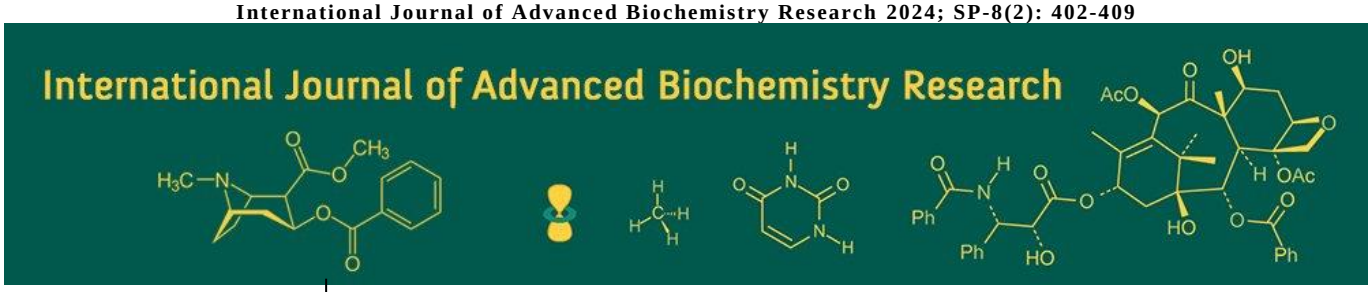

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Virological and molecular characterization of canine distemper virus passaged at high temperature towards development of thermostable vaccine candidate

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Abstract
Thermolabile nature of viruses is a major problem faced by most live attenuated vaccines. Addressing this issue will help increase the success rate of vaccination and reduce the economic burden on vaccination campaigns. In current study, the Canine Distemper Virus (CDV) belonging to India-1/Asia-5 lineage was adapted to grow at 41.5oC for assessing its suitability as thermostable vaccine candidate. The growth kinetics of thermotolerent virus was compared to that of original parent virus and it was found that the thermotolerent virus could grow only to a lower titre (4.5 logTCID50 per ml) in Vero cells as compared to the parent virus (5.61 logTCID50 per ml). When the coding sequences of structural genes of the virus M, N, H and F were compared before and after the high temperature passage, it was found that the H gene accrued the highest number of changes as compared to other genes of CDV. The mapped mutations in DNA and substitutions in amino acid sequences may shed light on the structural and functional mechanisms of virus adaptation at high temperature to be used as a strategy for creating recombinant thermostable vaccines.

Keywords: Thermostable vaccines, canine distemper vaccine, thermotolerant cells, gene mapping, growth kinetic study, growth curve

1. Introduction
Vaccine failure is a major threat to any vaccination campaign against infectious diseases. Even though the herd immunity can tackle vaccine failure to a certain level, many reports of morbidity among vaccinated individuals is a growing concern. The storage temperature Potency of vaccines is closely associated with mostly on the temperature they are exposed to (Bora et al., 2018) [1]. Consequently, cold chain is an indispensable component of almost all vaccination campaigns. Most vaccines, if cold chain is properly maintained at 2-8ºC, can achieve a longer shelf life, but many a times there is a breakage of cold chain on transit or accidental power failure (Dexiang and Debra 2009) [2]. A practical solution one can think of is the development of a vaccine formulation that can withstand high temperature. World Health Organization (WHO) encourages development of heat and freeze stable vaccines that can be stored for long time above +8ºC (WHO, 2015) [3], (Kumru *et al*., 2014) [4]. Vaccine failure most readily occurs in attenuated live vaccines. Being a replicative antigen, the viable biological nature of the antigen needs to be preserved. Although these vaccines are considered the most efficient in present situation, their efficiency largely depends upon the storage condition and proper management of cold chain. Live attenuated vaccine against Canine Distemper (CD), a main cause of systemic viral disease and death in domestic and wild carnivores, is our focus in this work. Its second highest fatality rate next to rabies, the accumulating reports of vaccine failure, reports of isolation of new distinct lineages from across the world, and inherent thermolabile nature of the virus make this vaccine a useful target to improve up on (Deem et al., 2000) [5], (Swati et al., 2015) [6], (Bhatt et al., 2019) [7], (Brussel and Karzon 1962) [8]. CD vaccines currently used are based on Onderstepoort, Rockborn or Lederle strain, developed and in use from as early as 1950s (Harder and Osterhaus 1997) [9].

Genetic divergence of the field virus from the vaccine can be a cause of vaccine inefficiency and in case of Canine distemper virus (CDV) it has been reported several times (Swati et al., 2015) [6],(Bhatt et al., 2019) [7]. In addition, the fact that CDV is the least thermostable component in the combined vaccines puts it at a risky position should any unexpected breakage of cold chain or shortfalls in transport or storage requirements occur (Brussel and Karzon 1962) [8], (Chappuis 1995) [10]. The present study is dedicated to addressing the above mentioned two technical problems in CDV vaccination in the field; we used an indigenous virus (CDV(Dog)/Bly/Ind/2018) divergent from the vaccine strain (Bhatt et al., 2019) [7] to develop a thermostable vaccine.

2. Materials and Methods

2.1 Cells and Virus

Vero cells obtained from ATCC and preserved in the repository of the Division of Biological Products, ICAR-IVRI Izatnagar were revived and used in this study. The freeze-dried indigenous CDV adapted in Vero cells (CDV(Dog)/Bly/Ind/2018; Passage - 60) was used as the parent virus in this study.

2.2 Developing thermotolerant Vero cells (Vero TT)

Vero cells were cultured in two different media *viz*; EMEM and IMDM supplemented with 10% fetal calf serum (FCS) and exposed to a temperature increment of 0.5 °C at each subculture. The temperature exposure started at 37 °C and continued until 42 °C. The cell morphology and growth were monitored visually under inverted light microscope. The cells were passaged and maintained up to passage level 10 at 41.5 °C (T10).

2.3 Development of thermostable CDV (CDV-Ta)

Vero-TT cells at passage T10 were infected with CDV-P60 at a moi of 0.01 by co-culture method. The infected cells were incubated at 37 ºC for the first 24 hours and thereafter the flask was transferred to 41.5 ºC. Media was changed every 48 hours until cytopathic effect (CPE) was observed. Further passages were carried out at 0.1 moi in a 25 °c flask. The presence of the virus in each harvest was confirmed by RT-PCR, cell-ELISA and titre of the virus was determined using Reed and Muench (1938) method. The virus was passaged 20 times in Vero-TT at 41.5 ºC to obtain CDV-Ta.

2.4 Confirmation of virus identity

2.4.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from the cell culture harvest of respective passage by trizol method and cDNA synthesis was done using Quantitect reverse transcription kit (#205311). PCR was done using SapphireAmp fast PCR mastermix (#RR350A) according to manufacturer's instructions with in-house designed diagnostic primers (N1 and N2) to demonstrate CDV specific amplicon of 497 bp.

2.4.2 Cell-ELISA

96-well cell culture plate infected with CDV-Ta was fixed with 80% chilled acetone in PBS and ELISA was performed using monoclonal antibodies isolated from hybridoma clone 2F8 developed in our lab (unpublished work). The wells having OD greater than twice the mean of cell control was considered as positive.

2.5 Virus Titration

Tissue culture infectivity dose ($TCID_{50}$) was used to estimate virus titres. In a 96 well plate, Vero cells were seeded at $1x10^4$ cells per well and incubated for 24 hours in order to obtain a monolayer. The preformed monolayers, were inoculated with 100 uL of 10-fold serially diluted (10^{-1} to 10^{-6}) virus (Both parent CDV P60 and thermotolerant CDV-Ta). EMEM containing 2% FBS was used for making the dilutions in deep well plates. Every dilution was inoculated in five replicates and the plates were incubated at 37 ºC in 5% CO_2 and 100% relative humidity. Media was changed with EMEM having 2% FBS every 48 hours. After 5 days of virus inoculation, each well was read as positive or negative based on the appearance of cytopathic effects typical for the CDV at 100x magnification with an inverted microscope. The titres were calculated using Reed and Muench method (Reed and Muench, 1938) [11] (Raut *et al.*, 2001) [12].

2.6 Comparison of growth kinetics of CDV-P60 virus and CDV-Ta

CDV-P60 was infected on monolayers of Vero cells in 25cc flasks at a moi of 0.01. After one hour of adsorption, the inoculum was removed from the flaks and same amount of 2% EMEM was added to the flasks. The time at which the inoculum was removed was taken as 0^{th} hour and from this point both cell free virus (CFV) released into the supernatant and cell associated virus (CAV) were harvested at time intervals of 0, 6, 12, 18, 24, 36, 48, 72, 96, 120, 144 and 168 hours of incubation. For CDV-Ta, the procedure was almost the same as in CDV-P60. However, the flasks were incubated at 41.5 ºC and the media used was serum free IMDM. The adsorption of virus was done at 37 ºC for 1 hour. The experiment was repeated twice and a line graph was plotted using the average of both values at each time points. The growth pattern of both the viruses was compared using growth kinetics study of the viruses. A curve for total virus produced at each time point was constructed; total amount was calculated from the separate titres by taking the sum of CAV and CFV at each time point. The time at which maximum titre obtained from this curve was suggested as the optimum harvesting time for the virus.

2.7 Sequencing and comparison of structural genes

The structural genes (M, F, N and H) of both the viruses (CDV-P60 and CDV-Ta) were amplified (Table 1) using EmeraldAmp® MAX HS PCR Master Mix (#RR310A) and PCR products were purified using MinElute gel extraction kit (#28604). The purified products were cloned into pJET1.2/blunt cloning vector using CloneJET PCR cloning kit (#K1232) using DH-5α cells. The plasmids isolation was carried out using GeneJET Plasmid Miniprep Kit (#K0503) and the Sanger's sequencing of isolated plasmids was outsourced (Eurofins Analytical Services India Pvt Ltd).

2.8 *In-silico* analyses of structural protein coding sequences

The translated nucleotide sequences generated for each structural gene from CDV-P60 and CDV-Ta were subjected to protein BLAST available in NCBI to check the identity of generated sequences. From the best aligned protein sequences, the coding region was annotated and taken for further analysis. The nucleotide and amino acid divergence for four structural proteins was calculated using BioEdit

software package and amino acid substitutions were recorded. ProtParam tool from Expasy was used to predict the physicochemical characteristics. Mainly the aliphatic index of the polypeptides was compared before and after high temperature treatment.

3. Results

3.1 Development of Vero-TT cells.

Exposure of Vero cells to sequentially increased temperature at each passage resulted in thermotolerant Vero cells. Two types of media were used in this study; the media routinely used for Vero cell culture in lab –EMEM and IMDM. Vero-TT cells grown in EMEM were characterized by increased cell necrosis and sloughing whereas in IMDM the cells were relatively healthy and fast growing (Figure 1; C). Even in IMDM the temperature could only reach 41.5 ºC even though the target was 42 ºC. As soon as the temperature crossed 42 ºC the cells started showing granulations, blebbing and necrosis before they eventually sloughed off. So, the final temperature was set 41.5 ºC to conduct further experiments in IMDM.

3.2 Development of CDV-TS

The procedure described elsewhere was followed for adaptation of virus at higher temperature with some modifications (Balamurugan *et al.*, 2014) [13]. The virus multiplication was evident by presence of visible CPE characteristic of CDV. The CPE in general was different in CDV P60 virus and CDV-T20 virus. While CPE by P60 virus was marked with widespread cell ballooning, formation of a few syncytia was the only indication of virus growth in case of CDV-T20 virus (figure 1). Syncytia formation has been described for Onderstepoort virus and various field strains of virus in primary cell cultures (Brussel and Karzon 1962) [8], (Rockborn, 1958) [14]. Thermostable CDV was took a longer time to elicit appreciable CPE as compared to CDV-P60 as it took more than 7 days to elicit 80% CPE at 41.5 °C. After each passage at 41.5 °C, the presence of the virus was confirmed by RT-PCR (figure 2) using diagnostic primers for N gene of CDV as well as cell-ELISA using monoclonal antibodies against CDV (CDV(Dog)/Bly/Ind/2018).

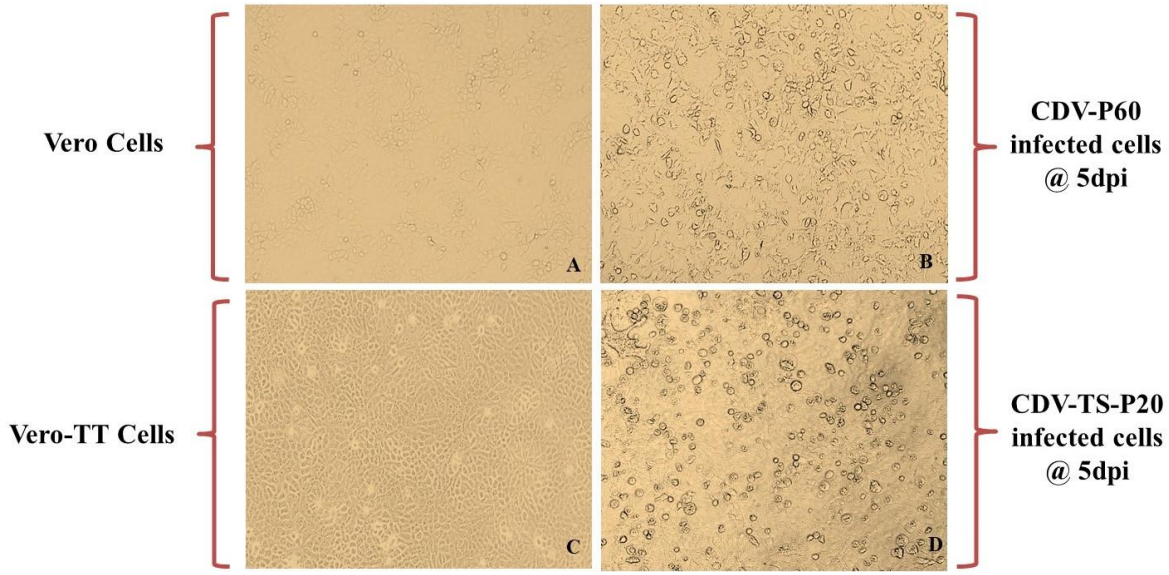

Fig 1: Comparative CPE of CDV(Dog)/Bly/Ind/2018 isolate in Vero and Vero TT cells: **A**-Healthy Vero cells (10x); **B**-Vero cells infected with CDV 5dpi (10x); **C**- Healthy Vero-TT cells (10x); **D**-Vero-TT cells infected with CDV-TS P20 5dpi (10x)

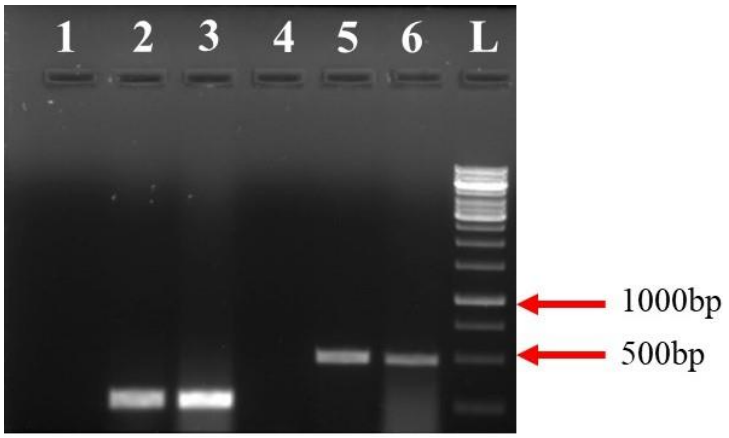

Fig 2: RT-PCR using diagnostic primers against N gene: Lane 1: NTC, β-actin gene; **Lane 2**: β-actin CDV-Ta (250 bp); **Lane 3**: β-actin CDV-P60 (250 bp); **Lane 4**: NTC for N gene; **Lane 5**: N gene of CDV-Ta (497bp); **Lane 6**: N gene of CDV-P60(497bp); **Lane L**: 1kb DNA ladder;

3.3 Comparative growth kinetics of CDV-P60 and CDV-Ta

Growth kinetics of CDV-P60 and CDV-Ta was compared to characterize the viruses in terms of growth characteristics. The growth curves (figure 3) were constructed using procedures described in various studies (Lan *et al.,* 2005a) [15], (Lan *et al.,* 2005b) [16], (Sreenivasa *et al.,* 2006) [17] with some modifications in terms of harvest time point. Both cell free and cell associated virus was collected at different time point and titre of virus was estimated. Eclipse period and Latent period were prolonged in case of CDV-Ta, which can be due to greater rate of degradation of cell free virus at higher temperature. Maximum titre was observed at 72 hours post infection (hpi) in CDV P-60, whereas it was at 96 hpi in CDV-Ta (time of harvest). Overall, cell associated virus was found to be more as compared to the virus in supernatant.

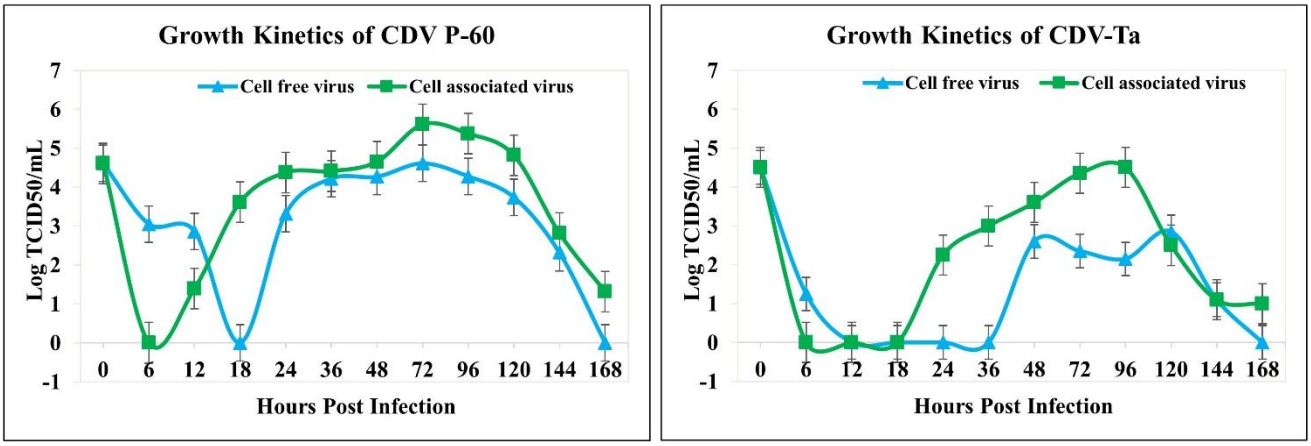

Fig 3: Growth curves of CDV P60 (A); CDV-Ta (B)

3.4 Sequence analysis of the structural genes

Thermostable virus was compared with CDV-P60 in terms of nucleotide and amino acid sequence differences in their structural genes. The structural genes (M, F, N and H) were amplified, cloned and sequenced (figure 4). Two sequences per each gene from CDV P-60 and CDV-Ta were annotated using MEGA 11. The coding sequence for each gene was annotated using protein database obtained from NCBI. Sequence comparison was done manually with the help of BioEdit software package. Both the sequences generated for a given gene were used to generate a consensus and percentage identity of the consensus sequences for each gene before and after high temperature passage were calculated. The M, N, H and F genes sequences of parent and thermostable CDV were 99.60 %, 99.40 %, 99.40 %, and 99.60 % identical, respectively when the nt. sequences of CDV P60 and CDV-Ta were compared. The amino acid differences reported in deduced amino acid sequence of respective genes are depicted in Figure 5-8.

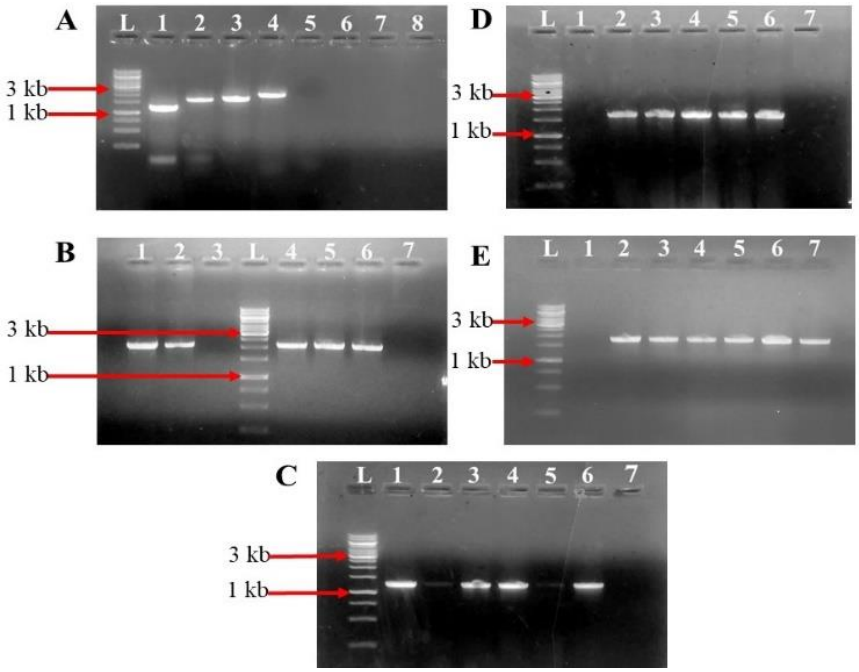

Fig 4: RT-PCR amplification of M, N, H and F genes of CDV-TS P-20. *Lane L:* 1 kb DNA ladder, *Lane 1:* M gene (1268 bp), *Lane 2:* N gene (1693 bp), *Lane 3:* H gene (1824 bp), *Lane 4:* F gene (2153 bp). *Lane 5-8*: NTC of respective gene

B-E: Colony PCR of M, N, H and F genes of CDV-TS P-20. **B:** F gene (2153 bp), **C:** M gene (1268 bp), **D:** N gene (1693 bp), **E:** H gene (1824 bp). *Lane L:* 1 kb DNA ladder, *Lane 1-6:* colony 1, 2, 3, 4, 5 of respective genes, *Lane 7*: NTC (Lane 1 in E)

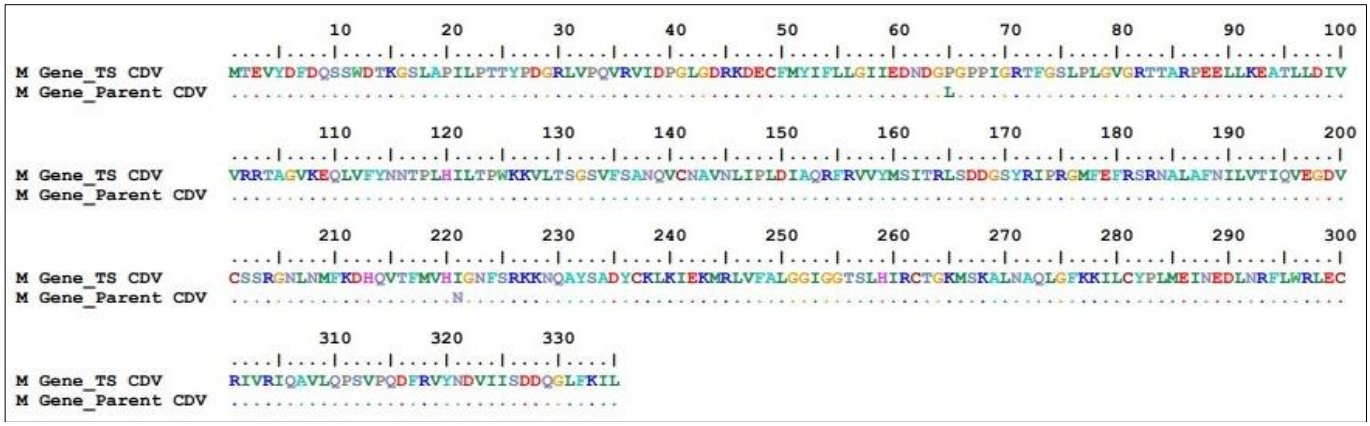

Fig 5: Sequence comparison of M protein

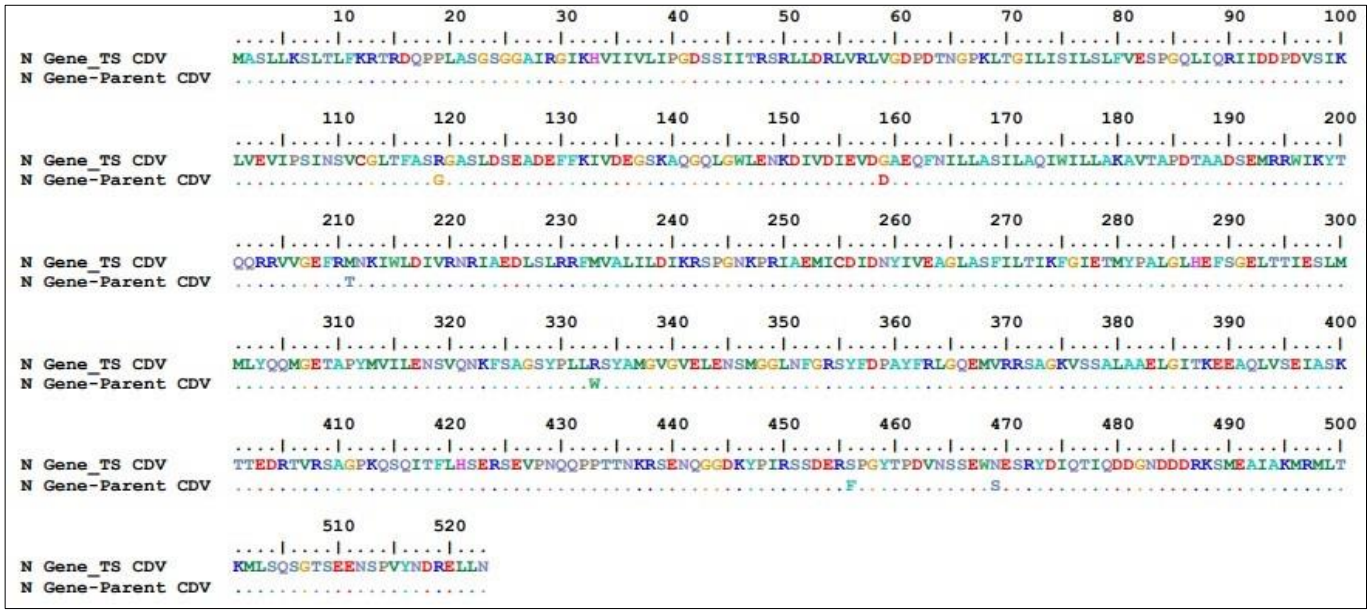

Fig 6: Sequence comparison of N protein

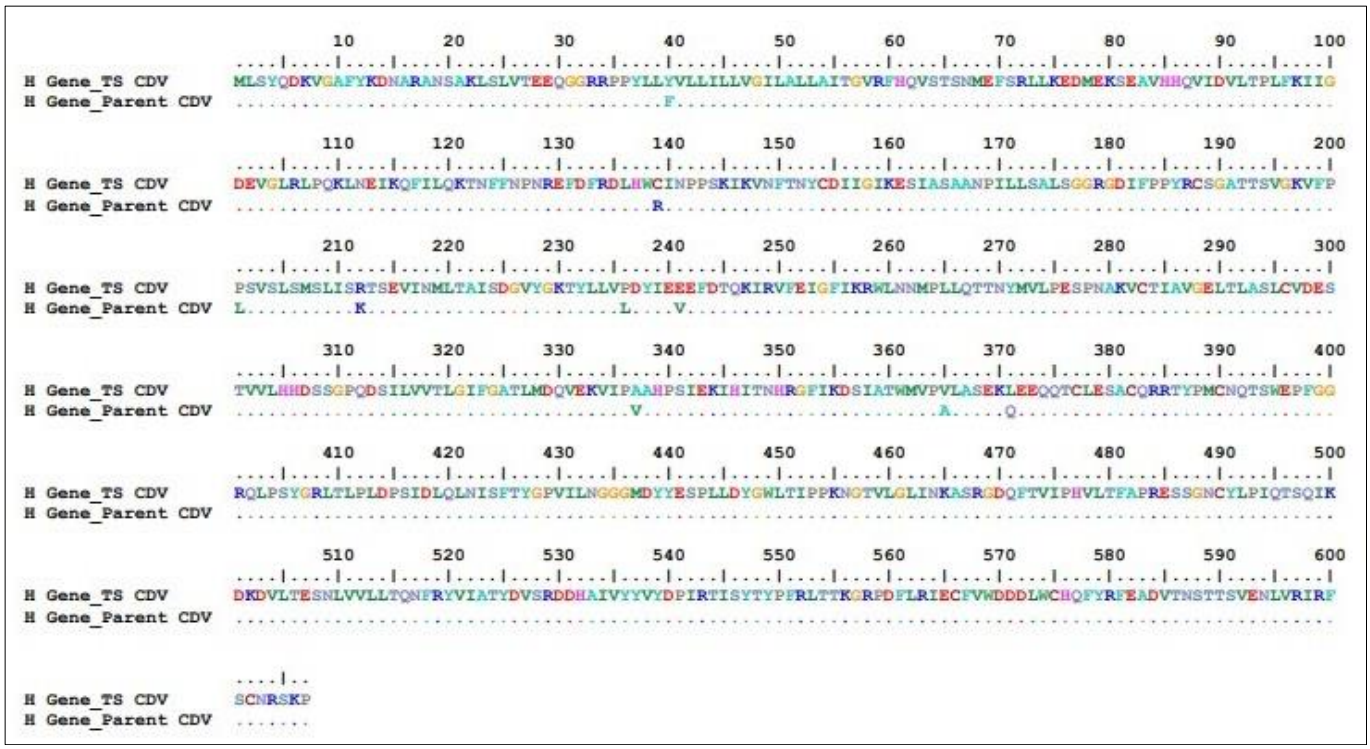

Fig 7: Sequence comparison of H protein

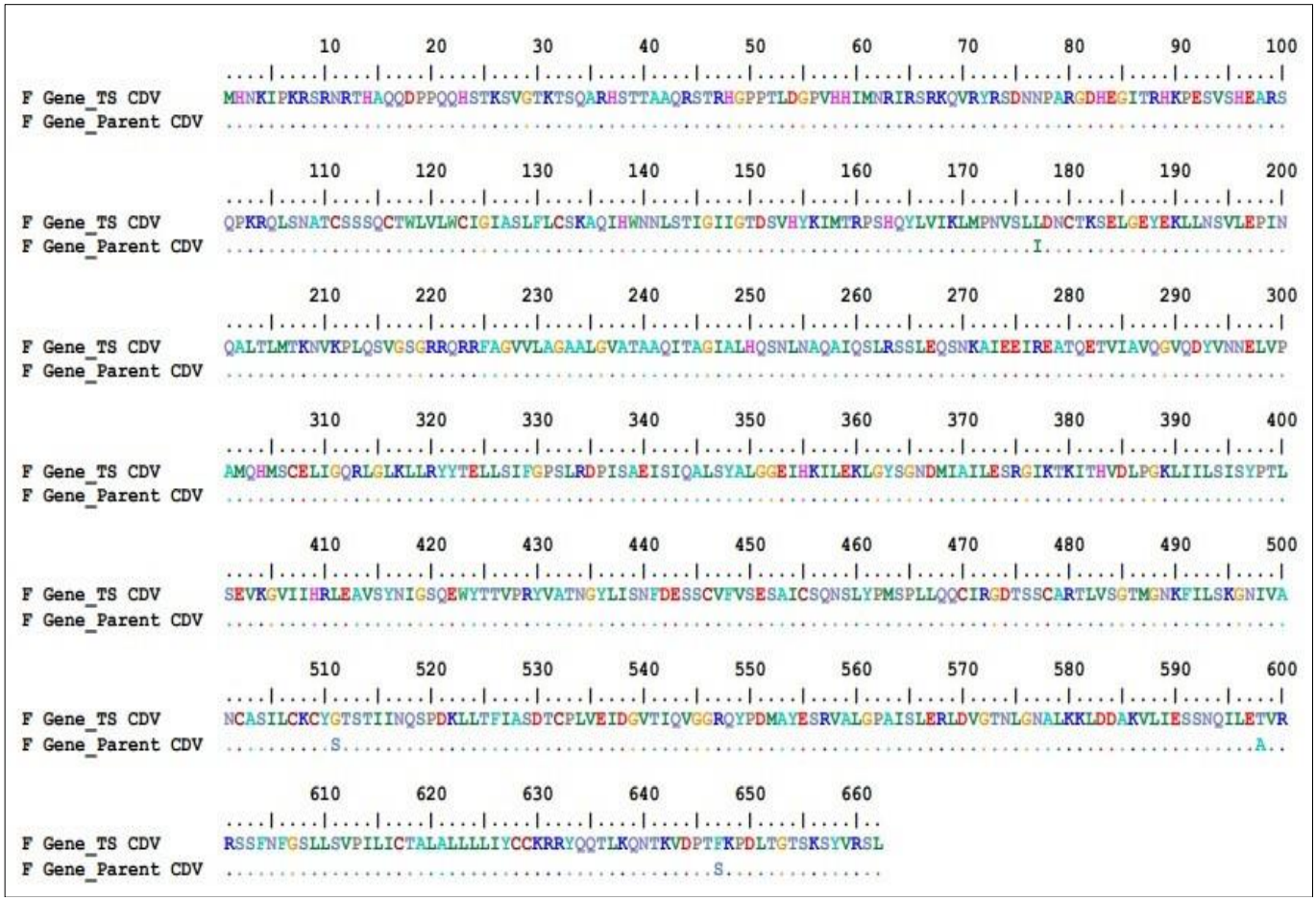

Fig 8: Sequence comparison of F protein

Table 1: List of the primers used in current study

	Sequence 5'-3'	Sense	Amplicon size	Target	Reference
1	CCTTCCAAAGCTGACTTGCATCATTG GATTTAGAGAATTTTGAAAAGACCCTG	+ -	1268 bp	M gene	[7]
2	ACAAGGTCAGGGTTCAGACCTACC ATGGYTGATCGGGTTTGTTGGACC	+ -	1693 bp	N gene	[In-house designed primer]
3	TGCTCTCCTACCAAGACAAGG TCAAGGTTTTGAACGGTTACATG	+ -	1824 bp	H gene	[8]
4	CAGACAAGCCCCATGCACAA TGGACTACCTGAGYCCTAAGT	+ -	2153 bp	F gene	[7]

Table 2: Comparison of proteins based on their aliphatic index

Protein	No of amino acids	MW(Da)	Theoretical pI	Aliphatic index
F protein (CDV parent virus)	662	72782.64	9.32	97.37
F protein CDV-TS	662	72842.74	9.32	97.22
H protein (CDV parent virus)	607	68449.79	6.21	98.39
H protein CDV-TS	607	68423.68	6.00	97.27
M protein (CDV parent virus)	335	37836.94	9.00	98.00
M protein CDV-TS	335	37819.95	9.00	98.00
N protein (CDV parent virus)	523	58066.12	5.01	94.17
N protein CDV-TS	523	58074.21	5.16	94.17

3.5 Comparison of structural proteins and its domains based on aliphatic index

structural proteins and its various structural and functional domains (locations obtained from Uniprot) were compared in terms of their aliphatic index before and after passaging at high temperature. Aliphatic index is reported to be significantly higher in thermostable proteins (Ikai, 1980) [18]. As in table 2 there wasn't any consistent or notable difference in the aliphatic index of either the proteins or their domains (data for the domains are not given).

4. Discussion and Conclusion

The efficiency of CD vaccine in the present scenario is questionable as various reports about morbidity among vaccinated animals from various parts of the world started accumulating. Wild type CDV that circulates in India is found to be different from any previously known Asian lineages *viz.*, Asia-1, Asia-2, Asia-3, and Asia-4. Moreover, it is different from vaccine types as well (Swati *et al.*, 2015) [6]. The strain used in the current study is a novel lineage of CDV circulating in domestic dog population in India

isolated in 2019. This new lineage is reportedly distinct from the strains used in vaccine as well as previously established strains (Bhatt *et al.*, 2019) [7]. A vaccine prepared using this strain shcould address the problem of vaccine failure in case the present vaccines fail to elicit enough immunity against currently circulating strains of the virus.

Inherently CDV is thermolabile; the half-life at 21 ºC, 37 ºC, 45 ºC and 56 ºC was 120 min, 60 min, 10 min and 2-3 minutes respectively (Brussel and Karzon 1962) [8]. Multiple passages applying a desired selection pressure is a conventional method used for adapting viruses to evolve desirable characteristics. Adapting the virus to temperature as high as 40ºC was attempted in other morbilliviruses such as PPRV and Rinderpest (Balamurugan *et al.*, 2014) [13] (Raut *et al.*, 2001) [12]. In present study, the CDV was passaged 20 times at 41.5 ºC and CDV-Ta was evaluated for its growth potential in comparison to the parent virus. In congruence to previous studies (Lan *et al.*, 2005a) [15] , (Lan *et al.*, 2005b) [16], Before 48 hours the virus was more in cell associated fraction and it was more in cell free fraction after 48 hours. In another study, wherein the growth kinetics of chick embryo adapted Lederle strain of CDV was studies in Vero cells, the titre of released virus never exceeded the titre of cell associated virus at any of the time points (Shishido *et al.*, 1967) [19]. The reason for same may be ascribed to the use of different cells to study the growth kinetics of the virus. However, in current study the virus was adapted in vero cells only in which the growth kinetics study was performed. The suggested optimum time point for harvesting the CDV based on current study is culture is 72 hpi which is similar to previous findings (Siddiqui *et al.*, 2021) [20].

In growth curve there was a difference in the maximum titre achieved at 0.01 moi. Peak titre of CDV-P60 was 5.61 log$TCID_{50}$ per ml but for CDV-Ta it was 4.5 log $TCID_{50}$ per ml. However, higher titres as high as 6 log$TCID_{50}$ per mL was achieved when the CDV-Ta was infected at higher moi such as 0.1. Also, the eclipse period and latent period of CDV-Ta was prolonged. These differences suggest that that virus is able to multiply at low titre at higher temperature but needs to achieve more fitness to survive at higher temperature. Structural genes, particularly HN gene are associated with thermostability of the virus as reported in a previous work based on chimeric NDV viruses (Wen *et al.*, 2016) [21]. However, no significant changes were reported in the aa sequence of all the structural proteins analysed in current study. These findings may be correlated with the low titre of virus with few changes acquired over passage regimen at higher temperature as observed in this study. Similar findings were also reported in PPR virus passaged at 40ºC up to 50 times (Balamurugan *et al.*, 2014) [13]. However, it is speculated that with more passages and more and more accumulating mutations the virus is expected to achieve fitness to grow at higher temperature. In such a virus, detailed research with comparison of aliphatic indices of structural proteins, it is possible to ascertain any relationship between thermostability of the whole virus and that of individual proteins. If the functional significance of the mutations in terms of thermostability can be found out with further detailed research, these mutations can be incorporated into other strains of the virus by gene editing as a way of creating thermostable vaccines without having to passage the candidate virus multiple times.

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6. Conflict of Interest

Authors declare no conflict of interests regarding this research, authorship, and/or publication of this article.

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