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Genetic variation of highly pathogenic Indian porcine reproductive and respiratory syndrome viruses after introduction in 2013

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ABSTRACT

To study its possible link to pathogenicity, the genomic variation in full ORF5 and ORF7 genes, and their encoded proteins in 26 field HP-PRRSV isolates from three major HP-PRRS outbreaks occurred in India, since 2013 was analysed. Sequence analysis and phylogenetic tree revealed involvement of genetically different strain in each outbreak of India rather persistence of a single strain. Analysis and comparison of N protein amino acid sequences of HP-PRRSV with VR2332 revealed consistent mutation at position 15D to N or K and 46 K to R in all the HP-PRRSV. GP5 protein showed consistent mutations at 29 positions from that of VR2332. The potential N-glycosylation sites in GP5 was found variable from 4–5 with one additional N-glycan moiety around the neutralizing epitope B. However, the 'decoy' epitope A was found highly conserved in all the HP-PRRSV.

Key words: GP5, HP-PRRS, India, N protein, ORF5, ORF7, Phylogeny

PRRSV belongs to the order Nidovirales, family Arteriviridae, genus A*rterivirus* and contains 5' capped and 3'-polyadenylated, single strand, positive sense RNA genome of approximately 15 kb in size. The viral genomic RNA contains 11 known open reading frames (ORFs). The replicase gene consists of the large ORFs 1a and 1b, are situated in the 5' -proximal three quarters of the polycistronic genome. The size of ORF1a is quite variable owing to the hypervariability in the central region of nonstructural protein 2 (nsp2) compared to the more conserved ORF1b. ORF1a and ORF1b encode two large nonstructural polyproteins, pp1a and pp1ab, with expression of the latter depending on a -1 ribosomal frame shift signal in the ORF1a/ORF1b overlap region. Further, the pp1a and pp1ab replicase polyproteins are processed into at least 14 nonstructural proteins (nsps) by 4 ORF1a-encoded proteinases residing in nsp1α, nsp1β, nsp2, and nsp4. Recently, a new ORF (TF) and -1/-2 programmed ribosomal frame shift signal was discovered in the central region of ORF1a, which expresses two novel proteins, nsp2TF and nsp2N (Fang *et al*. 2012, Li *et al*. 2014). The 3´-end of the viral genome contains eight relatively small genes, and these genes have both 5´- and 3´-terminal sequences overlapping with neighbouring genes, with the exception of ORF4/ORF5 of type 2 PRRSV. These genes encode four membrane-associated glycoproteins (GP2a, GP3, GP4, and GP5), three unglycosylated membrane proteins (E, ORF5a, and M), and a nucleocapsid protein (N).

The GP5 protein of PRRSV is one of the most important structural proteins exposed on the surface of the PRRSV and contains epitopes involved in virus neutralization and protection (Pirzadeh and Dea 1977, Gonin *et al.* 1999, Wissink *et al.* 2003, Ansari *et al.* 2006). It possesses an approximately 30aa N-terminal putative signal sequence (aa1–32), an ectodomain of approximately 35 residues (aa 33–63) with a variable number of potential N-glycosylation sites, a trans-membrane region (aa 64–134) and endodomain (aa 135–200). Comparison of the nucleotides and aa sequences of GP5 protein of a large no. of NA and EU strain identified a hypervariable region (aa 32–40), two variable regions (aa 57–70 and 121–130), three conserved regions (aa 41–56, 71–120 and 131–200) and multiple potential N-glycosylation sites. The hypervariable and immunodominant region of aa residues from 27 to 30 of GP5 is identified as non-neutralizing epitope A and the region between aa 37–45 in ectodomain of GP5 is identified as neutralizing epitope B. Epitope B is conserved among PRRSV isolates but not immunodominant (Ostrowski *et al*. 2002, Plagemann 2004).

The un-glycosylated N protein of NA PRRSV possesses 123 amino acids (aa) with a molecular weight of 15 kDa, encoded by ORF7 gene and is highly immunogenic. Pigs with infection of PRRSV mount a rapid antibody response directed mainly to the N protein and to a lesser extent to

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Table 1. List of gene sequences of Indian HP-PRRSV isolates used in this study.

Isolate ID/ Sample ID	Accession No.	Genotype	Country	Year
PRRSV/MZ/AZ/2/13	KJ624718 (ORF7)	2	India (Mizoram)	2013
	KM283188 (ORF5)			
	KU745395 (Nsp2)			
PRRS/MZ/AZ/35/13	KJ624717 (ORF7)	2	India (Mizoram)	2013
	KJ624716 (ORF5)			
	KU745394 (Nsp2)			
PRRS/MZ/AZ/36/13	KM283187 (ORF7)	2	India (Mizoram)	2013
	KM283189 (ORF5)			
	KU745393 (Nsp2)			
PRRS/MZ/AZ/55/13	KM283190 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/ 54/13	KM283191 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/53/13	KM283192 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/52/13	KM283193 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/ 51/13	KM283194 (ORF5)	2	India (Mizoram)	2013
	KU745397 (Nsp2)			
PRRS/MZ/AZ/ 50/13	KM283195 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/ 49/13	KM283196 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/AZ/ 46/13	KM283197 (ORF5)	2	India (Mizoram)	2013
	KU745396 (Nsp2)			
PRRS/MZ/AZ/ 34/13	KM283198 (ORF5)	2	India (Mizoram)	2013
PRRS/MZ/IND/1/15	KT844657 (ORF5)	2	India (Mizoram)	2015
	KT696491 (ORF7)			
	KU745399 (Nsp2)			
PRRS/MZ/IND/2/15	KT844654 (ORF5)	2	India (Mizoram)	2015
	KT696492 (ORF7)			
PRRS/MZ/IND/3/15	KT844658 (ORF5)	2	India (Mizoram)	2015
	KT844660 (ORF7)			
	KU745398 (Nsp2)			
PRRS/MZ/IND/4/15	KT844652 (ORF5)	2	India (Mizoram)	2015
	KT844661 (ORF7)			
PRRS/MZ/IND/5/15	KT844653 (ORF5)	2	India (Mizoram)	2015
	KT844659 (ORF7)			
	KU745402 (Nsp2)			
PRRS/MZ/IND/6/15	KT844655 (ORF5)	2	India (Mizoram)	2015
PRRS/MZ/IND/7/15	KT844656 (ORF5)	2	India (Mizoram)	2015
PRRS/MZ/IND/8/15	KU169894 (ORF5)	2	India (Mizoram)	2015
	KU169895 (ORF7)			
PRRS/MZ/IND/9/15	KU745401 (Nsp2)	2	India (Mizoram)	2015
PRRS/MZ/IND/10/15	KU745400 (Nsp2)	2	India (Mizoram)	2015
PRRS/IND/61/16	KX869639	2	India (Mizoram)	2016
PRRS/IND/66/16	KX869640	2	India (Mizoram)	2016
PRRS/IND/CH1/10/16	KX869641 (Nsp2)	2	India (Mizoram)	2016
	KX869653 (ORF5)			
	KX869647 (ORF7)			
PRRS/IND/CH2/10/16	KX869642 (Nsp2)	2	India (Mizoram)	2016
	KX869654 (ORF5)			

the M protein and are non-neutralizing (Loemba 1996). N protein has five domains of antigenic importance, identified for a reference genotype II strain. Four of them located at aa 30–52, 37–52, 69–112 and 112–123 and the fifth common conformational antigenic site is localized in the central region at aa 52–69 of the protein. The region between aa 37 to 52 is well conserved among isolates of both the genotype and is the most hydrophilic region of the protein.

Based on the genetic differences, PRRSV has been classified as type 1 or European-like strains and the type 2 or North American-like strains (Meulenberg *et al*. 1993, Nelsen *et al*. 1999). They share about 60% identity on the nucleotide level, while individual PRRSV isolates within genotypes can vary up to approximately 20% in nucleotide sequence. PRRSV undergoes remarkable genetic alteration, leading to high degree of genetic and antigenic diversity, which adds to the complexity of control and eradication of the disease (Yuan *et al*. 1999, Yuan *et al*. 2000, Yuan *et al*. 2001, Yuan *et al*. 2004, van Vugt *et al*. 2001).

Highly pathogenic PRRS (HP-PRRS) caused by HP-PRRS virus (HP-PRRSV), a novel variant of PRRSV, first emerged in pig populations of Southern China (Li *et al*. 2007, Tian *et al*. 2007, Tong *et al*. 2007, An *et al*. 2010, Zhou *et al*. 2008, Rajkhowa *et al*. 2015a). Subsequently the disease has spread to neighbouring Asian countries including Laos, Viet Nam, Cambodia, Bhutan and Myanmar resulting huge loss to the local pig husbandries.

The acute severe PRRS outbreaks that have been reported in 2013 and in 2015 from Mizoram, India, were identified as HP-PRRSV of Chinese origin (Rajkhowa *et al*. 2015a, 2015b and Rajkhowa *et al*. 2016). The disease has been contained so far within the state of Mizoram and not been reported from other region of the country. This article is confirming another major outbreak of HP-PRRS in Mizoram, India, bordering to Myanmar. In order to study the genetic evolutionary trend of Indian PRRSV, phylogenetic analysis on full ORF5, ORF7 and a 558 bp coding region of Nsp2 has been carried out. Further, the mutations in both GP5 and N proteins among the Indian and Chinese HP-PRRSV are analyzed and compared with VR2332 to find out the genetic diversity among them.

MATERIALS AND METHODS

Outbreak of an acute respiratory disease accompanied with reproductive problem in pig population of Champhai district of Mizoram state, India, was recorded during April to June, 2016. The disease with high morbidity and mortality rapidly spread to adjoining Lunglei and Lawngtlai district of Mizoram. Tissue samples comprising lungs, spleen, tonsil and lymph nodes (Inguinal, mesenteric and bronchial lymph nodes) were collected from total of 20 dead pigs of different age groups from three different disposal ground of Champhai district and stored at –80°C for diagnostic confirmation.

The tissue samples were tested by reverse transcription–PCR against PRRSV, CSFV (Greiser-Wilke *et al*. 1998) and also against PCV2 (Larochelle *et al*. 1999) by PCR. For RT-PCR, 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) following the manufacturer's instructions. The tissue samples were tested by reverse transcription–PCR against PRRSV genotype II

Table 2. List of PRRSV isolates retrieved from GenBank and used for the phylogenetic analysis.

Isolate ID/ Sample ID	Accession No.	Genotype	Country	Year of submission
RespPRRS Vaccine**	AJ223082	2	Denmark	1999
VR2332*	U87392	2	USA (Minesota)	2000
MN184A**	DQ176019	2	USA (Minesota)	
MN184C**	EF488739	2	USA (Minesota)	
CH-1a*	AY032626	2	China	1996
HB2**	AY262352	2	China	2002
F1**	AF030306	2	Taiwan	1998
HB1*	AY150312	2	China	2002
SHB*	EU864232	2	China (Guangdong)	2005
BJsy06*	EU097707	2	China (Beijing)	2006
SHH*	EU106888	2	China (Shanghai)	2006
GD*	EU109503	2	China (Beijing)	
JXA1*	EF112445	2	China	2006
07BJ*	FJ393459	2	China	2007
07QN*	FJ394029	2	Vietnam	2007
JNHS*	HM016158	2	China (Shandong)	2008
SX2009*	FJ895329	2	China	2009
09HUB1**	JF268682	2	China	2009
BB0907*	HQ315835	2	China (Guangxi)	2009
10 BJ-5	JQ663545	2	China (Beijing)	2010
GX1042*	JQ309822	2	China (Guangxi)	2010
FS*	JF796180	2	China (Guangdong)	2010
10GX-5*	JQ663562	2	China	2010
10GX-3*	JQ663560	2	China	2010
10HEB-2*	JQ663552	2	China	2010
10HEB-3*	JQ663553	2	China (Beijing)	2010
10JX*	JQ663540	2	China	2010
10QN*	JQ663556	2	Vietnam	2010
10BJ-1	JQ663541	2	China	2010
10BJ-2*	JQ663543	2	China	2010
10BJ-4*	JQ663544	2	China	2010
10FUJ-2*	JQ663547	2	China (Fujian)	2010
10FUJ-4	JQ663549	2	China (Fujian)	2010
10FUJ-5*	JQ663550	2	China (Fujian)	2010

Isolates with * mark were used for analysis of ORF5, ORF7 and Nsp2 sequences. Isolates with ** mark were used for analysis of ORF5 and ORF7 sequences only. Isolates without * mark were used for analysis of ORF7 and Nsp2 sequences only.

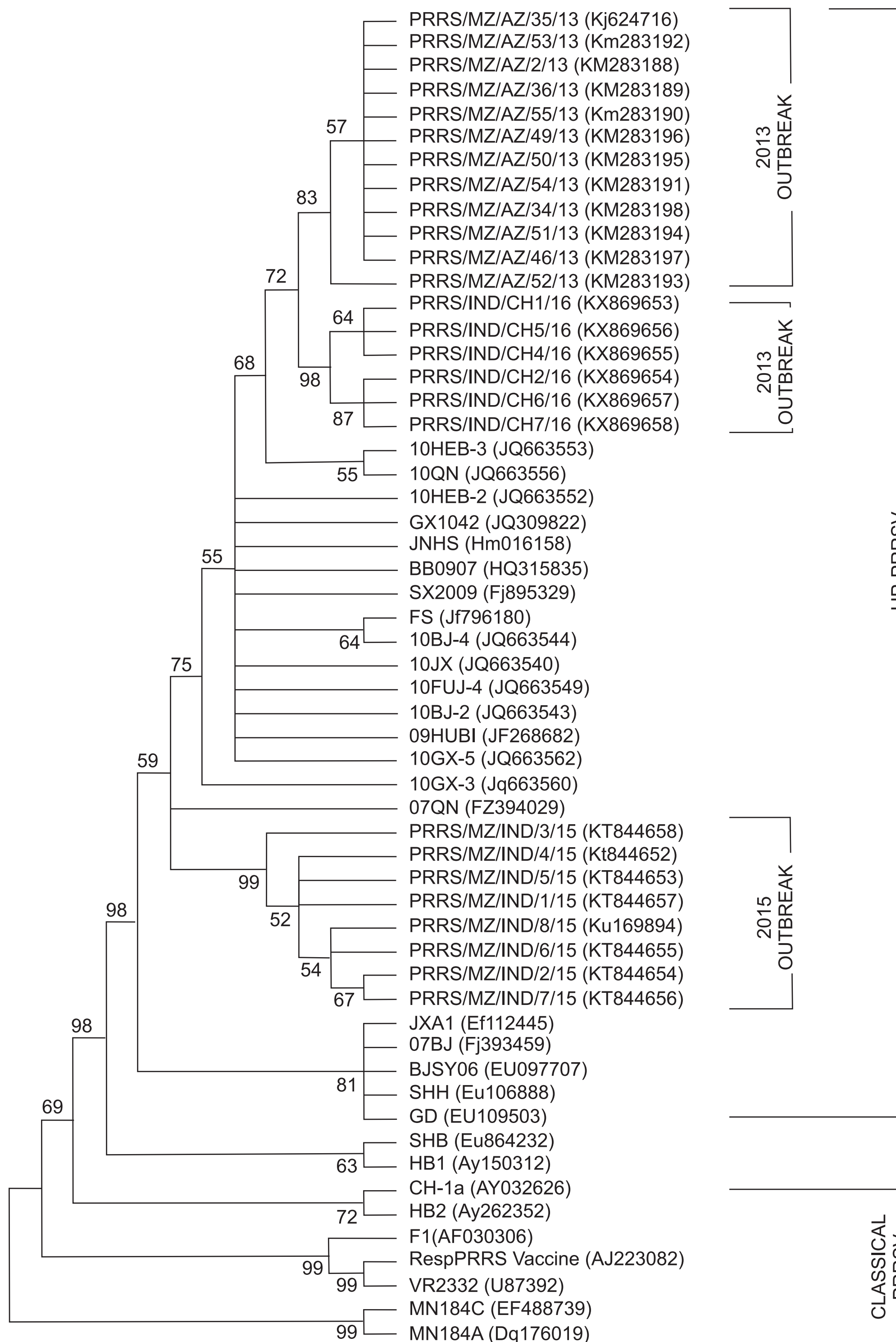

Fig. 1. Phylogenetic tree based on the analysis of ORF5 gene constructed using the neighbour-joining method.

targeting a 803 bp region covering the full ORF5 gene, a 592 bp region covering the full ORF7 gene of PRRSV and a 558 bp coding region of Nsp2 gene of PRRSV type2 to detect unique 30 amino acids deletion (Rajkhowa *et al.* 2015b). The amplified products were purified and cloned into pTZ57R/T vector using InsT/Aclone^{TM} PCR product cloning kit (Fermentas Life Sciences, Ottawa, ON, Canada) and recombinant plasmids containing gene fragments were subjected to DNA sequencing at DNA sequencing facility, South Campus, Delhi University, New Delhi, India.

Phylogenetic analysis was carried out using 6 nucleotide sequences each of full ORF5 (602 bp), full ORF7 (371 bp) and 558 bp coding region of Nsp2 from 6 field cases of 2016 outbreaks along with 34 (25HP-PRRSV, 2 intermediate genotype II, 7 classical genotype II) reference sequences retrieved from the NCBI GenBank nucleotide

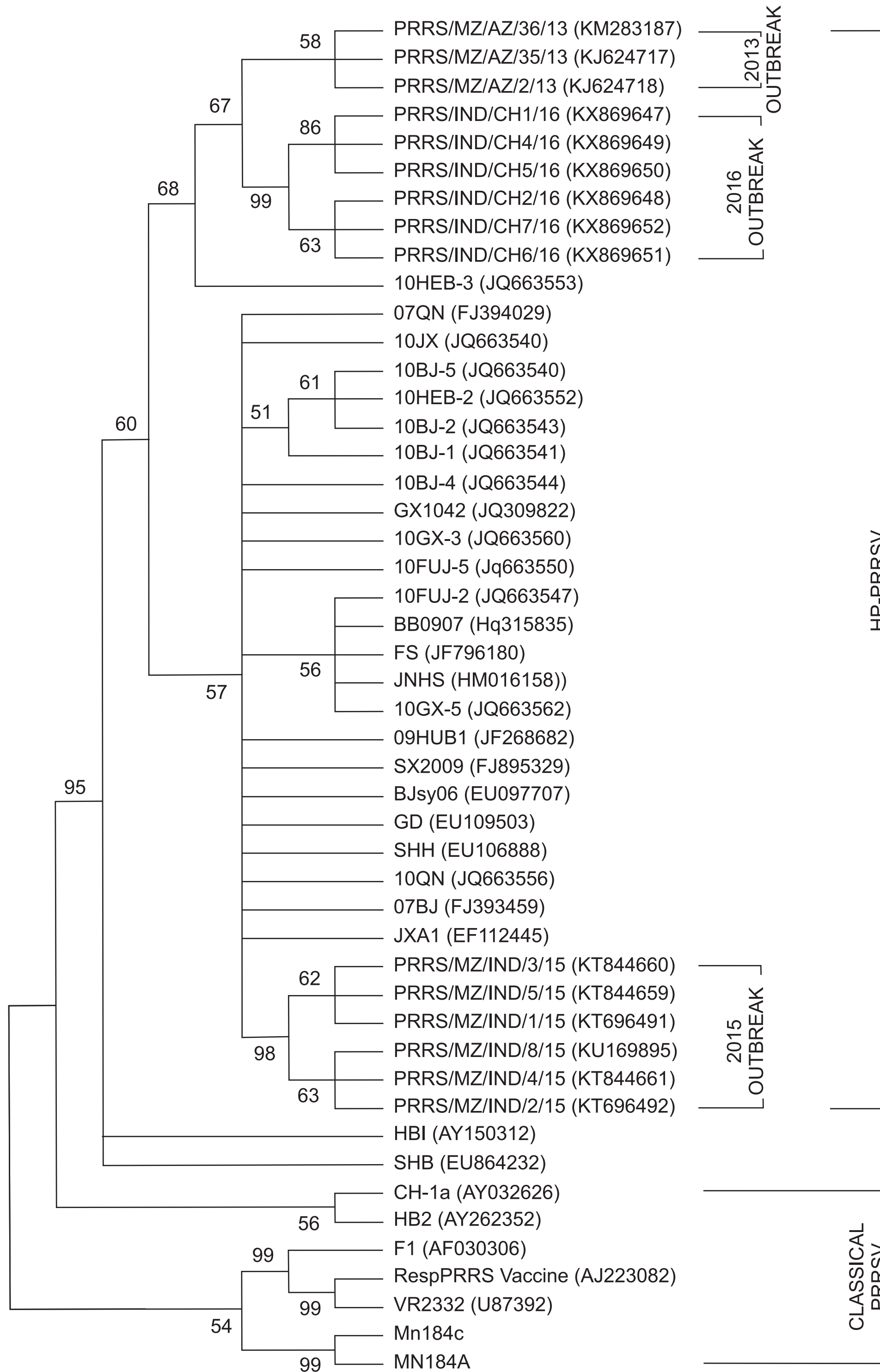

Fig. 2. Phylogenetic tree based on the analysis of ORF7 gene constructed using the neighbour-joining method.

database (Tables 1 and 2). To determine any variation of PRRSV from earlier outbreaks, our previously analyzed 20 sequences of ORF5, 9 sequences of ORF7 and 11 sequences of 558 bp region of Nsp2 were also included in the study. Phylogenetic and molecular evolutionary analyses were conducted using MEGA6 software with the neighbour joining method (Tamura *et al*. 2011). The robustness of the groupings in the neighbour–joining analysis was assessed with 1,000 bootstrap resampling. Multiple sequence alignment was generated by using Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool. The amino acid sequences of both GP5 and N protein were analyzed by using MEGA6.

RESULTS AND DISCUSSION

RT-PCR, sequence and phylogenetic analysis: Acute

Isolate code	Consistent mutation site (aa position) in Gp5 protein of Indian HP-PRRSV and Chinese HP-PRRSV isolates in reference to Vr2332																											
	3	6	10	9	11	16	23	24	25	34	35	39	58	59	66	91	92	94	101	102	121	127	137	161	164	170	185	189	200
VR2332	E	L	C	G	C	S	F	C	F	D	S	L	N	K	S	V	A	V	F	V	T	F	A	I	R	E	V	I	P
JXA1	G	L	C	C	C	F	F	Y	L	N	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	E	A	L	L
07QN	G	L	C	C	C	F	F	Y	L	N	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	E	A	L	L
10 HEB3	G	L	C	C	C	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	V	L	S	V	G	G	A	L	L
10 BJ4	G	L	C	C	C	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	E	A	L	L
PRRS/MZ/AZ/34/13	G	S	C	C	Y	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/AZ/46/13	G	S	C	C	Y	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/AZ/49/13	G	S	C	C	Y	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/AZ/51/13	G	L	C	C	C	F	F	Y	L	N	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/AZ/53/13	G	S	C	C	Y	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/AZ/55/13	G	S	C	C	Y	F	F	Y	L	N	N	I	K	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/1/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/2/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/3/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/4/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/5/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/6/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/7/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/MZ/IND/8/15	G	L	C	C	C	F	F	Y	L	N	N	I	K	N	T	A	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH1/16	G	L	S	C	C	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH2/16	G	L	S	C	C	F	S	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH4/16	G	L	S	C	C	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH5/16	G	L	S	C	C	F	F	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH6/16	G	L	S	C	C	F	S	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L
PRRS/IND/CH7/16	G	L	S	C	C	F	S	Y	L	S	N	I	Q	K	T	V	G	A	Y	Y	I	L	S	V	G	G	A	L	L

Fig. 3. Mutation site at GP5 protein in reference to VR2332.

respiratory disease with severe depression and high fever (40–42°C) with high morbidity and mortality was observed in pigs of all age groups during the outbreak period. Detailed post mortem examination of 20 dead pigs consistently revealed severe haemorrhagic, oedematous, non-collapsing lungs; enlarged, haemorrhagic, mesenteric and bronchial lymph nodes; congested, swollen spleen with areas of infarction. Tissue samples comprising spleen, lungs and lymph nodes from all the 20 dead pigs from the outbreak were tested by RT-PCR for PRRSV and CSFV and also for PCV2 by PCR. All the samples yielded positive results for PRRSV type 2 and were tested negative for both PCV2 and CSFV. The nucleotide sequences of full ORF5, ORF7 gene and 558 bp coding region of Nsp2 from six different cases were analyzed and submitted to the GenBank and accession no. was assigned (Table 1).

Sequence alignment results of 558 bp coding region of Nsp2 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 confirming the outbreak as HP-PRRS. All the 18 sequences (8 from 2016 and 5 each from 2013 and 2015 outbreak) of Nsp2 shared 94.61–100% nucleotide homology among themselves and showed high similarity with the recent HP-PRRSV isolates (2010) of China and Vietnam characterized as novel variant of HP-PRRSV. Among the HP-PRRSV reference isolates, 10HEB-3 (JQ663553) of China and 10QN (JQ663556) isolate from Vietnam showed maximum sequence homology of 94.97–98.39% and 94.43–97.85% respectively with Nsp2 of Indian PRRSV. Phylogenetic tree derived from the Nsp2 gene, however does not differentiate the sequences from three separate outbreaks and clustered together closely with Chinese HP-PRRSV.

Analysis of nucleotide sequences of full ORF5 and ORF7 from the outbreaks of 2016 showed 98.67–100% nucleotide identity among themselves but revealed variations with sequences from earlier outbreaks. ORF5 sequences from 2016 outbreak showed 93.93–97.18% sequence homology with sequences from 2013 outbreak, placed in the same cluster but in a separate group in the Phylogenetic tree (Fig. 1). While with sequences from 2015 outbreak, they showed 95.69–96.68% homology and placed completely in a different group away from 2013 and 2016 sequences. With classical PRRSV prototype VR2332, they showed only 87.87–88.54% sequence homology. Among the HP-PRRSV isolates, 10-QN (JQ663556) from Vietnam showed maximum sequence identity of 98.01-98.67% with sequences from 2013 and 2016 outbreaks in India and grouped closely together in the Phylogenetic tree. The full ORF7 sequences of 2016 outbreak revealed 98.65–100% sequence identities among themselves, 97.30–98.11% identities with the sequences of 2013 and only 91.03–96.23% homology with 2015 sequences. Phylogenetic tree

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                             1        10        20        30        40        50        60 62
VR2332 (U87392)              MPNNNGKQQKRKKGDGQPVNQLCQMLGKIIAQQNQSRGKGPGKKNKKKNPEKPHFPLATEDD
JXA1 (EF112445)              ..........K...N..............................R................
07QN (FJ394029)              ..........K...N..............................R...S............
CH-1a (AY032626)             ........R.K...N..................................S.............
HB1 (AY150312)               ....D.........N...............................................
HB2 (AY262352)               .............................................N.RS...........E.
10BJ-4 (JQ663544)            ..........K...S..............................R................
10HEB-3 (JQ663553)           ..............N..............................R................
MN184C                       ......R...K................R................S...S.............
MN184A                       ......R...K................R................S...S.............
PRRS/MZ/AZ/36/13 (KM283187)  ..............N..............................R................
PRRS/MZ/AZ/35/13 (KJ624717)  ..............N..............................R................
PRRS/MZ/AZ/2/13 (KJ624718)   ..............N..............................R................
PRRS/MZ/IND/2/15 (KT696492)  ......R...K...K...............S..............R................
PRRS/MZ/IND/1/15 (KT696491)  ......R...K...K...............S..............R................
PRRS/MZ/IND/3/15 (KT844660)  ......R...K...K...............S..............R................
PRRS/MZ/IND/4/15 (KT844661)  ......R...K...K...............S..............R................
PRRS/MZ/IND/8/15 (KU169895)  ......R...K...K...............S..............R................
PRRS/IND/CH1/16 (KX869647)   ..............N...........................R..RN...............
PRRS/IND/CH2/16 (KX869648)   ..............N..............................R...L............
PRRS/IND/CH4/16 (KX869649)   ..............N...........................R..RN...............
PRRS/IND/CH5/16 (KX869650)   ..............N...........................R..RN...............
PRRS/IND/CH6/16 (KX869651)   ..............N..............................R................
PRRS/IND/CH7/16 (KX869652)   ..............N..............................R................

                             63     70        80        90        100       110       120 123
VR2332 (U87392)              VRHHFTPSERQLCLSSIQTAFNQGAGTCTLSDSGRISYTVEFSLPTHHTVRLIRVTASPSA
JXA1 (EF112445)              ............................A.................Q.......A......
07QN (FJ394029)              ............................A.................Q.......A...---
CH-1a (AY032626)             .............................................................
HB1 (AY150312)               ............................A.........................A......
HB2 (AY262352)               ............................S.........A......................
10BJ-4 (JQ663544)            ............................A.................Q.......A......
10HEB-3 (JQ663553)           ..............................................Q.......A......
MN184C                       ......................................A......................
MN184A                       ......................................A......................
PRRS/MZ/AZ/36/13 (KM283187)  ..............................................Q.......A......
PRRS/MZ/AZ/35/13 (KJ624717)  ..............................................Q.......A......
PRRS/MZ/AZ/2/13 (KJ624718)   ..............................................Q.......A......
PRRS/MZ/IND/2/15 (KT696492)  ............................A.........................A......
PRRS/MZ/IND/1/15 (KT696491)  ............................A.........................A......
PRRS/MZ/IND/3/15 (KT844660)  ............................A.........................A......
PRRS/MZ/IND/4/15 (KT844661)  ............................A.........................A......
PRRS/MZ/IND/8/15 (KU169895)  ............................A.........................A......
PRRS/IND/CH1/16 (KX869647)   .............................................................
PRRS/IND/CH2/16 (KX869648)   ..................................................C..........
PRRS/IND/CH4/16 (KX869649)   .............................................................
PRRS/IND/CH5/16 (KX869650)   .............................................................
PRRS/IND/CH6/16 (KX869651)   .............................................................
PRRS/IND/CH7/16 (KX869652)   .............................................................
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Fig. 4. Amino acid sequences of ORF7 gene. The sequence of the VR2332 is used as reference. A dash indicates positions where the sequence is identical to that of the VR2332

generated from ORF7 sequences showed similar grouping as observed for ORF5 sequences (Fig. 2). Thus it is concluded that there are three genetically diverged HP-PRRSV strains that has caused outbreaks and circulating in pig population of Mizoram, India.

GP5 protein analysis: The analysis of GP5 protein of 20 isolates of Indian HP-PRRSV with 4 Chinese HP-PRRSV, when compared to VR2332 revealed consistent mutation in total 29 positions in both Indian and Chinese HP-PRRSV isolates. Maximum 9 mutations was observed in the signal sequence region (aa 1–32), followed by 8 mutation in transmembrane region (aa 64–134), 7 in endodomain region (aa 135–200) and 5 in ectodomain (aa 33–63). Interestingly the conserved site of aa 71–120 and aa 131–200 described for large number of NA and EU classical PRRSV strains showed mutation at 5 and 7 no. of amino acid positions respectively in HP-PRRSV. The epitope A region (aa 27–30), identified as 'decoy' epitopes is conserved, while the epitope B region, which was identified as neutralizing epitope showed substitution of aa 39L by I in HP-PRRSV (Fig. 3). GP5 B antigenic region of HP-PRRSV was recently identified as non-neutralizing and was linked to substitution of aa 39L to I (Leng *et al.* 2012).

The potential N- glycosylation sites of GP5 protein were analyzed (28) and found varied from 4 to 5 in both Indian and Chinese HP-PRRSV. An additional new N-glycan moiety at position aa 35 is observed in all the HP-PRRSV, when compared to VR2332. Another new site at position 34 was observed in Indian HP-PRRSV from 2015 outbreaks and 2006–2007 Chinese HP-PRRSV. Three of the Indian HP-PRRSV from 2016 outbreak has N-glycosylation site at position aa 33 like VR2332. The N_{30}, N_{44} and N_{51} sites were highly conserved like VR2332 in all the HP-PRRSV isolates.

Several studies earlier provided evidence that glycosylation of GP5 of PRRSV plays an important role in escaping or minimizing virus-neutralizing Ab response by

a N-glycan-shielding mechanism (Johnson *et al*. 2003, Wei *et al*. 2003). Particularly the presence of N-glycans in and around epitope B may reduce the immunogenicity of this critical neutralization determinant in the GP5 protein of PRRSV. Notably the new N-glycan moiety in HP-PRRSV was observed at position aa 35 in all HP-PRRSV along with a new site at position 34 in some of the HP-PRRSV, near the immunodominant epitope B. Thus, the heterogeneous glycosylation sites with an additional new N-glycan moiety in GP5 protein of HP-PRRSV, along with the conserved 'decoy' epitopes can impede and decrease the humoral immune response against GP5 by obscuring the critical neutralization site(s). This might contribute to high pathogenicity of HP-PRRSV.

N protein analysis: Analysis and comparison of N protein amino acid sequences derived from 14 Indian and 4 Chinese HP-PRRSV with VR2332 revealed consistent mutation at position 15 (aa D to N or K) and 46 (aa K to R) in all the HP-PRRSV. The Indian HP-PRRSV from 2015 outbreak showed maximum mutation of total 7 positions (aa 7, 11, 15, 31, 46, 91 and 117). Substitution of aa31 A by S, which is within the antigenic domain aa 30–52 was observed in Indian isolates from 2015 outbreak (Fig. 4). The region between aa 37-52, which is described as well conserved among isolates of both genotypes and is the most hydrophilic region of the protein showed substitution of aa 43 K by R in PRRS/IND/CH1/16&PRRS/IND/CH4/16, substitution of aa 46 K by R in all the HP-PRRSV isolates, aa 50 P by S and L in isolate 07QN and PRRS/IND/CH2/16 respectively and are not conserved in HP-PRRSV. In antigenic domain, aa 69–112, aa 91 T substituted by A in sequences of 2015 Indian isolates and aa 109 H is substituted by Q in sequence of 07QN, JXA1, 10BJ4, 10 HEB3 and 2013 Indian isolates. Replacement of aa 117 V to A was also observed in antigenic domain aa 112–123 in most of the HP-PRRSV. The common conformational antigenic site localized in aa 52-69 is found well conserved in all the HP-PRRSV isolates.

N protein has been targeted as a suitable candidate for the detection of virus specific Abs, since the early immunologic response following PRRS infection is directed against N protein and major antigenic determinants of N protein are highly conserved. The present findings showed two consistent mutations at amino acid position 15 and 46 in all the HP-PRRSV and mutations in the well conserved region between aa 37–52 at amino acid position 43, 46 and 50. Out of the five antigenic domains reported for N protein only the common conformational antigenic site localized in aa 52–69 is found conserved in all the HP-PRRSV isolates. Therefore, reactivity of the established diagnostics for the detection of virus specific Abs developed with classical PRRSV type II may need revaluation for detection of Abs against HP-PRRSV.

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