Journal of Aquatic Biology & Fisheries | 2020 | **8** | pp. 118-123

ISSN 2321–340X

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Molecular Surveillance Detects Betanodavirus Infection in Farmed and Wild Fishes of India

Binesh, C.P.*, Jithendran, K.P., Sujeet Kumar., Anandaraja, R. and Ambasankar, K.

Aquatic Animal Health and Environment Division, ICAR-Central Institute of Brackishwater Aquaculture
75, Santhome High Road, R.A Puram, Chennai, Tamil Nadu, India- 600 028
**Email: bineshkanayi@gmail.com*

Abstract

The disease, viral nervous necrosis caused by betanodaviruses is an emerging threat to the fish farming industry. The virus has a broad range of fish hosts and infects all age groups of fishes causing huge economic loss to the entrepreneurs. Acute and latent infections were described for this virus which may also remain dormant/persistent in carrier fishes/invertebrates. Estimation of the prevalence of the virus was attempted in this study using advanced molecular tools. A nested rt-PCR method followed by nucleotide sequencing and subsequent bioinformatics analysis was adopted. Samples were collected from hatchery, marine, coastal & low-saline farms and wild environments from eight sites along the east and west coasts of India. Samples included apparently healthy fishes along with moribund with clinical signs. Nodavirus infection was diagnosed in 19.7% of samples analysed. Prevalence ranged from 40.6% in marine farms, 29.5% in coastal farms, 23.6% in wild marine and 5.6% in wild coastal environments with no infection in the marine hatchery and low-saline farm samples. All the isolates belonged to RGNNV genotype which has the widest host range among all the species of *Betanodavirus*. This study also identified new susceptible fish species to betanodavirus infection naturally.

Keywords: Fish virus, *Betanodavirus*, Viral nervous necrosis, molecular surveillance, rt-PCR

1. Introduction

Viral nervous necrosis in fish is caused by *Betanodavirus* of family nodaviridae. The disease characterised by vacuolation in the central nervous system of the affected fish causes 100% mortality in larvae and juveniles within 2-3 days of contraction of the virus. Betanodavirus is an RNA virus with a bipartite genome which comprises a larger segment (3.1 kb) RNA1 and a smaller segment (1.4 kb) RNA2. RNA1 codes for RNA dependant RNA polymerase while RNA2 codes for viral coat protein. Over 100 fish species are reported susceptible to the virus so far. Asymptomatic infection without clinical manifestations of the disease has been reported in fishes (Haddad-Boubaker *et al*., 2013; Nishioka *et al*., 2016) which may aggravate into an acute disease under stress (Binesh, 2012). Infective virus particles were shown to be released into the environment from asymptomatically infected fishes causing infection into a naive host (Barker *et al*., 2002; Castric *et al*., 2001; Chi, Shieh, and Lin, 2003; Johansen *et al*., 2003; Johansen *et al*., 2002). Cohabitation experiments also proved the same (Korsnes *et al*., 2012). Trash fishes used as feed in farms could act as carriers of the virus (Gomez *et al*., 2010). Some crustaceans (Chi *et al*., 2000; Chi, Shieh, and Lin, 2003; Gomez *et al*., 2008b; Gomez *et al*., 2006) and molluscs (Kim *et al*., 2018; Volpe *et al*., 2018) were also found harbouring the virus by causing an infection leading to mortality. In bivalves, the infection does not cause disease; instead acts as a reservoir for betanodaviruses (Volpe *et al*., 2018; Volpe *et al*., 2017). Bivalve molluscs were observed capable of accumulating and shedding viable virions which can reinfect fishes upon exposure (Volpe *et al*., 2017).

In India, viral nervous necrosis caused by betanodavirus was reported from hatcheries of Asian Seabass (Azad *et al*., 2005; Parameswaran et al., 2008), farmed marine fishes (Jithendran and Binesh, 2011; John *et al*., 2014) and ornamental fishes (Binesh, 2013; Binesh *et al*., 2013; Jithendran *et al*., 2011). Other than acute disease, information on the prevalence of the virus in farmed and wild fishes, especially in asymptomatic forms are not documented.

2. Materials and Methods

2.1 Collection of samples

Samples were collected at random from marine, coastal and low saline environments during October 2007-October 2010, since there was no information available on the presence and distribution of betanodavirus in India. Hatchery-reared, farmed and wild fish-samples were collected from eight sampling sites along the Indian coast (Fig. 1; Table 1). Salinity and temperature of the water at the time of sampling were noted and tabulated along with the general health condition of sampled fishes. The marine hatchery in this study reared Asian Seabass (*Lates calcarifer*) while the marine, coastal and low-saline farms at various sites reared different fish species namely, *L. calcarifer, Mugil cephalus, Liza parsia, L. tade, Mystus gulio* and *Chanos chanos*. Wild samples from marine and coastal environments were purchased directly from local fishermen as and when available.

2.2 Molecular diagnosis

Brain and eyes of the fishes were dissected in the field and stored in RNA*later* TissueProtect Tubes (Qiagen) or TRIzol® Reagent (Invitrogen). Total RNA was extracted in the laboratory from approximately 20 mg of preserved

Received on: 04-02-2019; Revised on:16-09-2020; Accepted on: 05-12-2020

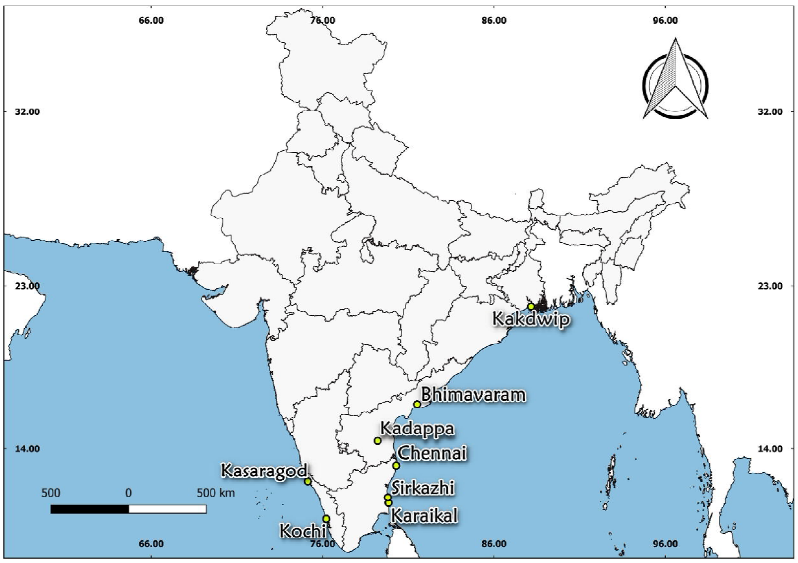


Fig. 1. Representation of sampling stations in the present study

tissue using TRIzol® Reagent (Invitrogen) following the manufacturer's instructions. The extracted total RNA was reconstituted in nuclease-free water and was stored at -70°C until used.

Previously published molecular screening protocol (Gomez et al., 2004) was adopted for nested rt-PCR in this study. The protocol included reverse transcription of extracted total RNA, primary PCR and nested PCR. Primers used for reverse transcription, PCR and nested PCR are summarised in Table 2. Reverse transcription was performed with betanodavirus specific primer BVNRT. Briefly, 2 µL extracted total RNA was incubated at 37 °C in reverse transcription reaction mixture containing 1X reaction buffer, 2.5 mM dNTPs, 0.5 pmols of primer BVNRT and M-MLV reverse transcriptase enzyme making up the total reaction volume to 20 µL. Primary PCR was performed in 20 µL with 1 µL cDNA from reverse transcription step, 2.5 mM dNTPs, 1 pmol each of forward and reverse primers (BNVUF1 and BNVUR1) and 0.5 U of DNA polymerase enzyme. Nested PCR was performed in the same way as primary PCR with nested PCR primers (BNVUF2 and BNVUR2) and primary PCR product as the template. Only the nested PCR results were documented in which 420 bp amplicons were generated. PCR products were visualised in 1.5% agarose gel stained with ethidium bromide. Prevalence of infection was calculated as the percentage of the fraction of infected samples to the total number of samples analysed.

2.3 Bioinformatics analysis

Representative PCR products from each host species from each location were extracted from agarose gels, purified and sequenced by outsourcing (Xcelris Labs Limited, Ahmedabad, India). All bioinformatics analyses were conducted in MEGA7 (Kumar, Stecher, and Tamura, 2016) unless specified otherwise. Raw sequences in forward and reverse directions were analysed, aligned, contigs were generated and submitted to NCBI GenBank (Table 1). They were analysed separately for similarity with other reported nodavirus sequences by nucleotide BLAST at NCBI website (https://blast.ncbi.nlm.nih.gov). Reference RNA2 sequences of the four *Betanodavirus* species were downloaded from RefSeq: NCBI Reference Sequence Database which hosts comprehensive, integrated, non-redundant, a well-annotated set of reference sequences including genomic, transcript, and protein. They included one sequence each of RGNNV, SJNNV, BFNNV and TPNNV with GenBank accession nos. NC 008041, NC 003449, NC 013459 and NC 013461 respectively. The reference sequences were aligned with the generated nucleotide sequences in this study using Clustal W module in MEGA 7. After predicting the best fit model, the phylogenetic analysis was carried out by the Neighbour-Joining method with the best fit model with 1000 re-samplings.

3. Results

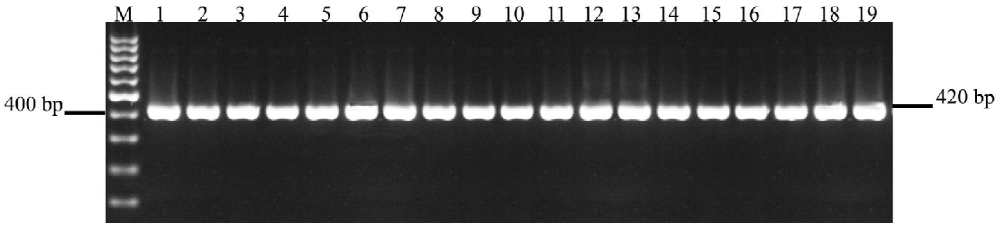
The samples included 258 fish samples belonging to 19 species (Table 1). Of them, 51 samples (19.7%) were positive for betanodavirus by molecular diagnosis (Table 1) generating 420 bp amplicons in nested PCR (Fig. 2). No positive cases were reported in samples of Asian Seabass collected from the marine hatchery (n=21) and low-saline farm (n=11). Prevalence of betanodavirus varied among the samples from different habitats (Fig. 3). In farms, it ranged from 40.6% (n=32) to 29.5% (n=44) while in the wild it ranged from 20.2% (n=114) to 5.6% (n=36) respectively in marine and coastal environments.

By rt-PCR and nucleotide sequencing, a total of 19 partial sequences of betanodavirusRNA2 were generated in this study (MG571288 - MG571306). In the nucleotide BLAST analysis, identity value of the hits ranged from 96-100%; the highest being towards isolates of *Redspotted Grouper Nervous Necrosis Virus* (RGNNV).

The calculated best fit model for phylogenetic relationships was Kimura-2 parameter method. In the Neighbour-Joining phylogenetic tree, constructed with five sequences of the four species of *Betanodavirus*, the 19 sequences generated in this study grouped with the RGNNV branch (Fig. 4).

Table 1. Details of collected fish samples and results of their analysis in the study

Sampling station	Fish species	Salinity (ppt)	Temp. ($^{o}_{C}$)	Health condition	Prevalence (%)	Accession no.
Hatchery samples					**0/21 = 0.0**	
Sirkazhi	*L. calcarifer*	32	28	Healthy	0/21 = 0.00	—
Marine farm samples					**13/32 = 40.6**	
Kasaragod	*L. calcarifer*	33	26	Healthy	5/8 = 62.5	MG571300
Kochi	*L. calcarifer*	26	28	Lethargic	4/4 = 100.0	MG571293
Karaikal	*L. calcarifer*	32	28	Lethargic	1/10 = 10.0	MG571298
Chennai	*L. calcarifer*	32	28	Healthy	3/10 = 7.5	MG571297
Coastal farm samples					**13/44 = 29.5**	
Kakdwip	*L. calcarifer*	14	26	Healthy	2/23 = 8.7	MG571306
	Mugil cephalus	14	26	Healthy	0/3 =0.0	—
	Liza parsia	14	26	Healthy	4/8 = 50.0	MG571302
	L. tade	14	26	Healthy	3/3 = 100.0	MG571303
	Mystus gulio	14	26	Healthy	1/1 = 100.0	MG571305
	Chanos chanos	14	26	Healthy	3/6 = 50.0	MG571304
Low saline farm samples					**0/11 = 0.0**	
Kadappa	*L. calcarifer*	4	28	Lethargic	0/11 = 0.0	—
Wild marine samples					**23/114 = 20.2**	
Kochi	*M. cephalus*	33	27	Healthy	0/12 = 0.0	—
	Epinephelus tauvina	33	27	Healthy	0/2 = 0.0	—
	Lepturacanthus savala	33	27	Healthy	0/1 = 0.0	—
Chennai	*Sardinella longiceps*	32	28	Healthy	2/5 = 40.0	MG571290
	L. calcarifer	28	25	Healthy	2/10 = 20.0	MG571294
	Amblygaster clupeoides	32	28	Healthy	1/3 = 33.3	MG571295
	Terapon jarbua	32	28	Healthy	0/1 = 0.0	—
	Synodus indicus	32	28	Healthy	0/1 = 0.0	—
	E. tauvina	32	28	Healthy	0/2 = 0.0	—
	Thryssa dussumieri	32	28	Healthy	3/4 = 75.0	MG571288
	T. setirostris	32	28	Healthy	0/1 = 0.0	—
	Leiognathus splendens	32	28	Healthy	2/4 = 50.0	MG571289
	Upeneus sulphureus	32	28	Healthy	2/5 = 40.0	MG571296
	M. cephalus	32	28	Healthy	3/26 = 11.5	MG571291
	Nemipterus japonicus	32	28	Healthy	0/6 = 0.0	—
	C. chanos	32	28	Healthy	2/9 = 22.2	MG571292
Bhimavaram	*L. calcarifer*	35	27	Healthy	6/22 = 27.3	MG571301
Wild coastal samples					**2/36 = 5.6**	
Karaikal	*M. cephalus*	13	27	Healthy	0/4 = 0.0	—
	Oreochromis mossambicus	14	27	Healthy	0/22 = 0.0	—
	L. calcarifer	6	25	Healthy	2/5 = 40.0	MG571299
Chennai	*Etroplus suratensis*	13	26	Healthy	0/3 = 0.0	—
	O. mossambicus	14	26	Healthy	0/2 = 0.0	—
TOTAL					**51/258 = 19.7**	



Lane M: 100 bp ladder
Lane 1 : Farmed *L. calcarifer* (Kasaragod)
Lane 2 : Farmed *L. calcarifer* (Kochi)
Lane 3 : Farmed *L. calcarifer* (Karaikal)
Lane 4 : Farmed *L. calcarifer* (Chennai)
Lane 5 : Farmed *L. calcarifer* (Kakdwip)
Lane 6 : Farmed *L. parsia* (Kakdwip)
Lane 7 : Farmed *L. tade* (Kakdwip)
Lane 8 : Farmed *M. gulio* (Kakdwip)
Lane 9 : Farmed *C. chanos* (Kakdwip)
Lane 10: Wild *S. longiceps* (Chennai)
Lane 11: Wild *L. calcarifer* (Chennai)
Lane 12: Wild *A. clupeoides* (Chennai)
Lane 13: Wild *T. dussumieri* (Chennai)
Lane 14: Wild *L. splendens* (Chennai)
Lane 15: Wild *U. sulphureus* (Chennai)
Lane 16: Wild *M. cephalus* (Chennai)
Lane 17: Wild *C. chanos* (Chennai)
Lane 18: Wild *L. calcarifer* (Karaikal)
Lane 19: Wild *L. calcarifer* (Bhimavaram)

Fig. 2. Representation of the amplified products specific to betanodavirusRNA2 in agarose gel stained with ethidium bromide. Lane M: 100 bp ladder; Lanes 1- 9: Farmed fishes; Lanes 10-19: Wild fishes

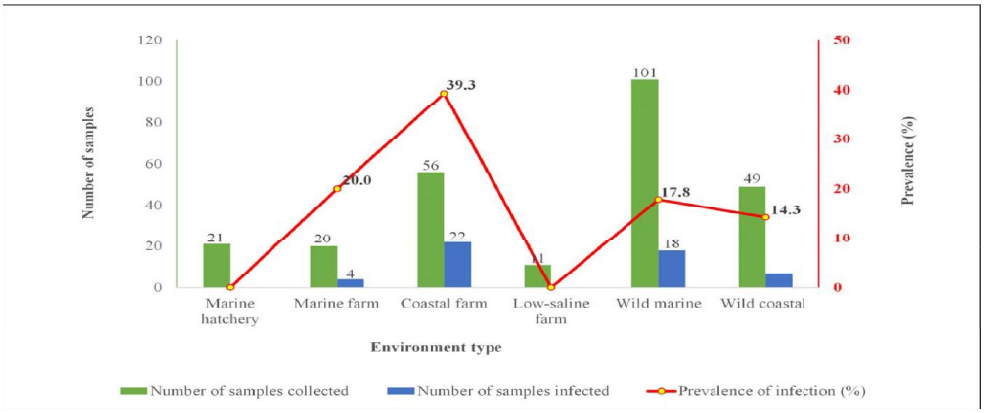


Fig. 3. Chart showing the prevalence of betanodavirus infection in fishes collected from different environments in this study

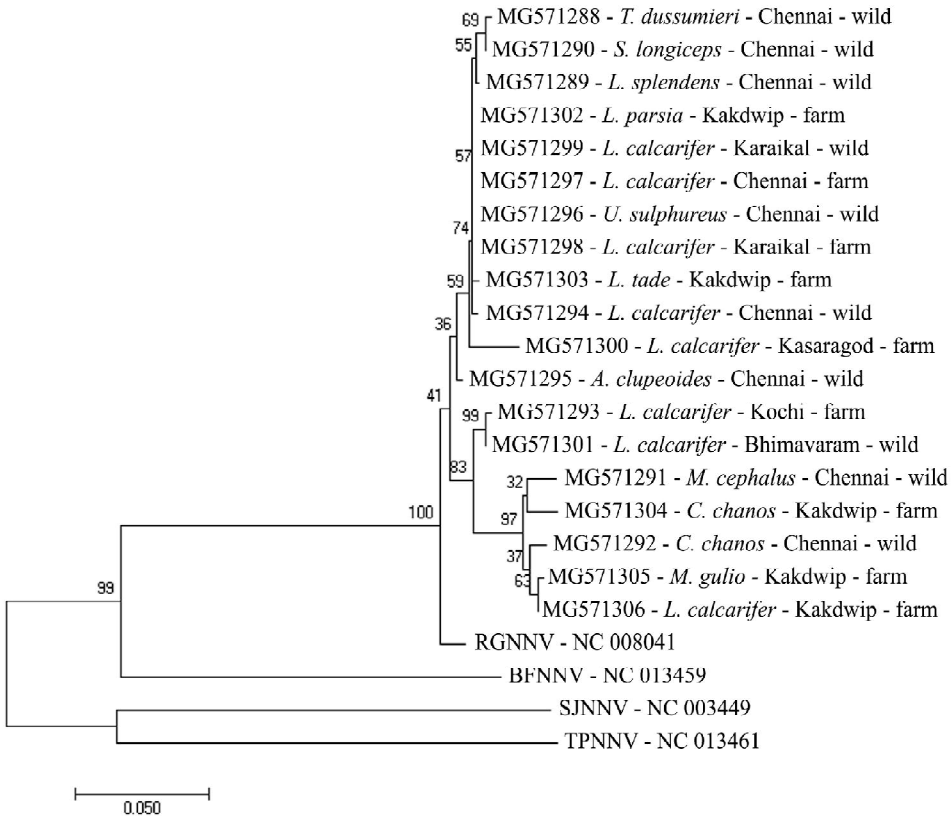


Fig. 4. Phylogenetic analyses of Indian isolates of *Betanodavirus*. The tree was drawn with members of the four species of *Betanodavirus* in MEGA7 using Neighbour-Joining method with 1000 re-samplings in the bootstrap test

Table 2. Details of nested *rt*-PCR primers used for surveillance in this study (Gomez *et al.*, 2004)

Primer name	Primer sequence (5'……3')	Position on RNA2*	Amplicon size (bp)
BVNRT[a]	GTGCCMRCHGGCAGCARRATYTG	990–1012	570
BNVUF1[b]	CAACTGACARCGAYCACACCTTCG	418–441	
BNVUR1[b]	CGDGGYTGCKSRTCRGARTARTA	968–988	
BVNUF2[c]	THCAAGCRACTCGYGGTGC	448-466	420
BNVUR2[c]	TGCCARTAVACRGCMCGKTCVACRTC	843–868	

***** GenBank accession number of template RNA2 was AY324870

[a] Used for reverse transcription; [b] Used for primary PCR; [c] Used for nested PCR.

4. Discussion

The molecular tool is considered as a confirmatory diagnostic test for VNN (OIE, 2018). An rt-PCR based molecular method using degenerate primers (Gomez *et al*., 2004) was used in this study due to its high specificity and sensitivity.

In this study, betanodavirus infection was diagnosed in fishes collected at random from marine and low saline environments and farms with an overall prevalence of 19.7%. Only a few lethargic cases of farmed fishes with VNN-like-disease-signs did yield a positive result in molecular diagnosis. This emphasises that the presumptive diagnosis must always be corroborated with a confirmative test. This study also forms the first report on the susceptibility of farmed *L.parsia, L. Tade* & *M.gulio* and wild *S.longiceps, A.clupeoides, T.dussumieri, L.splendens* and *U.sulphureus* to betanodavirus.

Compared to wild samples, the prevalence of infection was higher in farm samples (Fig. 3). This is a generally accepted trend as the occurrence of diseases always increases with farming activities. As the hatchery samples were free of the virus, it is highly suggestive of the ubiquitous presence of the virus in our environment. Betanodavirus is known to persist in farmed fishes or otherwise (Binesh and Jithendran, 2010; Chi, Wu, and Cheng, 2005; Johansen *et al*., 2003; Johansen *et al*., 2004; Johansen *et al*., 2002; Wu, Kai, and Chi, 2013) and may get unnoticed. Virions recovered from bivalves (Kim *et al*., 2018; Volpe *et al*., 2018; Volpe *et al*., 2017) were proved infective although infectivity of the virus detected in apparently healthy marine and freshwater ornamental fishes and marine invertebrates (Gomez *et al*., 2008a; Gomez *et al*., 2008b; Gomez *et al*., 2006) is unknown. Also, with the higher stocking density in farms, the infected-fishes/carriers interact more frequently with the rest of the population/community spreading the virus easily.

The variable region of viral coat protein gene encodes the C-terminal half of viral coat protein. This region is being used extensively for genomic classification of the virus followed by its first description (Nishizawa *et al*., 1997). All the isolates in this study shared high homology at the variable region indicating that they are the same irrespective of host, environment and nature of infection. In the BLAST analysis, they made high similarity hits with the RGNNV species of *Betanodavirus*. This confirms that the present virus isolates belong to RGNNV genotype. All the isolates in this study shared high homology at the variable region of viral coat protein gene which encodes the C-terminal half of viral coat protein. This region is being used extensively for genomic classification of the virus followed by its first description (Nishizawa *et al*., 1997). This indicates that all the isolates in the present study, irrespective of host, environment and nature of infection are con-specifics. In the neighbour-joining phylogenetic tree, all the present isolates were clustered into the RGNNV species branch indicating their con-specificity.

The wide spread distribution of betanodavirus as acute and asymptomatic infection in fishes is a cause of concern considering the recent growth of the aquaculture sector in India. Asymptomatic infection in farmed fishes may develop into acute disease under the influence of stress like repeated spawning, high stocking density & elevated temperature (Binesh, 2012). Most of the newly-found-susceptible wild marine fishes in this study are used as feed in semi-intensive farms facilitating the easy spread of the virus. Virus transmission through trash fishes used as feed is well documented (Gomez *et al*., 2010). Presently identified RGNNV genotype exhibits the widest host range of all *Betanodavirus* and can tolerate a wide range of salinity and temperature. Its ability to infect across salinity barrier is experimentally proven (Pascoli *et al*., 2016). Together, the situation warrants the design of proper strategies and policies to control and contain the threats caused by betanodavirus in the aquaculture sector of India. It is also essential to have a targeted surveillance programme for betanodavirus infection in the country. This may reveal the presence of any other genotypes and possible re-assortants in the region which will be essential for developing effective vaccination strategies.

Acknowledgements

The authors thank the Director, ICAR-Central Institute of Brackishwater Aquaculture for providing facilities to conduct the research. Financial support by the Department of Biotechnology, Govt. of India also is acknowledged.

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