Indian porcine reproductive and respiratory syndrome virus bears discontinuous deletion of 30 amino acids in nonstructural protein 2

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Received: 15 June 2016 / Accepted: 13 August 2016 / Published online: 27 August 2016
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Abstract Since its first outbreak in 2013, porcine reproductive and respiratory syndrome (PRRS) has established as an enzootic disease in pig population of Mizoram state, India. Our previous studies based on phylogenetic analysis of ORF5 and ORF7 gene sequences revealed close relationship of Indian PRRSV with the highly pathogenic variant of PRRSV (HP-PRRSV) of Chinese origin. Despite the control measures, second major outbreak of the disease was recorded in Aizawl district of Mizoram in 2015. The objective of the present study was to examine the origin of PRRSV isolates of 2015 outbreak, identification of deleted region in Nsp2 gene and determination of any genetic variation between 2013 and 2015 isolates of PRRSV. The outbreak was confirmed by the detection of PRRSV-specific antibodies in 57 out of 92 serum samples (61.96 %) and also by RT-PCR in 42 out of 42 necropsy samples (100 %). Nucleotide sequence analysis of Nsp2 coding region of Indian isolates and comparison with reference sequences revealed 90 nucleotides discontinuous deletion further establishes the closeness of Indian PRRSV to Chinese HP-PRRSV. Further, sequence and phylogenetic analysis of ORF5, ORF7 and Nsp2 genes of Indian

PRRSV from both 2013 and 2015 revealed that the outbreaks were caused by two different strains of HP-PRRSV closely associated with the Chinese 10 HEB-3 isolate and 07QN isolates of Vietnam origin respectively. The present study confirms that the Indian PRRSV is a highly pathogenic variant of PRRSV and this study serves as the basis for developing practical and effective control measures against this disease.

Keywords Pig · PRRS · Nsp2 · ORF5 · ORF7 · Phylogeny · India

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in 1987 in the United States of America and is characterized by reproductive failure in sows and respiratory tract illness in pigs [1, 15, 23, 26]. It became a pandemic disease in North America, Europe, and Asia in the subsequent years [3–6, 16, 31]. Now the disease occurs in most pig-producing countries and causes great economic loss to swine industry worldwide except for few countries including Switzerland, New Zealand and Australia [7, 21]. The causative agent, PRRSV, is an enveloped, single-stranded, positive-sense RNA virus with an approximate diameter of 50–65 nm and belongs to the family Arteriviridae in the order of Nidovirales [8, 31]. There are two distinct genotypes of PRRSV: genotype 1 (European) and genotype 2 (North American) which, share only about 60 % identity on the nucleotide level, while individual PRRSV isolates within genotypes varies approximately 20 % in nucleotide sequence [13, 19]. The genome size of PRRSV is about 15 kb possessing a 5'-capped structure, a 3'-polyadenylated tail, and at least nine

Electronic supplementary material The online version of this article (doi:10.1007/s13337-016-0341-9) contains supplementary material, which is available to authorized users.

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open reading frames (ORF1a, ORF1b and ORFs 2 to 7) flanked by 5'- and 3'-untranslated regions (UTR) [9, 20]. The GP5 is the most abundant enveloped N glycosylated protein encoded by ORF5 and is functionally important in terms of its role in virus neutralization. At the same time, it has been widely used for phylogenetic analysis because it contains more hypervariable regions compared to other genes, which allow discrimination of groups of strains in a meaningful manner [11, 17]. The ORF7 encodes for a nucleocapsid protein (N) encapsidating the viral RNA genome and is highly immunogenic in infected animals [27]. The nonstructural protein 2 (Nsp2) coding region is recognized as a variable gene, which undergoes significant genetic variation associated with natural point mutations and deletions [12, 14].

Highly pathogenic porcine reproductive and respiratory syndrome (HP-PRRS), characterized by high fever (40–42 °C), high morbidity (50–100 %) and mortality (20–100 %) in all ages of pigs, initially emerged in Southern China in 2006. The causative agent, HP-PRRS virus (HP-PRRSV) is a novel variant of PRRSV containing a unique discontinuous deletion of 30 amino acids (aa) in nonstructural protein 2 (Nsp2) [18, 29, 30]. Recent studies have suggested that HP-PRRSV has evolved from Chinese classical type 2 PRRSV like CH1a isolate [2]. However, what contributes to the increased pathogenicity of HP-PRRSV remains unclear. The genetic marker of HP-PRRSV, 30 aa deletion in Nsp2, is not linked to the increased virulence of HP-PRRSV [33]. In India, the first outbreak of PRRS in 2013 has caused huge economic loss through high morbidity and mortality in pig population of Mizoram state [24, 25]. Although there has been no record of major outbreak of PRRS in the year of 2014, the disease resurfaced in Aizawl district of Mizoram during the period March to June 2015. The aim of the present study was to examine the origin of PRRSV isolates of 2015 outbreak obtained from various farms of Mizoram state, India, and to identify any genetic variation between 2013 and 2015 isolates of PRRSV.

Materials and methods

Study area and sample collection

A total of 207 pigs of different age groups in two organized and 8 backyard farms from different locality in Aizawl district of Mizoram state, India, were studied during the outbreak period from March to June, 2015. Affected pigs were clinically examined and detailed necropsy examination was carried out in 42 dead pigs. A total of 92 serum samples were collected from pigs of different age groups of affected farms and were tested to detect PRRSV-specific antibodies using a commercial enzyme immunoassay kit (ELISA; IDEXX

PRRS X3, IDEXX Switzerland AG, 3097 Liebefeld-Bern, Switzerland). Serum samples were considered positive for PRRSV antibody if the optical density (OD) values of the sample to that of positive control was 0.4 or greater according to the manufacturer's instructions. Similarly representative tissue samples from lungs, spleen, tonsil and mesenteric lymph nodes were collected from individual dead pigs and stored at –80 °C for further studies.

RT-PCR, cloning and nucleotide sequencing

Total RNA was extracted from the tissue (lungs and spleen) using Trizol method (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of total RNA into cDNA was carried out using a cDNA synthesis kit (Fermentas Life Sciences, Ottawa, ON, Canada) according to the manufacturer's instructions. The tissue samples were tested by reverse transcription-PCR against PRRSV genotype II using the primer set PROF5-F: 5'-TGACACCTGA GACCATGAGG-3' and PROF5-R: 5'-GTGCAGAAGCC CTAGCAGTC-3' to amplify a 803 bp region covering the full ORF5 gene and PROF7-F: 5'-GATTGCGGCAAT GATAACC-3' and PROF7-R: 5'-GGCACAATGTCAAT CAGTGC-3' to amplify a 592 bp region covering the full ORF7 gene of PRRSV [25]. Based on nt sequence of Chinese HP-PRRSV reference strain 10HEB-3 (GenBank accession no. JQ663553), a primer set TKG-F: 5'-CTTCAGAAATGATGGCCCGG-3' and TKG-R: 5'-GTGCAGGGTTGGTGTCAATTT-3' was designed and used to amplify a 558 bp coding region of Nsp2 (nt position 2549 to 3106) to detect unique 30 amino acids deletion. The PCR cycling conditions were as follows: 95 °C for 2 min and 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min with a final extension for 5 min at 72 °C. The RT-PCR products were visualized in agarose gel and the size of the PCR product was compared to the GeneRuler™ 100 bp DNA Ladder (Fermentas Life Sciences, Ottawa, ON, Canada). The PCR products of size about 803, 592 and 558 bp for ORF5, ORF7 and Nsp2 respectively were considered as positive for PRRSV.

The amplified products of ORF5, ORF7 and Nsp2 of PRRSV positive samples were purified and cloned into pTZ57R/T vector using InsT/Aclone™ PCR product cloning kit (Fermentas Life Sciences, Ottawa, ON, Canada). The recombinant plasmids containing gene fragments were subjected to DNA sequencing at DNA sequencing facility, South campus, Delhi University, New Delhi, India.

Construction of phylogenetic trees

Phylogenetic trees were constructed using nucleotide sequences of 603 bp region corresponding to full ORF5

from 8 different field cases of 2015 outbreak, 372 bp region corresponding to full ORF7 from 6 field cases of 2015 outbreak and 558 bp coding region of Nsp2 from 5 field cases each from 2013 and 2015 outbreaks along with 45 (8 sequences of genotype I and 37 sequences of genotype II) reference sequences retrieved from the NCBI GenBank nucleotide database (Supplementary material 1). To determine any variation of PRRSV between the year 2013 and 2015 outbreaks, our previously analysed 12 sequences of ORF5 and 3 sequences of ORF7 from 2013 outbreak were also included in the study. Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 software with the neighbour joining method [28]. The robustness of the groupings in the neighbour-joining analysis was assessed with 1000 bootstrap resampling. Multiple sequence alignment was generated by using Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool.

Results

Epidemiological examination and RT-PCR

Acute respiratory disease with severe depression and high fever (40–42 °C) was observed in pigs of all age groups during the outbreak period. Affected pigs were completely anorexic continuously for 2–3 days but showed increased thirst and huddled together. Six of the pregnant sows in the population delivered either stillborn or weak piglets that died subsequently. Death of 42 pigs (20.28 %) of different age groups in different farms included in the study was recorded in the first 2 weeks from the onset of outbreak. Detailed necropsy examination of dead pigs consistently revealed severe haemorrhagic, oedematous, non collapsing lungs; enlarged, haemorrhagic, mesenteric and bronchial lymph nodes; congested, swollen spleen with areas of infarction. The serological analysis of 92 serum samples collected from pigs of different age groups of affected farms by enzyme immunoassay revealed 57 (61.96 %) serum samples positive for PRRSV-specific antibodies. Representative tissue samples (lungs, spleen) collected from all the 42 dead animals were tested positive by RT-PCR using the primers specific for ORF7.

Sequence and phylogenetic analysis

The nucleotide sequences derived from the clones of ORF5, ORF7 and Nsp2 were analysed and submitted to the GenBank. Accession numbers of Indian PRRSV isolates of 2013 and 2015 outbreaks and reference sequences are shown in supplementary material 1. All the 10 sequences of 558 bp coding region of Nsp2 showed high

similarity with the recent HP-PRRSV isolates (2010) of China characterized as novel variant of HP-PRRSV. They shared 94.09–96.42 % nucleotide identities with HP-PRRSV reference strain JXA1, while identities were 91.94–99.46 % among themselves. Among Indian PRRSV isolates, the isolate no. PRRS/MZ/IND/5/15 and PRRS/MZ/IND/9/15 showed only 92.10–92.46 % sequence homology with the others but 98.38 % homology among the two and the rest of 8 sequences showed 99.28–99.82 % similarity among themselves. Among the novel variants of Chinese HP-PRRSV, 10HEB-3 (GenBank Acc. No. JQ663553) showed maximum sequence homology of 98.39 % with Nsp2 of Indian PRRSV. Further, sequence alignment results also revealed the discontinuous deletion of 3 nucleotides at the position from 2778 to 2780 and 87 nucleotides deletion at the position from 2931 to 3017 corresponding to the classical PRRSV genotype 2 isolate VR2332 (Fig. 4a, b).

Sequence analysis of full ORF5 and ORF7 from the outbreaks of 2013 and 2015 revealed variations in nucleotide identity. ORF5 sequences of 2015 outbreak showed 99.34–100 % sequence identity among themselves but only 96.15–97.35 % identity with the sequences from 2013 outbreak. However, the 2013 sequences of ORF5 showed 98.83–100 % sequence identity within the group. Similarly, full ORF7 sequences of 2015 outbreak revealed 98.10–100 % sequence identities among themselves but only 95.93–97.58 % identity with the sequences of 2013. Within the ORF7 sequences of 2013 group showed 99.73–100 % identities among themselves. Interestingly, all the sequences of both ORF5 and ORF7 from 2015 outbreak showed maximum sequence homology (97.18–97.51 % in ORF5 and 97–98.39 % in ORF7) with the novel HP-PRRSV isolate 07QN (GenBank Acc. No. FJ394029) from Vietnam, while sequences from 2013 outbreak showed maximum identity (98.33–99.17 % in ORF5 and 99.19–99.46 % in ORF7) with the Chinese novel HP-PRRSV isolate 10 HEB-3.

In order to study the genetic evolutionary trend of Indian PRRSV, phylogenetic analysis on 20 sequences of ORF5, 9 sequences of ORF7 and 10 sequences of Nsp2 gene from both 2013 and 2015 outbreaks was carried out. Phylogenetic trees constructed using ORF5 and ORF7 sequences showed two distinct groups of Indian PRRSV separating the outbreaks of 2013 and 2015 but are grouped together with novel variant of Chinese HP-PRRSV. While 2013 sequences are clustered closely with the isolate 10HEB-3 of China, the 2015 sequences are placed closest to 07QN of Vietnam origin (Figs. 1, 2). However, phylogenetic tree derived from the Nsp2 gene does not differentiate the two groups but clustered together with Chinese HP-PRRSV (Fig. 3).

Fig. 1 Phylogenetic tree based on the analysis of ORF5 gene (603 bp) constructed using MEGA5 software by neighbour-joining method. It shows two distinct groups of Indian PRRSV separating the outbreaks of 2013 and 2015 but grouped together with novel variant of Chinese HP-PRRSV

Fig. 2 Phylogenetic tree based on the analysis of ORF7 gene (372 bp) constructed using MEGA5 software by neighbour-joining method. It shows two distinct groups of Indian PRRSV separating the outbreaks of 2013 and 2015 but grouped together with novel variant of Chinese HP-PRRSV

Discussion

The first outbreak of PRRS in 2013 in India occurred initially as severe acute respiratory disease with high fever affecting all age groups and caused up to 66.11 % mortality in affected pigs [24, 25]. The 2015 outbreak of HP-PRRSV was observed in the similar pattern but with less mortality. Due to inadequate compensation to the pig farmers following culling of infected animals, often the outbreaks

Fig. 3 Phylogenetic tree based on the analysis of Nsp2 coding region (558 bp) constructed using MEGA5 software by neighbour-joining method. It shows the Indian PRRSV clustered together with Chinese HP-PRRSV

were not reported and the farmers continue to rear both the sero-positive and pigs that have recovered from the acute disease. This is resulting persistence of the disease with growing incidences of reproductive failure in infected herd, which is a matter of great concern for control and prevention of the disease in rest of the pig population. HP-PRRS is now a big problem for Asian swine industry and becomes a huge threat to the world swine production with a major socioeconomic impact, affecting the livelihoods of millions of pig farmers. In recent past, HP-PRRS epidemics have also erupted in Southeast Asian countries, such as Vietnam (2007), Philippines (2007), Thailand (2009), Laos (2010), Cambodia (2010), Bhutan and Myanmar, causing huge economic loss to the local pig industries [10, 32]. In the present study, Indian Nsp2 nucleotide sequence analysis and comparison to reference sequences clearly demonstrated the presence of 90 nucleotides discontinuous deletion similar to HP-PRRSV of China (Fig. 4a, b). A

A		2777	2781
1	VR2332	AAGATTTGGCT	
2	CH-1a	AAGATTTTGCT	
3	HB1	AAGATTTGACT	
4	BJsy06	AAGA- - - AACT	
5	JXA1	AAGA- - - AACT	
6	07BJ	AAGA- - - AACT	
7	07QN	AAGA- - - AACT	
8	SX2009	AAGA- - - AACC	
9	10BJ-5	AAGA- - - AACT	
10	10GX-5	AAGA- - - AACT	
11	10GX-3	AAGA- - - AACT	
12	10HEB-2	AAGA- - - AACT	
13	10HEB-3	AAGA- - - AACT	
14	10JX	AAGA- - - AACT	
15	10QN	AAGA- - - AACT	
16	10BJ-1	AAGA- - - AACT	
17	10BJ-2	AAGA- - - AACT	
18	10BJ-4	AAGA- - - AACT	
19	10FUJ-4	AAGA- - - AACT	
20	10FUJ-5	AAGA- - - AACT	
21	PRRS/MZ/AZ/2/13	AAGA- - - AACT	
22	PRRS/MZ/AZ/35/13	AAGA- - - AACT	
23	PRRS/MZ/AZ/36/13	AAGA- - - AACT	
24	PRRS/MZ/AZ/46/13	AAGA- - - AACT	
25	PRRS/MZ/AZ/51/13	AAGA- - - AACT	
26	PRRS/MZ/AZ/1/15	AAGA- - - AACT	
27	PRRS/MZ/AZ/3/15	AAGA- - - AACT	
28	PRRS/MZ/AZ/5/15	AAGA- - - AGCT	
29	PRRS/MZ/AZ/9/15	AAGA- - - AGCT	
30	PRRS/MZ/AZ/10/15	AAGA- - - AACT	

Fig. 4 Alignment of Nsp2 nucleotide sequences of Indian PRRSV with reference sequences of North American and Chinese classical PRRSV and Chinese HP-PRRSV. **a** Alignment showing 3 nucleotides deletion in Indian PRRSV and Chinese HP-PRRSV from 2778 to 2780 with reference to VR-2332. **b** Alignment showing 87 nucleotides deletion in Indian PRRSV and Chinese HP-PRRSV from 2931 to 3017 with reference to VR-2332

unique 30 amino acid discontinuous deletion at position 481 and 533 to 561 in Nsp2 protein has been considered as the genetic marker for highly pathogenic variant of PRRSV [18, 29, 30]. Therefore, our results confirm the Indian PRRSV as HP-PRRSV of Chinese origin.

Despite the fact that almost 30 % of total pig population of India is present in North Eastern states of India including Mizoram, the region is yet to be self sufficient in production of pork and there is a huge gap between demand and supply for pork. This has resulted influx of pigs to the region from other states of India and also through porous international borders. The Mizoram state of India shares a

B

		2930	3018
1	VR2332	ACTGTGTCTCGACCGGTGACACCCTTGAGTGAGCCGATCCCTGTGCCCGACCCGCGCGTAAGTTTCAGCAGGTGAAAAGATTGAGTTCGGCGGGCGG	
2	CH-1a	ACTGTGTCCCACCGATGACGCCCTTGAGTGAGCCAATTTTGTGTCTGCACCCGCGACACAAATTCAGCAGGTGGAAGAAGCGAATCCGGCGGCAAA	
3	HB1	ACTGTGTCCCACCGATGACGCCCTTGAGTGAGCCAATTTTGTGTCTGCACCCGCGACACAAATTCAGCAGGTGGAAGAAGCGAATCCGGCGGCAAA	
4	BJsy06	ACTGTG-----	ACAA
5	JXA1	ACTGTG-----	ACAA
6	07BJ	CCTGTG-----	ACAA
7	07QN	ACTGTG-----	ACAA
8	SX2009	ACTGTG-----	ACAA
9	10BJ-5	ACTGTG-----	ACAA
10	10GX-5	ACTGTG-----	ACAA
11	10GX-3	ACTGTG-----	ACAA
12	10HEB-2	ACTGTG-----	ACAA
13	10HEB-3	ACTGTG-----	ACAA
14	10JX	ACTGTG-----	ACAA
15	10QN	ACTGTG-----	ACAA
16	10BJ-1	ACTGTG-----	ACAA
17	10BJ-2	ACTGTG-----	ACAA
18	10BJ-4	ACTGTG-----	ACAA
19	10FUJ-4	ACTGTG-----	ACAA
20	10FUJ-5	ACTGTG-----	ACAA
21	PRRS/MZ/AZ/2/13	ACTGTG-----	ATAA
22	PRRS/MZ/AZ/35/13	ACTGTG-----	ATAA
23	PRRS/MZ/AZ/36/13	ACTGTG-----	ATAA
24	PRRS/MZ/AZ/46/13	ACTGTG-----	ATAA
25	PRRS/MZ/AZ/51/13	ACTGTG-----	ATAA
26	PRRS/MZ/AZ/1/15	GCTGTG-----	ATAA
27	PRRS/MZ/AZ/3/15	ACTGTG-----	ATAA
28	PRRS/MZ/AZ/5/15	ACTGCG-----	ACAA
29	PRRS/MZ/AZ/9/15	ACTGCG-----	ACAA
30	PRRS/MZ/AZ/10/15	ACTGTG-----	ATAA

Fig. 4 continued

porous international border of 510 km with Myanmar. Hundreds of pigs regularly enter into Mizoram through the international border illegally, not only for slaughtering but also for breeding purpose. HP-PRRS was detected for the first time in Myanmar in February 2011, in Naypyidaw district of Mandalay province [22]. Subsequently it has spread to the other districts of the Mandalay region, killing more than 1000 pigs. Considering the fact that India was free from the disease till its emergence in Mizoram state in 2013 and our earlier study based on ORF5 and ORF7 gene sequences, the route of entry of first Indian HP-PRRS outbreak has been identified as this porous international border with Myanmar [24, 25]. Interestingly the analysis and comparison of ORF5 and ORF7 gene sequences from both 2013 and 2015 outbreaks revealed that the HP-PRRSV that has caused the outbreaks in 2015 is different from that of the HP-PRRSV of 2013 outbreaks. Thus it may be concluded that the HP-PRRSV of 2015 outbreaks is a result of second entry of a different HP-PRRSV strain closely associated to 07QN of Vietnam origin, through the same route. The emergence of highly virulent PRRSV strain has brought huge economical loss to the swine farmers of Mizoram state during both the outbreak periods. Government of India has declared several preventive measures including culling of infected pigs for control and containment of the disease following the first outbreak of the disease. However the illegal trade of pigs through the porous international border with Myanmar continues to be the main route of entry for HP-PRRSV in Mizoram, India.

Acknowledgments Authors are thankful to the Vice Chancellor, Central Agricultural University, Iroisemba, Imphal, Manipur for providing all necessary facilities to conduct the research work. Authors are also thankful to Department of Biotechnology, Government of India for funding the twinning project (C1-53), under which the work was carried out.

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