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Molecular analysis of NS1 gene of Indian protoparvoviruses

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ABSTRACT

Porcine parvovirus (PPV) is a predominant infectious agent responsible for infertility in pigs. Globally, it leads to colossal economic loss to the pig rearing communities, especially in the developing countries. The non-structural gene 1 (NS1) of PPV is responsible for the virus replication, transcription regulation and cytotoxicity. Variations in the NS1 gene could cause increased virulence of the virus and the enhanced virulence raises concern about the effectiveness of the PPV vaccines against newly emerging strains. So, in the present study, we collected 84 samples from different regions of Punjab and Guwahati (Assam) during 2019-2022. Out of 84 samples, 12 samples were positive for PPV-1. The NS1 gene was cloned and sequenced followed by analysis of Indian PPV-1 isolates to understand its evolutionary background, level of divergence and nucleotide/amino acid substitutions. The findings revealed that Indian PPV-1 isolates exhibit nucleotide substitutions with high percent sequence identity. Pairwise distance matrix values of the NS1 gene revealed that local PPV-1 isolates showed maximum divergence from sequences of Brazil, China, Korea and Germany. Furthermore, selection pressure analysis revealed that all the isolates were under positive selection. The findings of the current study warrant whole genome analysis of circulating PPVs in India to identify a putative vaccine strain for combating emerging PPVs.

Keywords: Characterization, Divergence, India, NS1 gene, Porcine parvovirus (PPV)

Pigs are reared worldwide to obtain valuable products for food (pork, bacon and gammon) and skin (Singh *et al*. 2019). Pigs are raised in unorganized as well as in organized farms especially in intensive commercial units in addition to commercial free-range enterprises (Ganaba *et al*. 2011). Although pig farming has a great potential, yet this business faces many problems time-to-time due to certain infectious diseases, which lead to high economic loss to the farmers. The infectious diseases occur due to poor biosecurity practices on the farm, uncontrolled trade and lack of awareness (Vander Waal and Deen 2018).

Porcine parvovirus causes reproductive diseases among sows (Streck and Truyen 2020). The virus also potentiates the effects of Porcine circovirus-2 (Sliz *et al*. 2015). In India, PPV was reported for the first time by Sharma and Saikumar in 2010 (Sharma and Saikumar 2010). At present, PPV is endemic in all pig-rearing countries of the world. It belongs to family Parvoviridae, subfamily Parvovirinae and genus *Ungulate protoparvovirus*. These are small, non-enveloped, single stranded DNA virus having genome size 4-6.3 kb (Liu *et al*. 2017). It encodes 3 non-structural as well as 3 structural proteins (Tamosiunas *et al*. 2014). Non-structural protein NS1 is responsible for replication of virus, transcription regulation and cytotoxicity. It also induces apoptosis via the mitochondria-mediated intrinsic apoptosis pathway, which causes host DNA damage. This damage leads to cell cycle arrest at the G1 and G2 phases and mitochondrial ROS accumulation that is responsible for mitochondrial damage which induces host-cell apoptosis (Zhang *et al*. 2019).

Previous phylogenetic analysis based on the NS1 gene indicated that the most recent common ancestor of PPV strains existed about 250 years ago and NS1 gene is under positive selection pressure (Ren *et al*. 2013). However, information is scanty on analysis of NS1 gene of PPV-1 from India. Therefore, we aimed to analyze the NS1 gene of Indian isolates of PPV to understand virus evolution.

MATERIALS AND METHODS

Sampling and processing: Clinical tissue samples (84) consisting of lungs, heart, lymph nodes, kidney, liver and/ or stomach content etc. were collected from aborted sows (stillbirth/ mummified/ aborted fetus) from Punjab state of India. The samples were processed for molecular detection of PPV through PCR as well as isolation of virus from PCR positive samples.

Primer designing: NS1 gene specific primers were designed from the sequence available in NCBI GenBank (accession no. JX568153) using Primer3

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input program. The primers (Forward primer: 5'-ATGCATCATTGGGGAAATGT-3' and Reverse Primer: 5'-GCAGGTTGGTGAAAGTTGGT-3') were targeting a 220 bp genomic region of NS1 gene of PPV-1.

Polymerase chain reaction: DNA was extracted from clinical samples using phenol: chloroform: isoamyl alcohol method (Sambrook and Russell 2006). PCR was carried out in a thermal cycler (Eppendorf) to detect PPV in the clinical samples using the following cycling conditions: Initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 60 sec and final extension at 72°C for 7 min. The reaction mixture consisted of 0.1U *Taq* DNA polymerase (Invitrogen), 1× PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs and 0.8 pmol each of forward and reverse primers. The amplified PCR products were visualized under ethidium bromide (0.5 µg/ml) stained 1.5% agarose gel and documented.

Virus isolation: Selected PCR positive tissue samples were used for virus isolation in the PK-15, ST and PS cell lines. These samples were given blind passages five times in cells for adaption. Infected cells were examined for cytopathic effects (CPE) like shrinkage, swelling and detachment of cell monolayer, if any. Positive cells with adaption of virus were confirmed through PCR. On the 7th day, these infected cells were harvested by 2-3 freeze-thawing cycles. Further, harvested cells were used for DNA isolation and reconfirmed through PCR.

Cloning of NS1 gene of PPV-1: PCR positive PPV DNA was used for amplification of the NS1 gene in a thermal cycler (Eppendorf) under PCR conditions as follows: Initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 45 sec, extension at 72°C for 1 min and a final extension step at 72°C for 7 min was given using Ampli Taq Gold^{Tm} 360 master mix (Thermo fisher scientific). After amplification, the NS1 gene specific product was run on the 1.5% agarose gel and purified by gel extraction kit (QIAGEN). The eluted products were cloned into pJET 1.2/ Blunt cloning vector (CloneJET PCR Cloning Kit, Thermo Fisher). The cloned plasmid was transformed in to *Escherichia coli* TOP10 competent cells. The transformed cells were selected on Luria Bertani (LB) agar plates containing ampicillin (100 µg/ml) and screened by colony PCR. Further, PCR positive clones were grown in LB broth containing ampicillin for plasmid isolation and restriction endonuclease (RE) digestion by BglII restriction enzyme to confirm the presence of insert in recombinant clones (Sambrook and Russell 2006). The plasmids confirmed through RE digestion were sequenced.

The sequences were analyzed with the retrieved NS1 gene sequences of PPV-1 from NCBI GenBank database for multiple sequence alignment, pairwise distance, selection pressure and phylogenetic analyzes using various bioinformatic tools such as MEGA7, Clustal Omega and online Data monkey server.

RESULTS AND DISCUSSION

PPV-1 is a leading cause of reproductive failure among swine population, characterized by embryonic/ fetal infection and death. The NS1 gene of PPV-1 contains lots of variations that might lead to increase of virulence of PPV-1, which raises concern about effectiveness of vaccines against PPV-1 in future. Till date, there are only few reports available on the NS1 gene based evolutionary analysis of PPV-1.

Initially analysis of NS-1 gene of PPV-1 revealed a biased mutation pattern (Shangjin *et al*. 2009). At the amino acid level, the target gene was having 50/662 (7.55%) polymorphic amino acid positions (Hao *et al*. 2011) and experienced negative selection pressure (Deng *et al*. 2020). Ren *et al*. (2013) did NS-1 gene based phylogenetic analysis of PPV-1 and reported that the target gene was having nucleotide substitutions (rate 3.03Ã×10^{-5}) and most recent common ancestor of PPV strains existed about 250 years ago. Aishwarya *et al*. (2016) targeted Indian NS1 gene sequences of PPV-1 and stated that the target sequence of Indian isolate had very close similarity with PPV sequences previously reported from India and China.

In the present study, out of 84 samples, 12 samples were positive for NS1 gene of PPV-1 through PCR, amplifying a 220 bp genomic region. Further, the PCR positive samples were grown in the PK-15/ST/PS cell lines where detachment of cells were observed in 5th day post infection in PK-15 and PS cell lines whereas on 7th day post infection in ST cell line indicating a slow growth of virus. The NS1 gene of selected PCR positive samples/isolates was cloned and screened by restriction digestion analysis (Fig. 1).

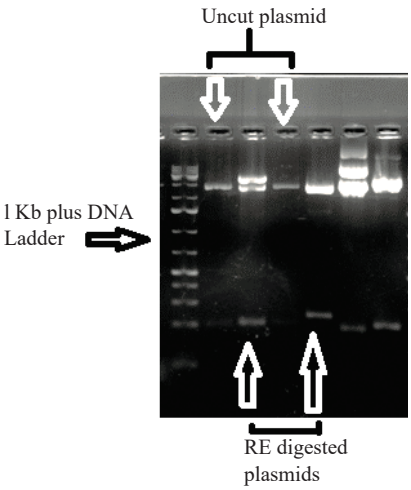

Fig. 1. Restriction digestion of NS1 gene of PPV-1. Lane 1: Gene ruler 1 Kb plus ladder; Lane 2, 4: undigested plasmids; Lane 3,5,6,7: RE (BglII) digested plasmids.

Multiple sequence alignment and pairwise distance matrix analysis: Multiple sequence alignment of NS1 gene sequences of Indian PPV-1 (11 sequences) as well as 19 retrieved sequences revealed 7 nucleotide substitutions in the partial NS1 gene sequence which comprised of 5 nucleotide transversions i.e. thymine replaced to adenine at

26th position, adenine replaced to thymine at 45th position, cytosine replaced to adenine at 46th and 102nd positions and adenine replaced to cytosine at 106th position. Similarly, 2 nucleotide transitions at position 15th and 79th positions (guanine replaced to adenine) were found.

The nucleotide sequences of the target gene (NS1) of Indian sequences with NCBI retrieved sequences showed 96.21-100% similarity. In our analysis, the NS1 gene sequences of Indian sequence Isl_11|LDH showed maximum distance (as per number of base substitutions) with respect to sequence with accession no. JN400522 with distance value of 0.0571, whereas, the remaining study sequences (1-10) from India showed divergence with that of sequence with accession no. JN400522 with distance values ranging between 0.024-0.037. The pairwise distance matrix showed that the sequences JN400522, GQ884039, JQ710891 and KY586143 indicated maximum distance from the rest of the isolates.

Selection pressure analysis: The selection pressure analysis assuming the null hypothesis (H_0) of neutral selection (*i.e.,* dN-dS=0) was tested against the alternative hypothesis (H_1) of positive selection/Darwinism selection (dN>dS). The results indicated that the sequence-pairs JN400522 (sequence was deposited from Germany) versus Isl_01|LDH (Asia), Isl_02|LDH, Isl_03|LDH, Isl_04|LDH, Isl_05|LDH, Isl_06|LDH, Isl_07|LDH, Isl_08|LDH, Isl_09|LDH, Isl_10|LDH have experienced positive selection.

Phylogenetic tree analysis: The best evolutionary model analysis revealed that Hasegawa-Kishino-Yano (HKY)+ Gamma distribution model was having the lowest Bayesian information criterion value (2333.33) whereas TN93+G model was having the lowest corrected Akaike's Information Criterion (AICc) value (1884.47).

NS1 gene based phylogenetic tree was constructed using maximum likelihood method, 1000 bootstraps and best evolutionary model (HKY) determining evolutionary linkages of PPV-1 (Fig. 2). The findings depicted that all the isolates were originating from a common ancestor but

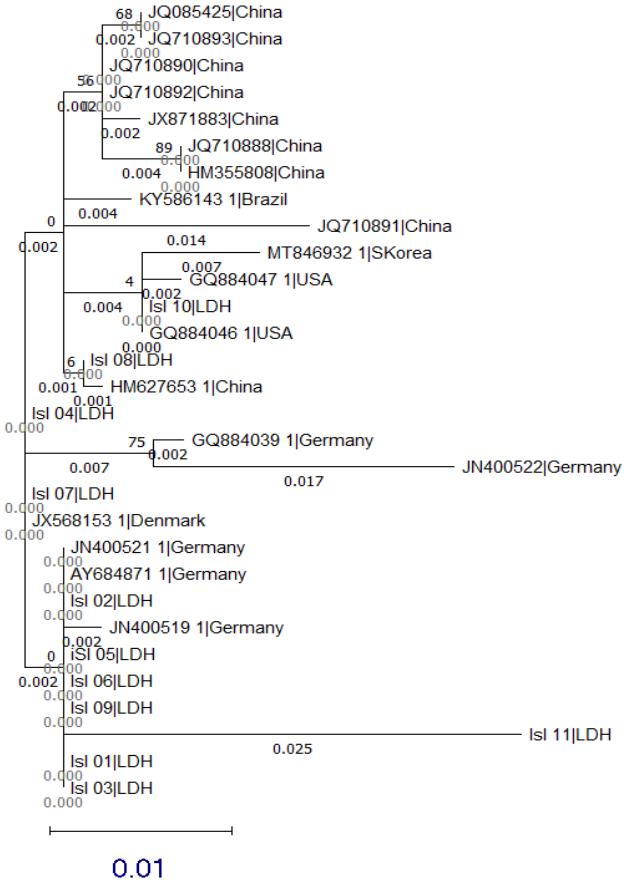

Fig. 2. NS1 gene based phylogenetic tree depicting evolutionary relationship between Indian PPV-1 sequences and other countries.

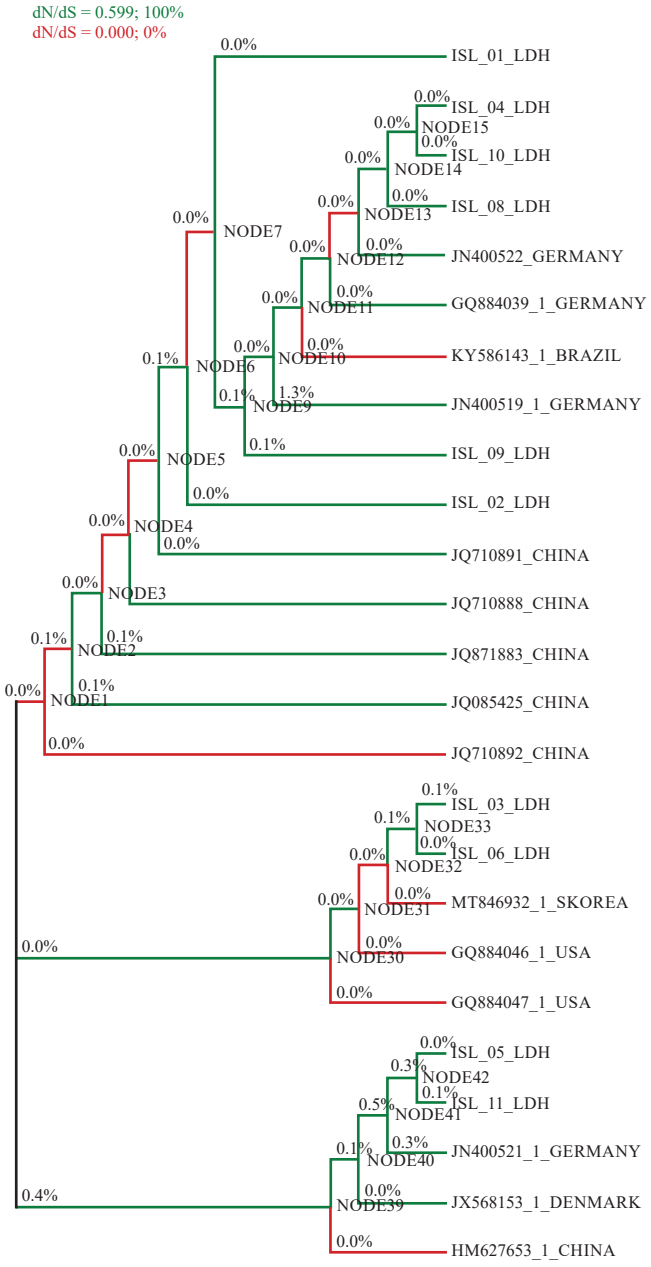

Fig. 3. Genetic algorithm (GA) branch depicting node wise experienced selection pressure on the tree of NS1 gene sequences of PPV1.

Table 1. Positively and negatively selected codons in IFEL analysis of NS1 gene of PPV-1

Codon	dS	dN	Dn/dS	p value
Positively selected codons				
23	1e-06	1.05894	1058940.00	0.065352
Negatively selected codons				
35	1.76756	0.0160177	0.091	0.0581392
45	1.76756	0.00474747	0.003	0.0731621
58	1.76756	0	0.000	0.011513
72	1.76756	0	0.000	0.0198276
88	1.76756	0	0.000	0.0247128
92	1.76756	0	0.000	0.0142403
117	1.76756	0	0.000	0.00607782
135	1.76756	0	0.000	0.0195851

due to the occurrence of genetic variations in the PPV-1 genome, its different strains emerged subsequently. These strains were clustered together based on genetic similarity. The phylogenetic analyses suggested that the Indian PPV isolates Isl01|LDH, Isl02|LDH, Isl03|LDH, Isl05|LDH, Isl06|LDH and Isl09|LDH shared maximum similarity and were clustered together in a clade, whereas Isl11|LDH showed maximum divergence within the clade. In this tree, majority of the Indian isolates occupied different positions in different clades, which depicted that there were more than one variant of PPV-1 circulating in swine population of India.

GA branching: The genetic algorithm branch (GA Branch), is a data extraction method that divides all the branches in the tree to get the specific sequences and performs multi-model assumptions to increase the accuracy of the result. It assigns the values of dN/dS to every branch of the phylogenetic tree. The GA Branch analysis of the PPV1_NS1 gene depicted that out of 3 rate classes, only 1 rate class had been observed with the best dN/dS score 0.585161 with the value of c-AIC 4419.06 and 19 branches showed the highest probability value of dN>dS, which depicted that all the nodes of this tree experienced positive selection pressure (Fig. 3).

IFEL (Internal Branch Fixed Effects Likelihood) analysis: The internal branch fixed effects likelihood (IFEL) is used to obtain good site-by-site substitution rate estimation. This type of selection pressure is confined to the internal branches of the tree. IFEL analysis of the PPV1_NS1 gene showed that out of 156 codons, only 1 codon was positively selected, whereas 8 codons were negatively selected (Table 1). By comparing results of GA branching with IFEL analysis, it was observed that in GA branching, all the nodes experienced positive selection pressure but in IFEL analysis, only one codon experienced positive selection. In IFEL analysis of NS1 gene of PPV1, maximum numbers of codons were found non-synonymous and while only 27 codons were found with synonymous substitutions.

The findings revealed that Indian PPV-1 isolates NSI gene depicted 5 nucleotide transversions and 2 nucleotide transitions with percent identity ranging between 96.21-100%. Pairwise distance matrix values of NS1 gene revealed that Indian PPV-1 isolates showed maximum divergence from Brazil, China, Korea and Germany (with distance value 0.057). Selection pressure analysis revealed that all the isolates experienced positive selection. The findings of current work warrant whole genome analysis of circulating PPVs in India to identify a putative vaccine strain for combining emerging PPV infections in the country.

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