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Molecular detection, epidemiology and clinico-pathological studies on first outbreak of porcine reproductive and respiratory syndrome (PRRS) in pig population of Mizoram, India

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS), an economically important viral disease of the pig industry worldwide, has brought great losses to the Chinese pig production in recent years, particularly following the emergence of the highly pathogenic PRRS virus (PRRSV). The Government of India has reported the first outbreak of the disease in pig population of Mizoram to OIE on 26 June 2013.The present study was carried out on molecular detection, epidemiology and clinico-pathological studies of the outbreak. Studies on 880 pigs revealed 65% morbidity and 63.11% mortality with highest mortality rate in pre-weaned piglets (81.06%) followed by pregnant sows (68%). Histopathological examination showed severe interstitial pneumonia and lymphoid depletion in the lymphoid organs. Diagnosis of the disease was confirmed by RT-PCR on tissue samples targeting the ORF–4 and ORF-7 genes of PRRSV. Sequencing of the amplified products revealed that the PRRSV detected in Mizoram belongs to the genotype II (North American type).

Key words: Epidemiology, Histopathology, Mizoram, Molecular detection, Porcine reproductive and respiratory syndrome, PRRS

Porcine reproductive and respiratory syndrome (PRRS) is characterised by a marked increase in late-term abortions, stillborn and birth of weak piglets; lowered farrowing rates, severe respiratory disease and high death rates in suckling and weaned pigs. The disease was first recognized in 1987 in the United States of America (Keffaber 1989), and subsequently became a pandemic disease in North America, Europe and Asia within the succeeding years (Bilodeau *et al*. 1991, Wensvoort *et al.* 1991, Benfield *et al.* 1992, Baron *et al.* 1992, Botner *et al.* 1994, Kuwahara *et al.* 1994).

The causative agent of porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped, single-stranded, positive-sense RNA virus (Meulenberg 2000, Wensvoort *et al.* 1991, Collins *et al*. 1992, Dea *et al.* 2000). There are 2 distinct genotypes of PRRSV: the European (Type I) and North American (Type II) (Meng *et al.* 1995). Both the genotypes of PRRSV cause the same disease symptoms but are antigenically different (Meng *et al*. 1995, Kapur *et al*. 1996, Nelsen *et al*. 1999, Meng 2000, Meulenberg 2000, Ropp *et al*. 2004, Stadejek *et al*. 2006). The PRRSV genome is approximately 15 kb in length and consists of a 5´-untranslated region (UTR), 9 open reading frames (ORFs), ORF1a, ORF1b, ORF2a, ORF2b and ORF3 to ORF7, followed by a 3´-UTR and a poly (A) tail (Meng *et al*. 1994, Dea *et al*. 2000, Meng 2000). The ORF1a and ORF1b located in the 5´-proximal part consist of approximately 75% of the genome and encode 13 putative non-structural proteins (nsp), nsp1a, nsp1ß, and nsp2 to nsp12 that are cleaved by proteolytic processing (Meulenberg *et al.* 1997, Snijder and Meulenberg 1998, Ziebuhr *et al*. 2000). The 3´-proximal part of the genome encodes seven PRRSV structural proteins that are translated from a 3´-coterminal nested set of 6 sub genomic mRNAs (Meng *et al*. 1996, Meulenberg *et al.* 1995). ORF2a and ORFs3–5 encode four membrane-associated Nglycosylated proteins (GP2a, GP3, GP4 and GP5) whereas ORF2b and ORF6 encode 2 non-glycosylated membrane proteins (E and M) (Dea *et al*. 2000, Meulenberg *et al.* 1995).The last ORF (ORF7) encodes for a nucleocapsid protein (N) encapsidating the viral RNA genome (Spilman *et al.* 2009).

By now, PRRS has become an endemic disease in the global swine production, except for several countries, including Sweden, Switzerland, New Zealand, and Australia claimed to be free of this disease (Motha *et al.* 1997, Cannon *et al.* 1998). India was considered as free from the disease till 26 June 2013, when the Government of India has reported the first outbreak of PRRS in pig population of Mizoram, to OIE. This article is reporting the epidemiology, clinical manifestation of the disease, pathological studies,

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molecular detection and genotyping of PRRSV from this first PRRS outbreak in pig population of Mizoram, India.

MATERIALS AND METHODS

Sample collection: Pigs (880) of different age groups from total 6 organized farms were studied from March 2013 to April 2013, following the report of a mysterious disease outbreak resulting heavy mortality in pig population of Mizoram. Affected animals were examined clinically at farm premises. Detailed necropsy examination was carried out in total 82 dead animals and gross lesions were recorded. Representative tissue samples from lungs, spleen, tonsil, mesenteric lymphnodes and kidney were collected and preserved both in 10% buffered formalin for histopathological studies and at –80°C for molecular analysis. Serum samples from affected pigs were also collected for diagnosis of the disease.

Histopathological examination: Fixed tissues were subjected to histopathological processing and staining as per Luna (1968). Haematoxyline and Eosin (H and E) stained individual sections were microscopically examined and the histopathological alterations were recorded.

RNA extraction, RT-PCR, cloning and sequencing: Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. Reverse transcription reactions were performed using random primer with the cDNA synthesis kit as per the protocol given by the manufacturer.

The tissue and serum samples were tested by RT-PCR against PRRSV by using a degenerating primer set, PRRSC-F and PRRSC-R (Toplak *et al*. 2011) that amplify a 300bp fragment of ORF7 gene of both PRRSV genotype I, II. Positive samples were again tested with RT-PCR, by using 2 genotype specific primer sets PRRA-4F and PRRA-4R for genotype 2 and PRRE-4F: and PRRE-4E for genotype 1, targeting the ORF4 gene (Madsen *et al*. 1998). All the samples were also tested for classical swine fever virus by using the primer set NTR-F and NTR-R, targeting the genomic region that encode the 5´ NCR (Greiser-Wilke *et al*.1998). The details of the primers used are given in the Table 1.

PCR was performed with 5pg -.5µg cDNA in a 25 µl volume of 10X Dream Taq^{TM} buffer 2.5µl, 2mM dNTP.25µl, 20pmol of each primer, 1.25 units Dream Taq^{TM} DNA polymerase. Cycling parameters were 95°C×2min, 95°C×1 min, optimal annealing temperature, 72°C×1 min and 72°C×5 min.

The amplified 300bp fragment of ORF7 and 548bp fragment of ORF4 of PRRS positive samples were purified, cloned in pTZ57R/T vector using InsT/AcloneTM PCR product cloning kit and the recombinant plasmids containing gene fragments were subjected to DNA sequencing by using an automated DNA sequencer.

RESULTS AND DISCUSSION

Clinical, gross and histopathological findings: Clinical examination of 880 pigs of different age group from 7 organized farms revealed 65% morbidity and 63.11% case fatality. Highest mortality rate was recorded in pre-weaned piglets (81.06%) followed by pregnant sows (68%).

The disease began with high rise of body temperature (105–107°F) with severe depression, ataxia and complete anorexia in pre-and post-weaned piglets. Affected piglets were huddled together and showed rough hair coat. The visible mucus membranes were slightly congested. Reddened patchy lesions on skin were observed in abdomen, inner thigh and tip of ear. Severely affected piglets manifested reddening of entire skin, coughing and difficulty in respiration (Fig.1).

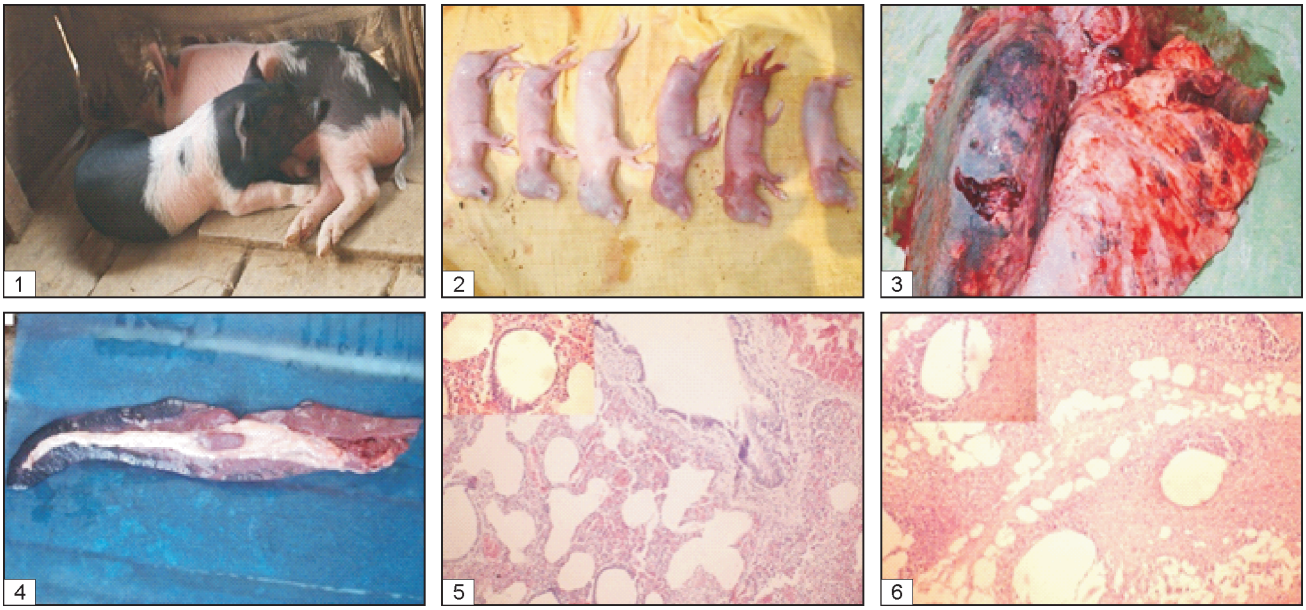
The signs were similar in older animals and pregnant sows were most severely affected. Sows were completely anorexic, depressed and showed increased thirst. Abortion, mummified fetuses, stillbirth and farrowing of weak piglets which eventually died were observed in pregnant sows (Fig.2).

Detailed post mortem examination was carried out in total 82 pigs (37 post weaned piglets + 34 pre weaning, aborted fetus, stillborn piglets +8 pregnant sows + 3 adult boar). Post mortem examination revealed cyanotic/ bluish to reddened patches on skin of ear and abdomen. Lungs showed severe haemorrhagic pneumonia with areas of suffusions and patecheations (Fig.3). All the lymph nodes were (particularly mesenteric lymph nodes, bronchial lymph nodes) severely congested, haemorrhagic and swollen. Spleen was swollen with patecheation and areas of infarction (Fig.4). Liver showed severe congestion.

Histopathological examination of lungs revealed severe interstitial pneumonia. The alveolar septa were thickened due to mononuclear cell infiltrations, congestion and

Table 1. List of primers

Primer identity	Sequence	Product size (expected)	Annealing temp. (standardized)
PRRSC-F	CCAGCCAGTCAATCARCTGTG	300bp	60 ºC
PRRSC-R	GCGAATCAGGCGCACWGTATG		
PRRA-4F	AGAATGGCTTCGTCCCTTCTTTTCTCCTC	548bp	60 ºC
PRRA-4R	CATTCAAATTGCCAACAGAATGGCAA AAAG		
PRRE-4F	GGAATGGCTGCGGCCACTCTTTTCTTC	508bp	60 ºC
PRRE-4E	GAGAACATCTCATATTGCCAAGAGAATGGC		
NTRF	5'-CTA GCC ATG CCC WYA GTA GG 3'	421 bp	50 ºC
NTRR	5'-CAG CTT CAR YGT TGA TTG T-3'		



Figs. 1–6. **1**. Affected piglets (2 months old) suffering from high fever shows reddening of skin and huddling together. **2**. Premature aborted foetuses. **3**. Oedematous, non-collapsing and haemorrhagic lungs of a boar died of PRRS. **4**. Spleen showing area of infarction. **5**. Section from lungs showing interstitial pneumonia characterized by thickening of alveolar wall due to mononuclear infiltration, congestion, haemorrhages, hyperplasia of pneumocyte II (inset, 400×) and bronchiolar epithelial cells. H and E. 200×. **6**. Section from mesenteric lymph nodes showing lymphoid depletion leading to cavity formation in lymphoid follicles (inset, 400×), congestion and haemorrhages H and E. 200×.

haemorrhages. Hyperplasia of pneumocyte II in alveolar wall and bronchiolar epithelial cells with peribronhiolar aggregation of mononuclear cells was also observed (Fig.5). Severe lymphoid depletion leading to cavity formation in the lymphoid follicles was observed in mesenteric lymph nodes with congestion of blood vessels (Fig.6). Lymphoid depletion with apoptotic lymphoid cells and congestion is also observed in tonsil. Spleen showed diffused haemorrhages, lymphoid depletion with histiocytic infiltration in the lymphoid follicles. Haemorrhages and congestion in the cortico-medulary junction was observed in kidney.

RT- PCR detection, cloning and sequencing: Tissue samples (lungs and spleen) from total 51 cases and 10 serum samples were first tested by RT-PCR by using the consensus primer set PRRSC-F and PRRSC-R targeting a 300bp region of ORF 7 gene. Tissue samples from 47 cases and 6 serum samples were tested positive for PRRS (Fig.7). The positive samples were again tested with another 2 sets of genotype specific primer PRRA-4F and PRRA-4R that amplify a 548bp fragment of American strain of PRRSV and PRRE-4F and PRRE-4R that amplify a 508bp fragment of European strain of PRRSV (Madsen *et. el*. 1998) to differentiate the genotype of PRRSV involved in causing the outbreak. All the PRRS positive samples have given amplification of 548bp fragment of American strain of PRRSV (Fig.7), whereas no result was obtained by using the PRRE-4F and PRRE-4R sets of primer. Samples were tested negative for CSF by RT-PCR.

The gene sequences derived from the clone of 300bp fragment of ORF7 and 548bp fragment of ORF4 were

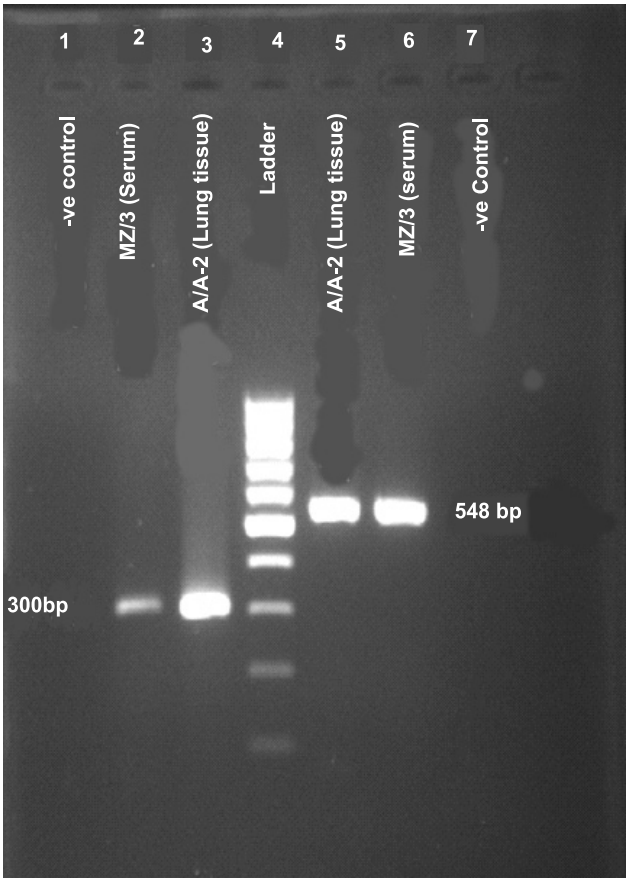


Fig.7. 1.5% Agarose gel electrophoresis showing amplified product of ORF7 [300 bp (L2 and L3)], ORF4 [548 bp (L5 and 6)], gene fragments of PRRSV.

analysed and submitted to the Genbank. Blast analysis of 300bp sequence of ORF 7 [Genbank accession no. KF208423 (PRRSV/MZ/AZ/3/13)] revealed 99% homology with the highly pathogenic PRRSV isolates of genotype 2 (North American type) from China, isolated during 2006–2010 (10–10 HEB-3, 10-10 BJ-5, 10-10 BJ-2) revealing the belonging of the PRRSV from Mizoram outbreak to the North American genotype (genotype II).The 548bp sequences of ORF4 [Genebank accession no.KF208420 (PRRSV/MZ/AZ/1/13), KF208421 (PRRSV/MZ/AZ/2/13), KF208422 (PRRSV/MZ/AZ/3/13)], showed maximum 89–98 % homology with the same Chinese isolates indicating variations in the nucleotides in ORF4 region of Mizoram strains.

Emergence of the variant strain of PRRSV, which is characterized as highly pathogenic PRRSV associated with porcine high fever syndrome (PHFS) (Tian *et al.* 2007), has resulted in significant economic losses in China and Vietnam. The North East Region (NER) of India, comprising 8 States namely Arunachal Pradesh, Asom, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Sikkim has 27.94% of the total pig population of India. Pig husbandry plays an important role in the socio-economic development of this NER of India, including Mizoram. Detection of PRRSV which belongs to genotype 2 (North American type), showed close link with the highly pathogenic PRRSV isolates from China and has caused heavy mortality in pig population of Mizoram, and is a matter of great concern for the entire pig population of NER, India. A thorough and systematic study on surveillance, epidemiology and diagnosis, is essential to know the magnitude of the disease and also for designing strategy to control and containment of PRRS in NER, India.

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