



Molecular detection and seroprevalence of classical swine fever virus from 2016 to 2018 in pigs of Mizoram, India

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ABSTRACT

Classical swine fever (CSF) is a fatal endemic disease of pig population of North eastern India in particular and India in general. Present study revealed molecular detection of CSFV and seroprevalence of the disease in pig population of Mizoram, India during 2016–2018. Serum samples from apparently healthy, unvaccinated pigs were collected in collaboration with the State Animal Husbandry and Veterinary Department, Mizoram and a total of 594 serum samples from 7 districts were subjected to detection of CSFV specific antibodies by indirect ELISA. A total of 206 (34.68%) serum samples were positive for CSFV antibodies by ELISA. District wise, Saiha district showed highest seroprevalence of the disease followed by Kolasib and Serchhip. Apart from this, during the same time period, CSFV suspected samples received in the Department of Veterinary Microbiology consisting of 269 serum samples, 10 whole blood and 83 tissue samples obtained from 8 districts of Mizoram were subjected to detection of NS5b and E² mRNA transcripts by nRT-PCR of which a total of 42 (11.60%) samples including serum (5.58%), tissues (27.71%) and whole blood (40%) were positive for the NS5b and E² mRNA transcripts, specific for CSFV. District wise analysis revealed that Aizawl has the highest percentage of positive samples of CSFV followed by Saiha and Lawngtlai district.

Keywords: CSFV, Mizoram, Pigs, Prevalence

Classical swine fever (CSF) is one of the most important viral disease of pigs worldwide and is highly contagious, hemorrhagic and multisystemic in nature. In all age groups of pigs, the disease may be presented as an acute, subacute, chronic, late onset, or inapparent depending upon various viral and host factors (Van 2004). The disease was first reported in Ohio, USA in 1833 (Hanson 1957) and since then, it has been reported in major parts of Asia, Europe, Central and South America, and parts of Africa (Pol *et al.* 2008), owing it to be listed as one of the notifiable diseases by World Organization for Animal Health (OIE). The disease is characterized by marked immune suppression with moderate to very high mortality (Moennig *et al.* 2003) leading to severe economic losses directly in the form of mortality, reduced growth, reproductive problems in the affected pigs and indirectly by the international trade of pork and pork products (Pol *et al.* 2008, Sarma *et al.* 2008). The disease was first reported in India in 1962 by Sapre *et al.* (1962) and since then, it has been considered as most

often reported endemic disease in India in all pig-producing areas (Malsawmkima *et al.* 2015).

In India, the North Eastern (NE) states constitute the major proportion (~ 28%) of the country's total pig population (Deka *et al.* 2008). An epidemiological study conducted in Assam, Nagaland and Mizoram by the International Livestock Research Institute (ILRI) reported that India faces huge losses (~ ₹ 2 billion) due to mortality, treatment and replacement costs every year (Bett *et al.* 2012). In another study by Singh *et al.* (2016), the economic losses due to CSF in India was calculated approximately ₹ 4.299 billion.

Prevalence of CSF in pigs of Mizoram and other parts of NER India is reported by various workers (Verma 1988, Barman *et al.* 2010, Rajkhowa *et al.* 2013, 2014, Roychoudhury *et al.* 2014, Malsawmkima *et al.* 2015). Bett *et al.* 2012 reported that the financial losses incurred from CSFV in Mizoram alone amounts to ₹ 36.35 million. Pork forming the major meat diet of Mizoram, and CSFV being the major cause of havoc for the pork industry in the state, regular surveillance is pertinent to monitor and control CSFV. Keeping this in mind, present study was aimed to determine the seroprevalence of CSFV and molecular detection of CSFV in pig population of Mizoram during March 2016–2018.

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MATERIALS AND METHODS

Ethical approval: Tissue samples for the present study were collected during post mortem study of dead animals which were suspected CSF infection. Serum and whole blood samples from live animals were collected following standard sample collection procedures inflicting minimum harm or stress to the animal by licensed Veterinarians and is approved by the Institutional Animal Ethics Committee, College of Veterinary Sciences & Animal Husbandry, CAU-Imphal, Aizawl, Mizoram.

Collection of samples: For the present study, samples were collected in two cohorts- Table 1 depicts serum samples (n=594) collected from apparently healthy pigs of different age groups without vaccination history from 7 districts of Mizoram, viz. Aizawl, Champhai, Saiha, Lawngtlai, Serchhip, Kolasib and Mamit during March 2016 to March 2018 in collaboration with the Animal Husbandry and Veterinary Department, Mizoram and were subjected to detection of CSFV specific antibodies by indirect ELISA (Table 1).

Apart from this, the second cohort samples depicted in Table 2 included serum (n=269), tissue (n=83) and whole blood (n=10) from symptomatic vaccinated animals from 8 districts which were separately collected and received in the Department of Veterinary Microbiology, CVSc and AH, Selesih for CSFV detection by nRT-PCR. Blood samples were collected either by intra cardiac puncture or from the ear vein in sterile vacutainer tubes with prescribed

anticoagulant. Tissue samples (fresh post mortem samples) from suspected cases and outbreaks were collected aseptically. The tissues including pieces of spleen, liver, lungs, heart and mesenteric lymph nodes were collected and transported to the laboratory under cold chain. All the specimens were stored at -80°C till further use.

Processing of the samples for molecular detection of CSFV: Total RNA was extracted from the tissue samples, whole blood and serum using TRIzol (Sigma, USA) as per the instruction of the manufacturer. The purity and quantification of the extracted RNA were analysed using a spectrophotometer (MultiScan Go, Thermo Scientific). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using a random primer as per the manufacturer's protocol.

RT-PCR based detection of NS5B and E² genes: The NS5B (Bjorklund *et al.* 1999) and E² (Lowings *et al.* 1996) glycoprotein encoding mRNA transcripts of CSFV were amplified by RT-PCR using specific oligonucleotide primers (Table 3). RT-PCR was performed in 25 μl reaction volume containing Dream Taq™ Buffer (10 $\times$), 200 μM of

Table 1. Seroprevalence of CSF in pig population of Mizoram during 2016 to 2018

Place of Collection	Serum samples tested	Positive for ELISA	Seroprevalence (%)
Aizawl	251	87	34.66
Champhai	174	30	17.24
Saiha	3	2	66.66
Lawngtlai	14	0	0
Lunglei	—	—	—
Serchhip	114	66	57.89
Kolasib	32	19	59.37
Mamit	6	2	33.33
Total	594	206	34.68

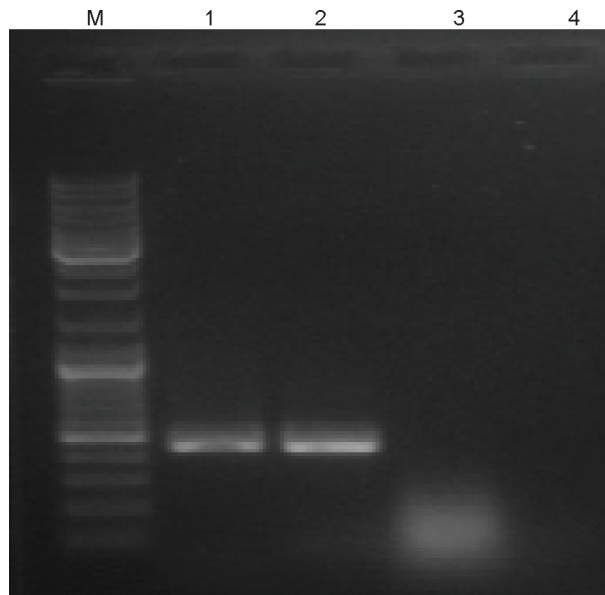


Fig. 1. Gel image of NS5B (~449 bp) partial fragment in 1.5% agarose gel. Lane M, 100 bp DNA ladder; 1,2, Positive samples; 3, Negative; 4, NTC.

Table 2. RT-PCR based detection of CSF virus in pig population of Mizoram during 2016 to 2018

Place of collection	Sera samples tested	+ ve for PCR	Tissue samples tested	+ ve for PCR	Whole blood tested	+ ve for PCR	Total samples tested	Total PCR +ve	Prevalence (%)
Aizawl	22	4	15	4	—	—	37	8	21.62
Champhai	90	2	47	15	10	4	147	21	14.28
Kolasib	18	1	—	—	—	—	18	1	5.55
Lawngtlai	18	2	9	2	—	—	27	4	14.81
Lunglei	32	1	1	—	—	—	33	1	3.03
Mamit	27	1	—	—	—	—	27	1	3.70
Saiha	31	4	6	2	—	—	37	6	16.21
Serchhip	31	—	5	—	—	—	36	—	—
TOTAL	269	15	83	23	10	4	362	42	11.60

Table 3. List of oligonucleotide primers used in the study

Primer identity	Sequence	Product size expected	Annealing temperature	Reference
<i>NS5bF</i>	5'-GAC ACT AGY GCA GGC AAY AG-3'	449 bp	56°C	Bjorklund <i>et al.</i> 1999
<i>NS5bR</i>	5'-AGT GGG TTC CAG GAR TAC AT-3'			
<i>E2-F</i>	5'-AGR CCA GAC TGG TGG CCN TAY GA-3'	671 bp	55°C	Lowings <i>et al.</i> 1996
<i>E2-R</i>	5'-TTY ACC ACT TCT GTT CTC A-3'			
<i>E2-IF</i>	5'-TCR WCA ACC AAY GAG ATA GGG-3'	271 bp	55°C	Lowings <i>et al.</i> 1996
<i>E2-IR</i>	5'-CAC AGY CCR AAY CCR AAG TCA TC-3'			

each dNTPs, 20 pM of each primer, 1.25 units of Dream Taq™ DNA polymerase and 0.5 µg of cDNA. The reactions were performed with cyclic conditions of 95°C × 2 min, 95°C × 1 min, optimal annealing temperature (Table 1), 72°C × 1 min and 72°C × 5 min. For E2 inner fragment (271 bp), a nested RT-PCR was performed using the RT-PCR product of the outer E2 fragment (671 bp) as template DNA with same thermal profile. A standard molecular marker (100 bp DNA ladder) was included in each gel. RT-PCR amplicons were separated by agarose gel electrophoresis in 1.5% agarose (Sigma, USA) at 80 V for 90 min in 1× TAE buffer. The gel was stained with ethidium bromide (0.5 µg/ml) and visualized through UV transilluminator and documented by gel documentation system (AlphaImager, USA).

Serology: All the serum samples were screened by commercial ELISA kit (IDEXX Laboratories) for detection of CSFV antibody as per the instruction of the manufacturer. Results obtained were expressed as positive and negative, based on the manufacturer's recommended cutoff value.

Statistical analysis: Chi square test was employed to find out the significance of differences between samples from different districts.

RESULTS AND DISCUSSION

Classical swine fever is a highly contagious a fatal endemic viral disease of pigs in India that has enormous implications on the vertical growth in the pig industry in India and more so in the North East as pig farming is a major contributor of the economy for most households and is the mainstay for marginalised backyard piggery farmers. Pork is a key component of the diet in Mizoram and around 80% of the households in NE rear pigs. An epidemiological study conducted in Assam, Nagaland and Mizoram by the International Livestock Research Institute (ILRI) reported that India faces huge losses (~ ₹ 2 billion) due to mortality, treatment and replacement costs every year. The projected economic losses from three North Eastern India states of Assam, Mizoram and Nagaland is ₹ 2224.30 million and from Mizoram singly, amounts to ₹ 36.35 million (Bett *et al.* 2012).

As depicted in Table 1, among the 7 districts which were sampled, all exhibited seroprevalence of CSFV except Lawngtlai. Total 34.68% pigs were seropositive for CSFV, where pigs from Saiha, Kolasib and Serchip exhibited higher prevalence (>50%) compared to other districts. In

an earlier study, Mukherjee *et al.* (2018) reported seroprevalence of CSFV in Meghalaya during 2014–2016 and indicated a declining incidence rate (76.4% in 2014, 66.09% in 2015, and 25.5% in 2016). Result of the present study also indicated a lower seroprevalence of CSFV in Mizoram compared to earlier reports from the states of West Bengal, Nagaland and Meghalaya (Nandi *et al.* 2011). The serum samples in Table 1 were a cohort of samples collected from unvaccinated pigs which were apparently healthy or showed no symptoms. In such cases, sero-positive status of animals reflects the incidence of the disease rather than post vaccination seroconversion (Nandi *et al.* 2011). Also, detection of virus-specific antibody in animals is an indirect evidence of virus persistence and implies that the pig population is a carrier and plays an important role in transmission of the virus. Prolonged viraemia, most times, leads to subclinical infections enabling animals to function as carriers which are nearly or completely asymptomatic, and, therefore, may be considered as reservoir hosts (Mukherjee *et al.* 2018). There is increased awareness of the pig farmers regarding CSFV which has encouraged sporadic vaccination in different parts of Mizoram. However, in majority areas of Mizoram, there is a shortfall in vaccine availability. Sarma *et al.* (2011) reported that while the vaccine doses required per year in NE is 7.64 million, the vaccine availability is a mere 0.04 million. This indicates a gap of 7.60 million doses per year.

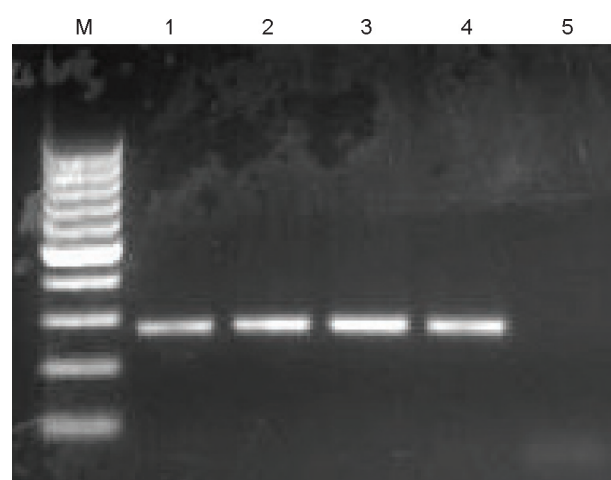


Fig. 2. Gel image of nested E2 (~271 bp) partial fragment in 1.5% agarose gel. Lane M, 100 bp DNA ladder; 1,2,3,4, Positive samples; 5, NTC.

The samples in Table 2 were a cohort of samples from suspected animals with vaccination history received in the Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Selesih. As depicted in Table 2, a total of 42 samples were found to be positive for the NS5b and E² mRNA transcripts specific for CSFV by nRT-PCR. Earlier, from the same laboratory, Malsawmkima *et al.* (2015) had also applied RT-PCR for detection of similar target genes for confirmation of clinical cases of CSF in pigs of Mizoram. Diagnosis of CSF mostly relies on confirmation by PCR targeting relatively conserved gene regions. Envelope of CSFV contains three glycoprotein E^{rns}, E¹ and E², where E² is a highly immunogenic major glycoprotein (Postel *et al.* 2012). The NS5B gene of CSFV encodes an RNA-dependent RNA polymerase (RdRp) with 70–75% identical amino acid sequences. It also has a conserved tertiary structure rather than primary sequence which makes it suitable for the differentiation of CSFV and non-CSFV *Pestivirus* (Zhang *et al.* 2005). In the present study, Aizawl district showed the highest percent of positive result (21.62%) followed by Saiha (16.21%), Lawngtlai (14.81%), Champhai (14.28%) Kolasib (5.55%), Mamit (3.70%) and Lunglei (3.03%).

While vaccination is the prerequisite preventive prophylactic measure for CSF, the vaccinated animals may have suffered from the disease due to vaccine failure which may occur due to an inappropriate titre of the vaccine, cold chain disruption or incorrect vaccine dose. In addition to these, PCV-2 when present as a coinfection, and being ubiquitous and immunosuppressive in nature, is capable of hindering the efficacy of CSFV vaccination (Huang *et al.* Nandi *et al.* 2011) and Varte *et al.* (2018) has reported the prevalence of PCV-2 in pigs from all the districts of Mizoram.

In the present study, majority of the CSF suspected animals (Table 2) exhibited similar typical clinical signs which were in corroboration with observations by previous studies (Rahman *et al.* 2001, Sarma *et al.* 2011, Rajkhowa *et al.* 2013). Highly virulent strains are capable of causing 100% mortality, while, moderate virulent strains are responsible for sub-acute illness in piglets and abnormalities in foetuses, and avirulent strains do not cause any measurable damage to both pigs and foetuses. This concludes that pathogenicity and virulence of a viral strain play a crucial role in determining the manifestation of the disease in an animal, however, other host factors, like age, immune status and breed of pigs may also influence the clinical picture of disease (Malsawmkima *et al.* 2015). In the present study, suspected animals which died showed various course of the disease and succumbed to death within 10 to 45 days after appearance of clinical signs.

The detection of CSFV antibodies in apparently healthy unvaccinated animals (Table 1) and the high probability of detection by nRT-PCR in suspected vaccinated animals (Table 2) outlines the endemicity of CSFV in pigs in Mizoram. Although genomic and phylogenetic studies on CSFV virus have been done throughout the country, there

is paucity on the epidemiology of the disease (Nandi *et al.* 2014). There are few reports regarding CSFV infections/outbreaks in pigs in Mizoram and other parts of India. Along with CSFV, PRRS and PCV-2 are other important transboundary diseases which are wreaking havoc in the pig industry in Mizoram through massive mortality, lowering productivity and reproductive failure (Rajkhowa *et al.* 2015, Varte *et al.* 2018). CSFV has also incurred huge economic losses in neighbouring pig producing states like Meghalaya, Assam and Nagaland. Persistence of such disease conditions in pig population of this region is one of the major factors which retards growth of pig husbandry. The disease can be controlled if proper control and preventive measures are in place. Restricted and proper monitoring of movement of pigs within national and state boundaries, strengthening infrastructure and capacity for diagnosis, meeting demand for vaccines, maintenance of farm hygiene and surveillance programs are crucial and should be prioritised to check the continued repercussions of this disease on pig industry of the state.

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